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Genetic analysis of inherited canine neurological disorders

PhD Thesis submitted by

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Abstract

Comparable to human medicine, neurological disorders in dogs represent a category of diseases with often progressive and severe phenotypes. These conditions can drastically impair normal life and lead to lasting disabilities, movement disorders, or even premature death. In recent years, the availability of medical care for companion animals has reached levels that are comparable with the standards in human medicine. However, many neurological diseases remain untreatable for humans and their canine companions alike. Simultaneously, the unique population structure of modern dog breeds with very little genetic variation within specific breeds facilitates the fast spreading of spontaneous genetic variants that can lead to the development of diseases, such as inherited neurological disorders. On one hand, this makes dogs valuable models for studying such spontaneously emerging genetic diseases, but on the other hand breeders need methods like genetic tests to stop new variants from spreading.

In this thesis, I analyzed the genetic background of seven diverse inherited canine neurological phenotypes. My research involved different genetic mapping methods, whole genome sequencing analysis, and genotyping of selected subpopulations with Sanger Sequencing. Incidentally, all seven investigated phenotypes turned out to be inherited in a fully penetrant, autosomal recessive mode.

A deletion of the complete *SELENOP* gene in Belgian Shepherd dogs led to a selenium deficiency which was in turn associated with juvenile onset cerebellar ataxia. Another ataxia phenotype was investigated in Nova Scotia Duck Tolling Retrievers and associated with a missense variant in *SLC25A12*. A third type of hereditary ataxia, more precisely an exercise induced dystonia-ataxia syndrome, was studied in Weimaraner dogs. I was able to connect this phenotype with a candidate causative frameshift variant in *TNR*, coding for the extracellular matrix protein tenascin, which is specific to the central nervous system.

Another type of neurological disease that was studied as part of this work was a hereditary sensory and autonomic neuropathy (HSAN) in mixed breed dogs, which was associated with a variant in *SCN9A*, a known human candidate gene for HSAN and congenital insensitivity to pain. The large and heterogeneous group of inherited errors of metabolism yielded candidate causative variants for three different phenotypes. *ACADM* and *MFF* were both associated with phenotypes affecting the metabolism and homeostasis of mitochondria in neurons and throughout the body. The variants were specific to Cavalier King Charles Spaniels with medium-chain acyl CoA deficiency and Bullmastiffs with mitochondrial fission encephalopathy, respectively. Lastly, a *MYO5A* frameshift variant showed the expected co-segregation for a

monogenic autosomal recessive defect in a litter of miniature Dachshunds with a single puppy suffering from coat color dilution and neurological defects.

The identification of these seven candidate causative variants enabled genetic testing and controlled breeding. The long-term goal of eradicating the corresponding disorders from the target breeds and thus increasing breed- and overall animal welfare will become achievable with the availability of such tests. Most of the variants we identified were in known candidate genes for human disorders. However, *SELENOP* variants have never been reported as the cause for human neurological disorders. This thesis, therefore, demonstrates that genetic analysis of inherited canine diseases continues to offer the chance of gaining new biological insights that might be valuable for human medicine in the future.

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Introduction

Since the beginning of their domestication, dogs have always been subject to selection of behavioral and physical traits which have been advantageous for the tasks that humans wanted their companions to perform. With the development of society and more prosperity, the selection of purely aesthetic characteristics was introduced as well, thus leading to increasing divergence of size, coat color or character between certain individuals. Strict selection programs arose with time, allowing only breeding of a limited number of animals which embodied the desired traits in the best possible way. The result of this process, which can be seen today, is the development of currently 356 incredibly diverse dog breeds, which are recognized by the world canine organization FCI [1]. This arguably makes the dog the mammalian species with the greatest phenotypical variance on the planet [2].

This variance between specific breeds conversely is represented by very little heterogeneity within specific breeding populations. As only a restricted quantity of desirable animals are used to breed for the next generation, and as often only matings inside the same breed are recommended or even allowed, line breeding, inbreeding, and the use of popular sires frequently become necessary tools in a breeder's toolkit to maintain breed standards [3]. Unfortunately, this artificial selection process favors additional co-selection of undesirable, and often detrimental genetic mutations. Predispositions of certain breeds for specific diseases are ultimately the consequence of this breeding process [4]. Additionally, as breeds often descend from only a few founding dogs, it is often assumed that all dogs affected by a similar disease in a breed carry the same genetic defect. Aforementioned breeding practices often favor the spontaneous emergence of autosomal recessively inherited diseases. A combination of short generation times, constantly evolving medical standards in companion animal medicine, and the shared living environment between humans and pets, ultimately make the dog an optimal model to study such inherited diseases and thus also benefit human research [5,6].

Inherited neurological diseases

The brain and spinal cord (central nervous system, CNS), as well as the nerves branching of from the spinal cord (peripheral nervous system, PNS), are what make up the body's nervous system. The transmission of electrical and chemical signals between the brain and the rest of the body is what enables and controls basic functions such as breathing, as well as complex processes of thought and movement alike. Consequently, a neurological disease arises, where this network is disturbed or disrupted in some way. The two main structures most commonly affected by these disturbances are the different types of neurons, and the protective

and supportive non-neuron cells called glia, which maintain the function of neurons, for example by producing the insulating myelin sheath (Figure 1).

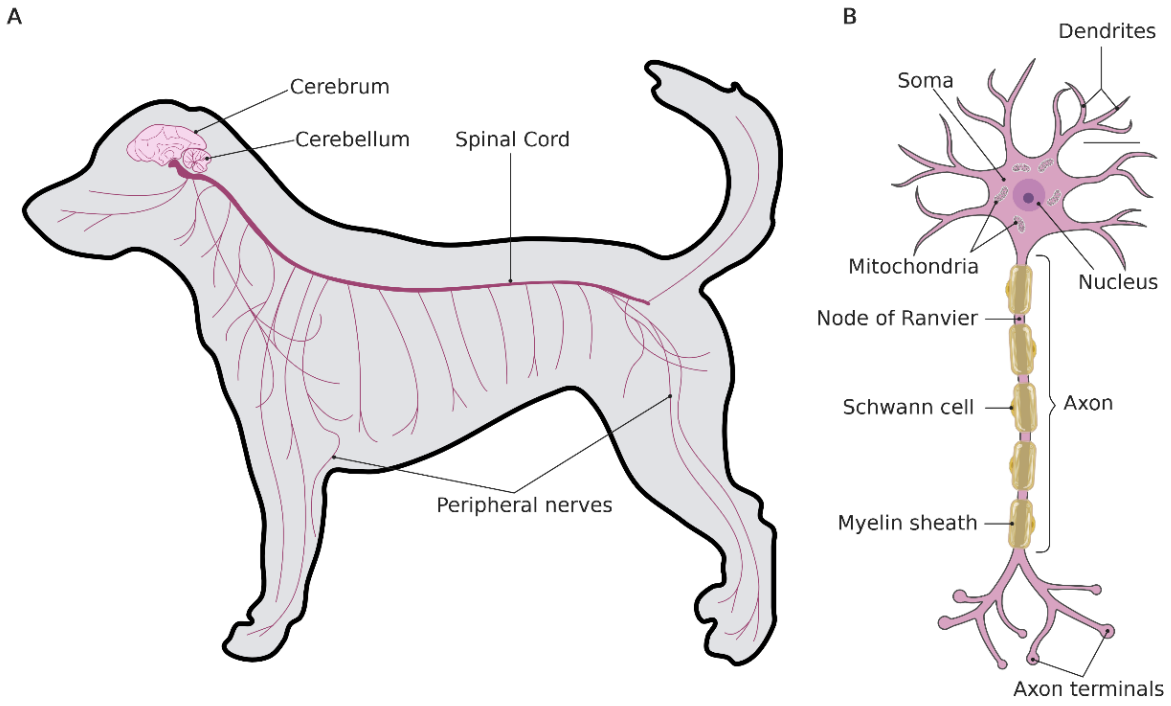

Figure1. Schematic depiction of the canine nervous system. (**A**) The CNS consists of brain (cerebrum, cerebellum, and brainstem) and spinal cord, and the PNS is comprised of the peripheral nerves which connect CNS and the rest of the body. (**B**) Structural parts of a peripheral nerve with a myelinated axon. Created with BioRender.com

The human genome contains an estimated 20,000-25,000 protein coding genes. A third of those genes are expressed primarily in the brain or during brain development [7]. It is therefore not surprising, that almost one third of rare diseases in humans are classified as neurologic and neurodevelopmental disorders, or as intellectual disabilities [8]. Molecular characterization and thus genetic diagnosis can nowadays be achieved in up to 40% of human patients, thereby potentially also refining therapeutic decisions [8]. With the availability of more accurate, fast, and affordable sequencing techniques, genetic characterization of inherited diseases is becoming a more and more indispensable part of a complete diagnostic workup in human paediatrics [9,10]. This development is expected to spread to veterinary medicine in the near future [11]. Since dogs share most protein-coding genes with humans [12], discoveries of novel gene-phenotype relationships in one species may serve as a model for the other. Such new genes involved in neuropathological pathways are still being discovered in human and veterinary medicine alike. This suggests that genetic characterization will not only serve as a diagnostic tool in the future, but that dogs will continue to serve as valuable model organisms for human research for time to come.

In canines and humans alike, genetic defects in neurodevelopment or progressive neurodegeneration often lead to severe phenotypes and impaired quality of life, or even early death [13]. Such defects in the PNS are called peripheral neuropathies and are categorized

according to the type of affected nerve pathway [3,14]. In contrast, CNS disorders can be divided into various overlapping categories, for example ataxias and cerebellar degenerations, as well as leukodystrophies, encephalopathies, neuroaxonal dystrophies, or lysosomal storage diseases [13]. If the affected pathway is metabolic, all these disease categories may additionally overlap with the classification as a neurometabolic disorder [15]. The categories of Mendelian neurological diseases, in which most of the described pathogenic canine variants can be found, are described in more detail in the following chapters.

Hereditary ataxia

Ataxia is a neurological sign that describes the loss or lack of coordinated voluntary muscle movements. It may include difficulty with walking and balance, inability to perform tasks that require precision and accuracy, a wide-based gait, as well as tremors [16–18]. Additionally, speech may be slurred, and patients report blurred vision due to nystagmus. Clinical signs may arise due to degeneration or dysfunction in cerebellar structures with their afferent and efferent connections (cerebellar ataxia, CA), the proprioceptive sensory pathway, or the vestibular system [16]. Sensory ataxia is distinguished by the presence of the Romberg's sign, which means that, in contrast to CA, patients show near normal coordination as long as they can visually observe what they are doing. Vestibular ataxia is often marked by vertigo, dizziness, nystagmus, and hearing impairment [16].

Human hereditary ataxias are usually classified according to their mode of inheritance. They range from the comparatively common autosomal recessive Friedreich's ataxia (OMIM 229300) and the over 45 diverse dominantly inherited spinocerebellar ataxias (OMIM PS164400), to neurometabolic disorders such as coenzyme Q10 deficiencies (OMIM PS607426) [19]. In this wide range of different phenotypes, ataxia can be the sole symptom or just one of many different consistent clinical features. Thus, the diagnostic process and the identification of the most appropriate functional candidate genes can be very long and challenging [20]. According to data collected by the International Parkinson and Movement Disorder Society, variants in at least 28 genes lead to pure or relatively pure forms of ataxia [21–23]. A further 125 disease-associated genes give rise to complex ataxias or disorders, in which ataxia frequently coexists with other predominant or consistent movement disorder features. Finally, over 100 genes are known to present with complex non-ataxia phenotypes, that may occasionally include ataxia nonetheless [21–23]. These additional entities include candidate genes for phenotypes like spastic paraplegia (OMIM PS303350), Leigh syndrome (OMIM 256000), or neuronal ceroid lipofuscinosis (NCL) (OMIM PS256730), thus again highlighting the genetic variability of hereditary ataxia. However, not all these numbers can be assigned to unique genes, as different variants in the same gene might lead to diverse

phenotypes. One such example are variants in *SPTBN2* leading to unique dominant (OMIM 600224) and recessive (OMIM 615386) inherited forms of spinocerebellar ataxia [23].

Similar to historical classification systems in humans [24], canine hereditary ataxia is still classified according to neuropathological findings [25,26]. Three of the categories all involve degeneration of neurons, which can be restricted to the cerebellar cortex (cerebellar cortical degeneration), involve the medulla and/or spinal cord with or without a cerebellar component (spinocerebellar degeneration), or can be characterized by multifocal degeneration with a predominant (spino)cerebellar component. Two further categories include “cerebellar ataxias without significant neurodegeneration” and “episodic ataxias”. Episodic ataxias are, as the name implies, of episodic nature but again without significant accompanying histopathological changes [25,26]. Finally, the non-ataxia centered, complex phenotypes with occasional ataxia development, such as NCL, must be considered in veterinary medicine and human medicine alike [21–23].

Accurate neuropathology is immensely helpful when looking for candidate genes causing similar changes in humans. However, such a system may also lead to difficulties during the investigation. This is illustrated by the example of spongy degeneration and cerebellar ataxia subtypes 1 and 2 (SDCA1 and SDCA2) in Belgian Shepherd dogs, which show a similar clinical disease progression in closely related dogs, but are caused by variants in two different genes, namely *KCNJ10* and *ATP1B2* [27,28]. Although both affected genes function in cation trafficking, their temporally different roles lead to additional clinical signs and histopathological lesions in SDCA2 affected puppies, resulting in a different ataxia classification of the two genes [26].

Driven by the developments in human genetics, and by the efforts of many veterinarians and breeders, the number of candidate variants for neurological disorders causing ataxia in dogs has increased considerably over the last decade and is still rapidly expanding (Table 1). New forms of canine cerebellar ataxia are regularly reported, but often remain genetically unsolved [26]. For this reason, episodic ataxias are excluded from the list below, as the only report in veterinary medicine to date was made in a single Bichon Frise, and no candidate causative was investigated therein [29].

Table 1. List of canine genetic variants (OMIA#), in which ataxias are or can be part of the phenotype, plus corresponding diseases (OMIM#) caused by variants in their human orthologs.

Gene	MOI[1]	VT[2]	OMIA# [30]	Breed	Corresponding Human diseases	OMIM# [31]
Cerebellar Cortical Degeneration						
RAB24	AR	MS	001913-9615	Gordon Setter Old English Sheepdog	-	612415
SEL1L	AR	MS	001692-9615	Finnish Hound	-	602329
SNX14	AR	SS	002034-9615	Vizsla	Spinocerebellar ataxia 20, AR	616354
SPTBN2	AR	FS	002092-9615	Beagle	Spinocerebellar ataxia 5, AD Spinocerebellar ataxia 14, AR	600224 615386
VMP1	AR	MS	002602-9615	Australian Working Kelpie	-	611753
Spinocerebellar Degeneration						
CAPN1	AR	MS	001810-9615	Parson Russell Terrier	Spastic paraplegia 76, AR	114220
KCNJ10	AR	MS FS	002089-9615	Belgian Shepherd Jack Russell Terrier Parson Russell Terrier Smooth-Haired Fox Terrier	Enlarged vestibular aqueduct (digenic) SESAME syndrome	600791 612780
SCN8A	AR	MS	002194-9615	Alpine Dachsbracke	Familial myoclonus 2 Cognitive impairment with or without CA Developmental and epileptic encephalopathy 13 Benign familial infantile seizures 5	618364 614306 614558 617080
SLC12A6	AR	FS	002279-9615	Belgian Shepherd	Agenesis of the corpus callosum with peripheral neuropathy AD-CMTax-*SLC12A6*	218000 620068
Multifocal degeneration with predominant (spino)cerebellar component						
ATP1B2	AR	FS	002110-9615	Belgian Shepherd	-	182331
HACE1	AR	FS	002522-9615	Norwegian Elkhound	Spastic paraplegia and psychomotor retardation with or without seizures	616756
PNPLA8	AR	FS	000827-9615	Australian Shepherd	Mitochondrial myopathy with lactic acidosis	251950
SERAC1	AR	-	-	Chinese crested dog Kerry Blue Terrier	3-methylglutaconic aciduria with deafness, encephalopathy, and Leigh-like syndrome	614739
Ataxia without neurodegeneration						
GRM1	AR	FS	000078-9615	Coton de Tulear	Spinocerebellar ataxia 13, AR Spinocerebellar ataxia 44, AD	614831 617691
ITPR1	AR	IRE	002097-9615	Italian Spinone	Gillespie syndrome Spinocerebellar ataxia 15, AD Spinocerebellar ataxia 29, AD	206700 606658 117360
KCNIP4	AR	MS	002240-9615	Norwegian Buhund	-	608182
Other phenotypes that include ataxia as part of the clinical spectrum						
ATF2	AR	MS	001471-9615	Standard Poodle	-	123811
LGI2	ADI	NS	001596-9615	Lagotto Romagnolo	-	608301
VLDLR	AR	FS	001947-9615	Eurasier	Cerebellar hypoplasia, impaired intellectual development, and disequilibrium syndrome 1	224050

[1]MOI: mode of inheritance; AD: autosomal dominant; ADI: autosomal dominant with incomplete penetrance, AR: autosomal recessive, MI: mitochondrial
[2]VT: variant type; FS: frameshift, MS: missense, NS: Nonsense; SS: splice site, IRE: intronic repeat expansion; diverse variants in different breeds can lead to multiple entries.

Leukodystrophies

Derived from the words “leuko” = white, and “dystrophy” = wasting, leukodystrophies are inherited and often progressive disorders that specifically affect the white matter of the CNS. [32]. This means that leukodystrophies are diseases which center around problems in development and maintenance of myelinated axons, oligodendrocytes, astrocytes, microglia, and blood vessels surrounding these structures [32]. As many of these disorders can also affect nonwhite matter regions of the nervous system, they are sometimes also referred to as leukoencephalopathies [33].

Oligodendrocytes form myelin sheaths by wrapping their plasma membrane around the axon in a complex feat of membrane synthesis [34]. They are in communication with their surrounding environment and thus, disruptions in astrocytes or axons may also affect oligodendrocytes and myelin.

In contrast to primary neuronal disorders in humans, which usually present with cognitive decline and seizures, most human patients with leukodystrophies present with motor symptoms, such as stagnation or even regression of motor skills, and delays in motor milestones [35]. Other symptoms which vary across the over 60 described leukodystrophies/ leukoencephalopathies include cerebellar degeneration, progressive ataxia, altered proprioception, and various extra-neurological findings [35,36].

Canine forms of hereditary CNS white matter disorders have been reported in many breeds, but no consensus classification as in human medicine was devised so far. Disease reports include “degenerative myelopathy” [37], “hypomyelination of the CNS” [38], “fibrinoid leukodystrophy” [39], or “leukoencephalomyelopathy” [40], and candidate variants have been identified for at least eight distinct diseases (Table 2).

Table 2. List of canine genetic leukodystrophy variants (OMIA#), plus corresponding diseases (OMIM#) caused by variants in their human orthologs

Gene	MOI[1]	VT[2]	OMIA# [30]	Breed	Corresponding Human diseases	OMIM# [31]
Alexander disease (astrocytopathy)						
GFAP	AD	MS	001208-9615	Labrador Retriever	Alexander disease	203450
Degenerative myelopathy						
SOD1	AR	MS	000263-9615	Belgian Shepherd Boxer Bernese Mountain Dog Chesapeake Bay Retriever German Shepherd Dog Hovawart Pembroke Welsh Corgi Rhodesian Ridgeback	Amyotrophic lateral sclerosis 1, AD and AR Progressive spastic tetraplegia and axial hypotonia, AR	105400 618598
Hypomyelinating leukodystrophy						
PLP1	XLR	MS	000770-0615	Springer Spaniel	Pelizaeus-Merzbacher disease Spastic paraplegia 2, XLR	312080 312920
TSEN54	AR	MS	002215-9615	Standard Schnauzer	Pontocerebellar hypoplasia type 2A Pontocerebellar hypoplasia type 4	277470 225753
VPS11	AR	MS	002152-9615	Rottweiler	Hypomyelinating leukodystrophy	616683
Hypomyelination of the central nervous system						
FNIP2	AR	FS	000526-9615	Weimaraner	-	612768
Leukoencephalomyelopathy						
CYTB	MT	MS	002684-9615	Australian Cattle Dog Shetland Sheepdog	-	516020
NAPEPLD	AR	FS MS	001788-9615	Great Dane Leonberger Rottweiler	-	612334

[1]MOI: mode of inheritance; AD: autosomal dominant, AR: autosomal recessive, MT: mitochondrial, XLR: X-linked recessive

[2]VT: variant type; FS: frameshift, MS: missense; diverse variants in different breeds can lead to multiple entries.

Charcot-Marie-Tooth disease (CMT)

CMT, also referred to as hereditary motor and sensory neuropathy (HSMN), is the most common form of peripheral neuropathy with Mendelian inheritance in humans [41]. The hallmark of CMT is progressive atrophy of the peroneal muscles leading to muscle weakness, without significant pain or sensory symptoms [41]. Other common shared clinical features of most CMT types include hollow foot arches (pes cavus), a reduction or loss of tendon reflexes, and possible skeletal anomalies [19]. However, clinical signs vary, and patients may additionally show pyramidal signs, inflammation of the optic nerve, deafness, or developmental disability [42].

Classification of the CMT subtype is partially done according to motor conduction velocity of the median nerve [43]. The distinction between demyelinating type (low conduction velocity), axonal type (low potential amplitudes) and intermediate type corresponds to conduction velocities of <38 m/s, >38 m/s, and 25-45 m/s, respectively [43]. Another important part of CMT classification is the mostly autosomal dominant inheritance. Almost 90% of human CMT cases are due to de novo variants in *PMP22*, *GJB1*, *MFN2* and *MPZ* [44], but over 70 different genes with different modes of inheritance have been implicated in the disease overall [19] (OMIM PS118220). Recent proposals for the classification of CMT subtypes consider inheritance, affected gene, and electrophysiological phenotype [42]. For example, a de novo occurring duplication of the *PMP22* gene causing a demyelinating CMT would be called “AD-CMTde-*PMP22dup*”, thus making the condition easier to understand for patients and neurologists alike [19,42].

Similar to humans, signs of HSMN in dogs are typically first apparent in the pelvic limbs and consist of decreased reflexes, weakness, and muscle atrophy [14]. An important additional feature of many canine HSMN is laryngeal paralysis. In affected animals, degeneration of the recurrent laryngeal nerve can occur together with the loss of other myelinated nerve fibers, and thus lead to loss of sensory innervation in esophagus and trachea [45]. Clinical signs range from mild inspiratory stridor and altered barking to exercise and heat intolerance, severe respiratory insufficiency, and death [14,45]. Laryngeal paralysis can also be present in dogs without any other clinical signs and may even be the only clinical abnormality in the early disease process [14]. To date, variants in six different genes have been associated with CMT-like phenotypes in dogs. Laryngeal paralysis is described as part of the clinical presentation in all the associated diseases (Table 3).

Table 3. List of canine peripheral neuropathies with described candidate causative variants (OMIA#), plus corresponding diseases (OMIM#) caused by variants in their human orthologs.

Gene	MOI[1]	VT[2]	OMIA# [30]	Breed	Corresponding Human diseases	OMIM# [31]
HMSN/CMT- like phenotypes. All named 'polyneuropathy' in dogs, due to additional laryngeal paralysis						
NDRG1	AR	MS FS	002120-9615	Alaskan Malamute Greyhound	AR-CMTde-*NDRG1*	601455
ARHGEF10	?	FS	001917-9615	Leonberger Saint Bernard	Slowed nerve conduction velocity	608236
RAB3GAP1	AR	SS FS	001970-9615	Alaskan Husky Black Russian Terrier Rottweiler	Martsolf syndrome 2 Warburg micro syndrome 1	619420 600118
GJA9	AD	FS	002119-9615	Leonberger	-	611923
SBF2	AR	SS	002284-9615	Miniature Schnauzer	AR-CMTde-*SBF2*	604563
CNTNAP1	AR	MS	002301-9615	Labrador Retriever Leonberger Saint Bernard	Hypomyelinating neuropathy 3 Lethal congenital contracture syndrome 7	618186 616286
HSAN – like phenotypes						
RETREG1	AR	IV MS	002032-9615	Border Collie Mixed Breed	HSAN type IIB	613115
lncRNA upstream *GDNF*	AR	RE	001514-9615	English Pointer English Springer Spaniel French Spaniel German Shorthaired Pointer	Susceptibility to Hirschsprung disease 3	613711
Sensory neuropathy without known motor or autonomous components						
MTTY	MT	FS	001467-9615	Golden Retriever	-	590100

[1]MOI: mode of inheritance; AD: autosomal dominant, AR: autosomal recessive, MT: mitochondrial, ?: unclear
[2]VT: variant type; IV: inversion, FS: frameshift, MS: missense, RE: regulatory, SS: splice site; diverse variants in different breeds can lead to multiple entries.

Hereditary sensory and autonomic neuropathies (HSAN)

Different from the predominantly motoric decline in CMT [41], the group of peripheral neuropathies called HSAN arise due to degeneration or developmental defects in sensory neurons [46]. Affected humans are born without the capacity to sense pain or lose the ability to perceive it, often resulting in severe consequential injuries [46,47]. To make matters worse, these lesions may then stay unnoticed, which can lead to serious infections that can even require amputation. Painless fractures are difficult to manage, especially in children who have never learned avoidance behaviours [46].

Eight different subtypes of HSAN are described in the scientific literature [48]. HSAN1 and HSAN7 follow an autosomal dominant inheritance and generally manifest later in life, while the other forms are usually congenital and more severe. The distinction between the recessive forms of HSAN is made by presence of different additional problems, such as absent lacrimation and dysautonomic crisis in HSAN3, or anhidrosis in HSAN4 [49]. Overall, at least 26 genes have been reported to cause either HSAN or one of seven forms of congenital insensitivity to pain (CIP), an overlapping phenotype with not always clearly specified differences [47,50].

Self-injury is a feature of HSAN, which is not only described in human patients, but also in canines [3,46]. Dogs that gradually lose their pain perception, frequently show progressive mutilation of the paws. The corresponding diseases are therefore commonly referred to as

“acral mutilation syndromes” by clinicians [3]. HSAN have been described in many different breeds, and candidate causative variants have been identified in *RETREG1* for Border Collies and mixed breed dogs [51,52], as well as in a regulatory region upstream of *GDNF* for different hunting dog breeds [53] (Table 3). However, at least eight known forms of canine HSAN remain genetically unresolved, highlighting the need for further studies in this field [3].

Neurometabolic disorders

Inborn errors of metabolism (IEM), in a very general sense, result in pathologic phenotypes by blockage of any metabolic pathway [54]. With a prevalence ranging from 1 in 784 [55] to 1 in 2555 [56], IEM may be rare as single disorders, but altogether they show considerable representation throughout western populations. The immense class of IEM currently circumscribe over 1400 human disorders [54]. Over 85% of IEM have influence on neurodevelopment or neurodegeneration, leading to at least partial neurological phenotypes. A recent system proposed three big and comprehensive categories of IEM: Small molecule disorders, complex molecule disorders, and disorders primarily involving energy metabolism [15,57].

Small molecule disorders are characterized by the fact, that they affect small and diffusible, water-soluble molecules, that can be readily measured using chromatography, such as amino acids, organic acids, or acylcarnitines [57]. These molecules can then either accumulate and lead to build-up of toxic compounds, or they may be deficient due to defective synthesis or faulty transportation of molecules through membranes [57]. For instance, accumulation of toxic products is demonstrated in *L2HGDH* defects, where L-2-hydroxyglutaric acid (L2-HGA) accumulates in various tissues and leads to apoptosis of cerebellar structures in humans and companion animals [58–60].

Contrary to small molecules, complex molecules are defined as non-water-soluble or diffusible, such as phospholipids, sphingolipids, or glycosaminoglycans. Lack of said molecules often lead to severe congenital syndromic phenotypes. An example is *SERAC1*, which functions in phosphatidylglycerol remodelling. Genetic variants leading to deficiency of the produced protein result in 3-methylglutaconic aciduria, sensorineural deafness, encephalopathy, and Leigh syndrome (=MEGDEL syndrome) in humans (OMIM 614725). On the other hand, accumulation of complex molecules over time results in storage of these compounds in the cellular cytoplasm or in lysosomes and causes lysosomal storage disease, such as NCL [61].

Lastly, disorders involving energy metabolism are characterized by an overlapping clinical spectrum including hypoglycaemia, generalized hypotonia, failure to thrive, unexpected infant death and many more [15]. This third category of IEM can be divided in the three subgroups

energetic molecule transporter, cytoplasmic defects, and mitochondrial defects. One such mitochondrial defect known in dogs arises due to the deletion of a single amino acid in *MFN2* in Schnauzer-Beagle crosses [62]. The encoded mutant mitofusin 2 is expected to be destabilized and unable to perform its physiological task. Contacts of mitochondria to the endoplasmic reticulum, autophagosome formation, and mitochondrial transport in axons are diminished and neuroaxonal degeneration follows [62].

As more links between canine IEM and their disease-causing genetic variants are made, the mode of classification of these diseases also becomes more complex. Diseases can often be assigned to more than one group of disorders, as there is a certain overlap between the diverse nomenclature systems used in human and veterinary medicine. Many phenotypes can be classified as IEM, as well as other disease classes. The aforementioned *SERAC1* can be assigned to complex molecule disorders, as well as to hereditary ataxia class of multifocal degeneration with predominant (spino)cerebellar component (Table 1, OMIM 614725). Variants in *RETREG1* lead to HSAN through accumulation of parts of the endoplasmic reticulum and subsequent cell death (Table 3, OMIM 613115). This mechanism of disease is also consistent with a designation as a metabolic complex molecule IEM (Table 4). Dysfunctional *TECPR2*, *PLA2G6*, and the above mentioned *MFN2* all lead to neuroaxonal dystrophy in dogs but are all concomitantly classifiable as IEM (Table 4). Conclusively, many diseases can be added on the list of IEMs with known genetic defect in dogs. Therefore, the below table only includes genes, that have not already been mentioned in previous chapters for other categories of diseases (Table 4). Advances in canine genetic research in the coming years will help to unravel this complex interplay between neurological disease and the involved metabolic pathways and dogs will continue to be useful models for similar human phenotypes.

Table 4. List of canine neurological IEM with described candidate causative variants (OMIA#), plus corresponding diseases (OMIM#) caused by variants in their human orthologs. Known candidate genes for lysosomal storage disease forms in dogs are separated according to the type of accumulated material (Platt 2018).

Gene	MOI[1]	VT[2]	OMIA# [30]	Breed	Corresponding Human diseases	OMIM# [31]
Small molecule disorder						
ALDH5A1	AR	MS	002250-9615	Saluki	Succinic semialdehyde dehydrogenase deficiency	271980
CHAT	AR	MS	002072-9615	Old Danish Pointing Dog	Congenital presynaptic myasthenic syndrome 6	254210
CHRNE	AR	FS	000685-9615	Jack Russell Terrier Heideterrier	Congenital presynaptic myasthenic syndrome 4A Congenital presynaptic myasthenic syndrome 4B Congenital presynaptic myasthenic syndrome 4C	605809 616324 608931
DNM1	AR	MS	001466-9615	Chesapeake Bay Retriever Curly-Coated Retriever Labrador Retriever	Developmental and epileptic encephalopathy 31A Developmental and epileptic encephalopathy 31B	616346 620352
GLRA1	AR	FS	000689-9615	Miniature Australian Shepherd	Hyperekplexia 1	149400
L2HGDH	AR	MS SL	001371-9615	Staffordshire Bull Terrier Yorkshire Terrier	L-2-hydroxyglutaric aciduria	236792
SLC19A3	AR	FS	001097-9615	Alaskan Husky Yorkshire Terrier	Thiamine metabolism dysfunction 2	607483
SLC6A5	AR	FS	001594-9615	Irish Wolfhound Spanish Greyhound	Hyperekplexia 3	614618
Complex molecule disorder, general						
PIGN	AR	MS	002084-9615	Soft Coated Wheaten Terrier	Multiple congenital anomalies-hypotonia-seizures syndrome 1	614080
PLA2G6	AR	MS	002105-9615	Papillon	Infantile neuroaxonal dystrophy 1 Neurodegeneration with brain iron accumulation 2B Parkinson disease 14	256600 610217 612953
TECPR2	AR	MS	001975-9615	Spanish Water Dog	HSAN type 9 with developmental delay	615031
Complex molecule disorder, lysosomal storage disease, glycoproteinosis						
FUCA1	AR	FS	000396-9615	English Springer Spaniel	Fucosidosis	230000
MANBA	AR	MS FS	000626-9615	German Shepherd Mixed breed dog	Mannosidosis beta	248510
Complex molecule disorder, lysosomal storage disease, mucopolysaccharidosis						
ARSG	AR	MS	001503-9615	American Staffordshire Terrier	Usher syndrome type 4	618144
NAGLU	AR	FS	001342-9615	Schipperke	Mucopolysaccharidosis type 3B	252920
SGSH	AR	IFD NS	001309-9615	Dachshund New Zealand Huntaway Dog	Mucopolysaccharidosis type 3A	252900
Complex molecule disorder, lysosomal storage disease, neuronal ceroid lipofuscinosis						
ATP13A2	AR	MS SS	001552-9615	Australian Cattle Dog Tibetan Terrier	Kufor-Rakeb syndrome Spastic paraplegia 78, AR	606693 617225
CLN5	AR	NS FS	001482-9615	Australian Cattle Dog Border Collie Golden Retriever	NCL5	256731
CLN6	AR	MS	001503-9615	American Staffordshire Terrier	NCL6A NCL6B, Kufs type	601780 204300
CLN8	AR	WGD NS MS FS	001506-9615	Alpenländische Dachsbracke Australian Shepherd English Setter German Shepherd Saluki	NCL8 NCL8, northern epilepsy variant	600143 610003
CNP	ADI	FS	002591-9615	Dalmatian	Hypomyelinating Leukodystrophy 20	619071
CTSD	AR	MS	001505-9615	American Bulldog	NCL10	610127
MFSD8	AR	FS	001962-9615	Chihuahua Chinese Crested dog	NCL7 Macular dystrophy with central cone involvement	610951 616170
PPT1	AR	SS MS	001504-9615	Cane Corso Italiano Dachshund	NCL1	256730
TPP1	AR	FS	001472-9615	Dachshund	NCL2 Spinocerebellar ataxia 7, AR	204500 609270

Table 4 - continued

Gene	MOI[1]	VT[2]	OMIA# [30]	Breed	Corresponding Human diseases	OMIM# [31]
Complex molecule disorder, lysosomal storage disease, sphingolipidosis						
GALC	AR	MS IFI	000578-9615	Cairn Terrier Irish Setter West Highland White Terrier	Krabbe disease	245200
GLB1	AR	FS MS	000402-9615	Alaskan Husky Mame Shiba Portuguese Water Dog Shiba Inu	GM1-gangliosidosis type 1 GM1-gangliosidosis type 2 GM1-gangliosidosis type 3 Mucopolysaccharidosis type 4B	230500 230600 230650 253010
HEXA	AR	MS	001461-9615	Japanese Chin	GM2-gangliosidosis, several forms Tay-Sachs disease Hex A pseudodeficiency	272800
HEXB	AR	FS IFD	001462-9615	Toy Poodle Shiba Inu	Juvenile and adult forms of Sandhoff disease	268800
Complex molecule disorder, lysosomal storage disease, vacuolar hybrid type						
ATG4D	AR	MS	001954-9615	Lagotto Romagnolo	-	611340
Disorders involving energy metabolism						
MFN2	AR	IFD	002153-9615	Mixed breed dog	AR-CMTax-*MFN2* AD-CMTax-*MFN2* HSAN6a Multiple symmetric Lipomatosis with or without peripheral neuropathy	609260 617087 601152 151800

[1]MOI: mode of inheritance; ADI: autosomal dominant with incomplete penetrance, AR: autosomal recessive
[2]VT: variant type; FS: frameshift, IFD: in frame deletion, IFI: in frame insertion, MS: missense, NS: Nonsense; SL: Start codon lost, SS: splice site, WGD: Whole gene deletion; diverse variants in different breeds can lead to multiple entries.

Aim and hypothesis of the thesis

The goal of my thesis was to identify candidate disease-causing variants in rare canine neurological disorders to facilitate genetic testing. Such tests create the possibility to improve the overall health status of these breeds through targeted breeding against the identified variants. An additional aim was to provide new candidate genes for similar human disorders that do not yet have a known genetic cause. To accomplish said goals, I was working under the hypothesis that these disorders follow a simple Mendelian inheritance pattern.

Specifically, I investigated the following neurological phenotypes in detail:

1. CNS atrophy with cerebellar ataxia in Belgian Shepherd dogs
2. Coat color dilution and neurological defects in a Miniature Dachshund
3. Cerebellar degeneration and myositis complex in Nova Scotia Duck Tolling Retrievers
4. Mitochondrial Fission Encephalopathy in Bullmastiffs
5. Medium chain Acyl CoA dehydrogenase deficiency in Cavalier King Charles Spaniels
6. Congenital insensitivity to pain in mixed breed dogs
7. Exercise induced paroxysmal movement disorder in Weimaraner dogs

Results

Deletion of the *SELENOP* gene leads to CNS atrophy with cerebellar ataxia in dogs

Journal:	PLoS Genetics
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PLOS GENETICS

RESEARCH ARTICLE

Deletion of the *SELENOP* gene leads to CNS atrophy with cerebellar ataxia in dogs

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Data Availability Statement: All genome sequence data are publicly available. Accessions are given in the S2 Table. All SNV genotyping data are provided with the manucript as S1 File.

Abstract

We investigated a hereditary cerebellar ataxia in Belgian Shepherd dogs. Affected dogs developed uncoordinated movements and intention tremor at two weeks of age. The severity of clinical signs was highly variable. Histopathology demonstrated atrophy of the CNS, particularly in the cerebellum. Combined linkage and homozygosity mapping in a family with four affected puppies delineated a 52 Mb critical interval. The comparison of whole genome sequence data of one affected dog to 735 control genomes revealed a private homozygous structural variant in the critical interval, Chr4:66,946,539_66,963,863del17,325. This deletion includes the entire protein coding sequence of *SELENOP* and is predicted to result in complete absence of the encoded selenoprotein P required for selenium transport into the CNS. Genotypes at the deletion showed the expected co-segregation with the phenotype in the investigated family. Total selenium levels in the blood of homozygous mutant puppies of the investigated litter were reduced to about 30% of the value of a homozygous wildtype littermate. Genotyping >600 Belgian Shepherd dogs revealed an additional homozygous mutant dog. This dog also suffered from pronounced ataxia, but reached an age of 10 years. *Selenop*$^{-/-}$ knock-out mice were reported to develop ataxia, but their histopathological changes were less severe than in the investigated dogs. Our results demonstrate that deletion of the *SELENOP* gene in dogs cause a defect in selenium transport associated with CNS atrophy and cerebellar ataxia (CACA). The affected dogs represent a valuable spontaneous animal model to gain further insights into the pathophysiological consequences of CNS selenium deficiency.

Author summary

We studied a form of inherited ataxia in a family of Belgian Shepherd dogs that we termed CNS atrophy and cerebellar ataxia (CACA). Clinical signs were evident at 2 weeks of age and the affected puppies had to be euthanized at 4 weeks of age. The pedigree of the index family with 4 affected and 4 unaffected puppies suggested autosomal recessive inheritance.

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Using a purely positional cloning approach, we identified a complete deletion of the *SELENOP* gene as the most likely causative variant. *SELENOP* encodes selenoprotein P, a protein with multiple selenocysteine residues, which is required for the transport of selenium into the CNS. Selenium measurements in affected dogs demonstrated blood selenium levels of about 30% compared to normal control dogs. Genotyping a cohort of additional Belgian Shepherd dogs with unexplained ataxia identified another CACA case that had a relatively stable clinical condition and reached an age of 10 years. *Selenop*$^{-/-}$ knock-out mice show a related but not identical ataxia phenotype. Our finding of a *SELENOP* gene deletion in CACA affected dogs identifies a spontaneous animal model to gain further insights into the pathophysiological consequences of CNS selenium deficiency.

Introduction

Ataxias are a heterogeneous group of neurological disorders characterized by irregular and clumsy movements, decreased coordination, tremors, wide-based stance and dysarthria [1]. They are often caused by dysfunction of the cerebellum and then termed cerebellar ataxia [2]. In human medicine, numerous forms of ataxia can be differentiated based on the specific phenotype and mode of inheritance [3–5]. Pathogenic variants causing isolated or syndromic ataxia have been identified in more than fifty genes [4,5].

Dogs share many homologous inherited diseases with humans including different forms of cerebellar ataxia. Veterinary neurology and veterinary diagnostic imaging approaches for dogs made significant advances during the last years enabling comparative investigations that are expected to benefit humans, dogs and other companion animals alike. Currently, less than twenty causative genetic variants for canine forms of cerebellar ataxia are known, but this number is continually growing [6,7].

In Belgian Shepherd dogs, a missense variant in *KCNJ10* encoding a potassium channel causes spongy degeneration with cerebellar ataxia, subtype 1 (SDCA1, OMIA 002089–9615) [8,9]. The clinically similar SDCA2 in Belgian Shepherd dogs is due to a SINE insertion into *ATP1B2* encoding the beta 2 subunit of the Na^+/K^+ transporting ATPase (OMIA 002110–9615) [10]. Finally, we recently identified a variant in *YARS2* encoding the mitochondrial tyrosyl-tRNA synthetase as candidate causative variant for cardiomyopathy and juvenile mortality (CJM, OMIA 002256–9615) [11]. While CJM is not primarily a neurologic disease, it is characterized by a highly variable clinical phenotype that may also include gait abnormalities. Another form of ataxia in Belgian Shepherds was clinically and histopathologically characterized, but the underlying genetic defect remained unknown [12].

In 2020, a Belgian Shepherd breeder reported a litter with four ataxic puppies. The aim of this study was to characterize the clinical and histopathological phenotype and to identify the underlying causative genetic defect for this presumably new form of ataxia.

Results

Clinical description

A Belgian Shepherd litter of the Malinois variety with 8 offspring was investigated. One male and three female puppies presented with ataxia. The other four puppies and the parents were clinically inconspicuous (S1 Fig). Clinical examination was performed on day 27 after birth. The affected puppies showed truncal wobbling, intention tremor, general elevated muscle tone, reduced swallowing reflex, and short episodic spastic fits in variable intensity (Fig 1 and

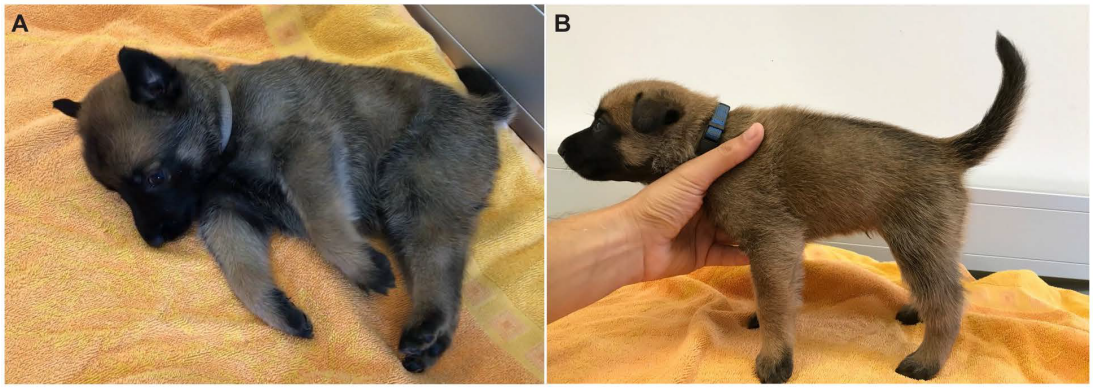

Fig 1. Clinical phenotype of the affected puppies. A Female affected puppy in lateral recumbency unable to stand up with severe tremor and episodic fits. **B** Male affected puppy with increased muscle tone of the trunk and neck as well as moderate tremor. More details of the clinical phenotype can be seen in the supplementary data (S1 Video and S2 Video).

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S1 and S2 Videos). First obvious signs were observed on post natum day 12 to 14 and developed progressively thereafter. The four affected puppies gained less body weight (mean 1.8 kg) in comparison to the four unaffected littermates (2–3 kg). They were euthanized on day 27 after birth due to animal welfare reasons.

Necropsy and histopathological examination

During necropsy no gross lesions were detectable except for mild anemia. Histologically, all four animals showed similar lesions in brain and spinal cord to variable extent. In the cerebellum, all cortical layers were atrophic with depletion of Purkinje cells and granule cells (Fig 2A and 2B). Neuroaxonal degeneration was present in midbrain, brain stem and spinal cord. Myelin content was severely diminished in the white matter of brain and spinal cord (Fig 2C and 2D). Gliosis was evident in affected regions showing activation and increased numbers of astrocytes and microglial cells, respectively (Fig 2E–2H). Based on the clinical signs and histopathological changes we propose to designate this phenotype as CNS atrophy with cerebellar ataxia (CACA, OMIA 002367-9615).

Genetic analysis

The occurrence of ataxia, muscle spasm and fits in multiple puppies from the same litter with healthy parents suggested autosomal recessive inheritance (S1 Fig). We obtained genomic DNA from all ten dogs of this family and performed parametric linkage analysis in the family as well as autozygosity analysis in the four affected puppies. A single ~52 Mb segment on chromosome 4 or roughly 2.2% of the 2.4 Gb dog genome simultaneously showed linkage with a maximum LOD score of 2.31 in the family and shared homozygous genotypes in the four available cases. The exact coordinates of the critical interval were Chr4:28,708,283–80,608,758 (S1 Table).

We sequenced the genome of one of the affected dogs and searched for private homozygous variants by comparing the variants from the case with 735 control genomes (S2 Table). The automated variant calling identified a total of 2.6 million homozygous variants in the genome of the sequenced case, but no private protein-changing variant in the critical interval (S3 Table).

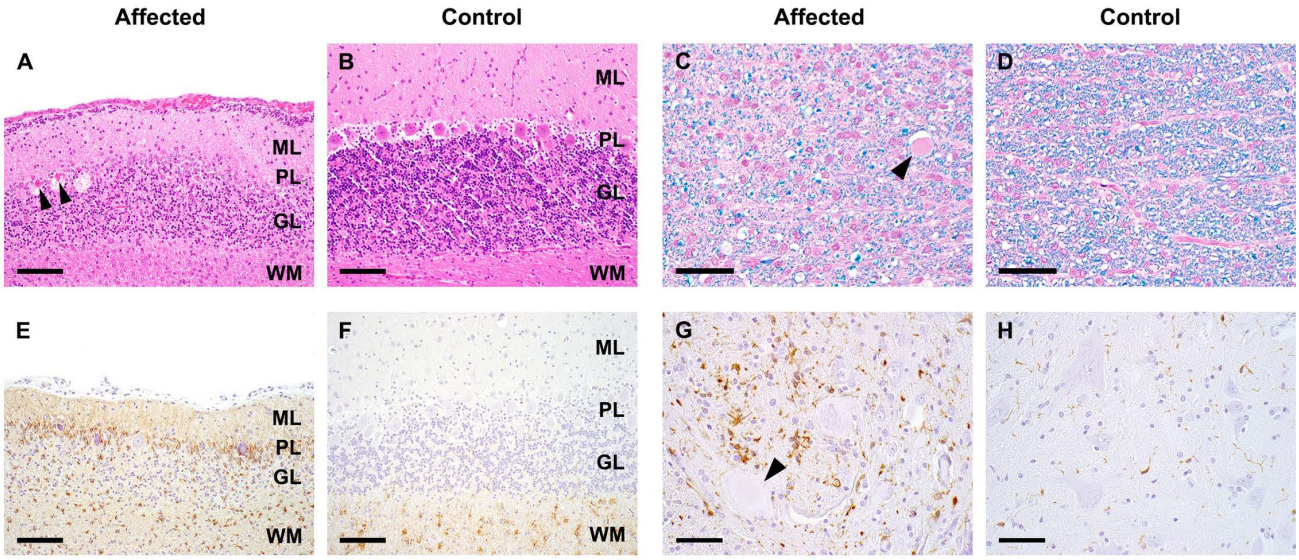

Fig 2. Histopathological examination. A, B Cerebellum: Marked attenuation of all cortical layers and necrotic Purkinje cells (arrowheads) (**A**) compared to control (**B**). ML: molecular layer, PL: Purkinje cell layer, GL: granule cell layer, WM: white matter; HE-staining, bar = 100 μm. **C, D** Spinal cord white matter: Diminished myelination (blue staining) and axonal degeneration (arrowhead) (**C**) compared to control (**D**); HE-LFB staining, bar = 50 μm. **E, F** Cerebellum: Increased numbers and activation of astrocytes particularly in Purkinje cell and granule cell layer (**E**) compared to control (**F**); GFAP-IHC, bar = 100 μm. **G, H** Spinal cord gray matter: Increased numbers and activation of microglia associated with neuronal degeneration (arrowhead) (**G**) compared to control (**H**); Iba1-IHC, bar = 50 μm.

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Our automated variant calling pipeline considered only single nucleotide variants (SNVs) and small indels. A visual search for structural variants that would have been missed during the initial analysis detected a single structural variant involving protein coding exons in the critical interval. This variant, Chr4:66,946,539_66,963,863del17,325, represents a deletion removing the complete protein coding sequence of the *SELENOP* gene. More specifically, the deletion breakpoints are located ~6.5 kb upstream of the transcription start site of *SELENOP* and within the 3'-UTR of the last exon of *SELENOP* (Fig 3). The deletion was present in homozygous state in the sequenced case and absent from 735 control genomes that were visually inspected in IGV.

We genotyped the deletion in a cohort of 668 Belgian Shepherd dogs. This cohort included the index family with four CACA-affected ataxic dogs, 13 ataxia cases with known pathogenic variants from our earlier SDCA1 and SDCA2 studies [9,10], as well as 20 other unexplained ataxia cases from the Vetsuisse biobank. The genotypes at the deletion co-segregated with the CACA phenotype as expected for a monogenic autosomal recessive mode of inheritance in the index family (S1 Fig).

None of the previously reported SDCA1 and SDCA2 cases carried the *SELENOP* deletion. However, one of the archived unexplained ataxia cases from our biobank was also homozygous for the deletion (Table 1). This dog developed ataxia as a puppy and died at 10 years of age. It reportedly came from a litter with a total of 10 puppies, of which three were euthanized due to severe ataxia at a few weeks of age. The additional case was distantly related to the four affected puppies from the index family (S1 Fig).

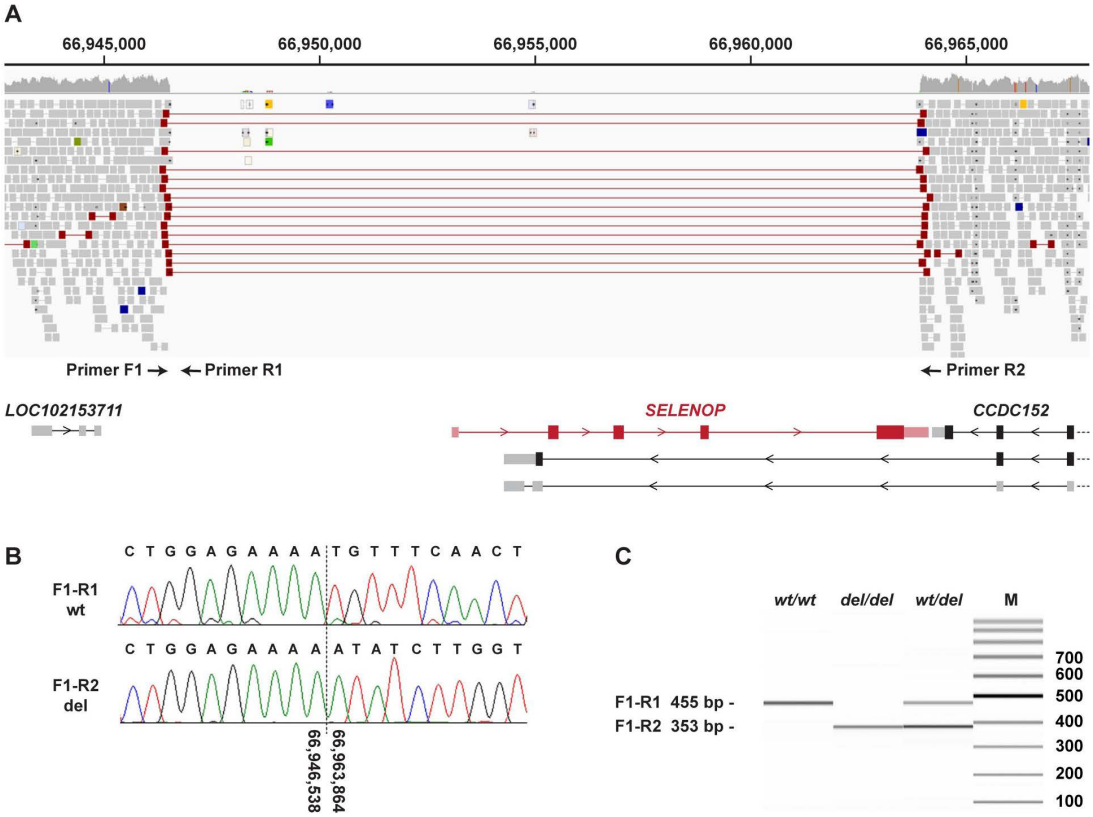

Fig 3. Details of the Chr4:66,946,539_66,963,863del17,325 variant. A WGS short-read alignments of an affected dog indicate a homozygous deletion of 17,325 bp. The position of three PCR primers for an allele-specific genotyping assay is indicated. The deletion harbors the entire coding region of *SELENOP*. The neighboring *CCDC152* gene has three annotated transcripts, of which only one is evolutionarily conserved (on top in the figure). The deletion does not extend into this canonical isoform X1 of the *CCDC152* gene (CanFam3.1, NCBI annotation release 105). The deletion affects 3'-exons of two alternative *CCDC152* transcript isoforms whose biological significance is unknown and which are not annotated in humans. **B** Sanger sequencing of the diagnostic PCR products confirmed the deletion breakpoints. **C** Fragment size analysis of the PCR amplification products obtained from genomic DNA of a healthy control (*wt/wt*), an affected dog (*del/del*) and a heterozygous carrier (*wt/del*).

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Selenium measurement

We measured total selenium content from frozen blood samples of the eight puppies. Selenium levels were reduced by ~25% in heterozygous dogs and by ~70% in homozygous dogs (P_{ANOVA} = 0.00011, Fig 4 and S4 Table).

Table 1. Association of the *SELENOP* deletion with ataxia in 668 Belgian Shepherd dogs.

Phenotype	*wt/wt*	*wt/del*	*del/del*
Ataxia cases from the investigated index family (n = 4)	-	-	4
Ataxia cases affected by SDCA1 or SDCA2 (n = 13)	13	-	-
Previously unexplained ataxia cases (n = 20)	19	-	1
Controls (n = 631)[a]	593	38	-

[a]These dogs do not include any of the 735 control genomes, which were used for the initial analysis.

https://doi.org/10.1371/journal.pgen.1009716.t001

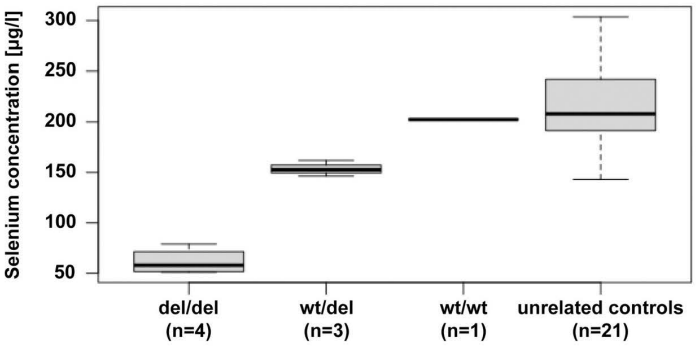

Fig 4. Selenium concentrations in the blood of dogs with different *SELENOP* genotypes. The values were derived from the eight puppies of the index family (first three columns) and a cohort of 21 unrelated control dogs (right column).

https://doi.org/10.1371/journal.pgen.1009716.g004

Discussion

In this study, we identified a ~17 kb genomic deletion on chromosome 4 harboring almost the entire *SELENOP* gene in Belgian Shepherd dogs with CACA. *SELENOP* encodes selenoprotein P, which functions in storing and transporting selenium [13–18]. Selenoprotein P is the only known protein in vertebrates containing multiple selenocysteine residues [19]. It is primarily synthesized in the liver and secreted into the blood stream [13]. Incorporation of selenium into selenoprotein P prevents the toxic effects of free selenium and the majority of plasma selenium is bound in selenoprotein P [13]. Studies in *Selenop*$^{-/-}$ knockout mice demonstrated that selenoprotein P is required for the transport of selenium into the brain and other organs [15–18]. Both blood and brain selenium levels were decreased by approximately 70%-80% in *Selenop*$^{-/-}$ mice [15]. Selenoprotein P delivery through the blood-brain barrier is mediated by the low density lipoprotein receptor-related protein 8 (LRP8) [20]. Additionally, astrocytes are able to newly synthesize selenoprotein P [21], which is thought to be transferred to neurons via LRP8. Selenoprotein P can be degraded via the lysosomal pathway to provide selenium for the *de novo* synthesis of other selenoproteins [22]. Selenium is required in several enzymes such as the glutathione peroxidases and thioredoxin reductases that protect cells from oxidative stress [13]. In addition to its established role in selenium transport, it was hypothesized that selenoprotein P might also be directly involved in signaling processes in the brain [23,24].

Similar to the findings in *Selenop*$^{-/-}$ mice [15], blood selenium levels in homozygous mutant dogs were reduced to about 30% of the value in a homozygous wildtype littermate. Unfortunately, due to a lack of suitable tissue samples, we could not directly measure the selenium level in brain of the affected puppies. However, previous studies in *Selenop*$^{-/-}$ mice demonstrated that their selenium levels in brain were reduced to a similar relative level as those in plasma [15].

Selenop$^{-/-}$ mice develop ataxia and neurodegeneration when fed low selenium diets [15,16]. Neurological function can stabilize, but not return to normal, when adequate selenium in the diet is reinstituted [17]. *Selenop*$^{-/-}$ mice that were fed a diet containing $\geq$0.25 mg selenium/kg did not develop neurological dysfunction [17]. *Selenop*$^{-/-}$ mice show axonal and neuronal degeneration in thalamus, mesencephalon, brainstem and cerebellar white matter [23]. Lesions in these regions are present at weaning and progress when animals are fed low selenium diets. Further lesions develop in the somatosensory cortex and striatum associated with astrogliosis 12 days post weaning upon low selenium diets [25].

Histological lesions present in mesencephalon, cerebellar white matter and brain stem in the *SELENOP*$^{-/-}$ dogs were similar to the lesions in *Selenop*$^{-/-}$ mice [25,26]. However, in contrast to the phenotype described in *Selenop*$^{-/-}$ mice, dogs showed cerebellar atrophy with attenuation of all cerebellar layers and widespread loss of Purkinje cells and granule cells. Cerebellar lesions have been reported in mice with a deletion of the *Trsp* gene encoding selenocysteinyl tRNA [27]. *Trsp*$^{-/-}$ mice have a completely abrogated neuronal selenoprotein biosynthesis and show cerebellar hypoplasia/atrophy with Purkinje cell death and decreased granule cell proliferation similar to the homozygous *SELENOP*$^{-/-}$ dogs. Similar cerebellar lesions and a wide spread loss of myelin have also been reported in human patients with progressive encephalopathy caused by hypomorphic variants in the *SEPSECS* gene encoding the O-phosphoseryl-tRNA:selenocysteinyl-tRNA synthase, the key enzyme in the sole biosynthetic route to selenocysteine [28].

To the best of our knowledge, there are no reports of human patients with neurological dysfunction caused by genetic variants in *SELENOP* or other spontaneous *SELENOP* mutants in any mammalian species. The deletion in the investigated dogs removed the entire coding sequence of *SELENOP* and may thus assumed to be a true null allele. Consequently, the dogs investigated in this study provide a valuable spontaneous animal model to further study the effects of a complete deficiency of selenoprotein P.

While this study demonstrates the value of clinical veterinary genetics to identify potential spontaneous domestic animal models, we also have to acknowledge some limitations of our study. The studied dogs were privately owned and represented clinical cases. Therefore, the initial sample collection was necessarily limited and precluded e.g. a more detailed characterization of selenium levels in different tissues. A more detailed characterization of the phenotype and possibly modifying genes under controlled dietary selenium intake might further enhance the value of this animal model in the future. However, this will require targeted matings of carrier dogs or the generation of genome-edited *SELENOP* deficient dogs in an experimental setting.

The clinical phenotype in *SELENOP*$^{-/-}$ dogs was highly variable in severity. Two out of four affected puppies of the index family showed short fits with tonic muscle spasm of the trunk, neck, and limbs. Intention tremor was observed in all four puppies. Ability to walk was variable, too. One dog remained in lateral or sternal recumbency and any attempt to walk resulted in immediate falling. The remaining three puppies were able to walk with moderate to severe ataxia as well as activity and stress depended extensor muscle spasms. The retrospectively identified fifth case reached an age of 10 years and apparently showed a milder and more or less stable clinical condition. It seems possible that the amount of dietary selenium intake has an influence on the clinical variability. In light of the findings in *Selenop*$^{-/-}$ mice, feeding a diet with high and constant selenium levels might be beneficial to affected puppies, if their disease is diagnosed early enough.

Conclusions

This study identified a deletion of *SELENOP* in Belgian Shepherd dogs with autosomal recessive CNS atrophy and cerebellar ataxia (CACA). Our findings enable genetic testing, which can be used to avoid the unintentional breeding of further affected puppies. The studied dogs might serve as a translational spontaneous animal model to better understand the pathophysiological consequences of *SELENOP* deficiency.

Materials and methods

Ethics statement

All examinations were performed after obtaining written informed owner´s consent according to ethical guidelines of the University of Veterinary Medicine Vienna. Blood samples were

collected with the approval of the Cantonal Committee for Animal Experiments (Canton of Bern; permit BE 71/19). All animal experiments were done in accordance with local laws and regulations.

Necropsy, histology, immunohistochemistry

A full necropsy was performed on all four puppies and samples of brain, spinal cord, sciatic nerves, striated muscle and visceral organs (heart, lung, thymus, liver, kidney, spleen, gastrointestinal tract, pancreas, adrenals and coeliac ganglion) were fixed in 4% neutral buffered formalin for histologic examination. Organ, muscle and nerve samples as well as coronary sections of brain and cervical, thoracic, and lumbar spinal cord were embedded in paraffin and cut at a thickness of 2 μm. All sections were stained with hematoxylin and eosin (HE) and slides examined by light microscopy under a BX 53 Olympus microscope. Furthermore, brain and spinal cord were stained with a combination of HE and Luxol fast blue (HE-LFB) to determine myelination. Brain and spinal cord lesions were compared to juvenile control dogs without CNS lesions, a female Husky puppy with a weight of 1.9 kg and a female French Bulldog puppy with a weight of 2 kg, respectively.

For the detection of astrocytes and microglia immunohistochemistry (IHC) was performed using primary antibodies against glial fibrillary acidic protein (GFAP, Dako, cat# Z0334, dilution 1:10000) and ionized calcium-binding adapter molecule 1 (Iba1, Wako, cat# 019–19741, dilution 1:1250). IHC was performed automatically in an autostainer (Lab Vision AS 360, Thermo Scientific, Freemont, USA) using a secondary antibody formulation conjugated to an enzyme-labelled polymer (Bright Vision Goat anti Rabbit HRP, ImmunoLogic, cat# DPVR 110 HRP). Di-amino-benzidine was used as chromogen and sections were counterstained with hematoxylin.

Animal selection for genetic analysis

This study was conducted with 668 Belgian Shepherd dog samples. In addition to the Belgian Shepherd index family consisting of four affected and four unaffected full siblings as well as their parents, the genetic study included 658 dogs from different European countries that were donated to the Vetsuisse biobank. 20 of those dogs represented ataxia cases with unknown genetic etiology. Another 13 ataxia cases were due to known pathogenic variants for SDCA 1 or SDCA2. For the remaining 625 dogs, we had no reports of specific neurologic diseases. These were designated as population controls.

DNA extraction

Genomic DNA was extracted from EDTA blood and hair samples according standard methods using the Maxwell RSC Whole Blood DNA and the Maxwell RSC Blood DNA Kits in combination with the Maxwell RSC instrument (Promega, Dübendorf, Switzerland).

Linkage analysis and homozygosity mapping

Genotype data for the ten members of the index family were obtained with Illumina CanineHD BeadChips by Geneseek/Neogen (S1 File). For all dogs, the call rate was > 95%. Using PLINK v1.9 [29], markers that were non-informative, located on the sex chromosomes or missing in any of the 10 dogs, had Mendel errors or a minor allele frequency < 0.05, were removed. The final pruned dataset contained 95,207 markers. To analyze the data for parametric linkage, an autosomal recessive inheritance model with full penetrance, a disease allele frequency of 0.5 and the Merlin software [30] were applied.

For homozygosity mapping, the genotype data for the four affected dogs were used. Markers that were missing in one of the four cases, markers on the sex chromosomes and markers with Mendel errors in the family were excluded. The --homozyg and --homozyg-group options in PLINK were used to search for extended regions of homozygosity > 1 Mb. The output intervals were matched against the intervals from linkage analysis in Excel spreadsheets to find overlapping regions (S1 Table). All positions correspond to the CanFam3.1 reference genome assembly.

Whole genome resequencing

An Illumina TruSeq PCR-free library with ~500 bp insert size was prepared from one affected dog (MA509). We collected 169 million 2 x 150 bp paired-end reads on a NovaSeq 6000 instrument (17x coverage). Mapping to the CanFam3.1 reference genome assembly was performed as described [31]. The sequence data were deposited under study accession PRJEB16012 and sample accession SAMEA7198602 at the European Nucleotide Archive.

Variant calling

Variant calling was performed using GATK HaplotypeCaller [32] in gVCF mode as described [31]. For private variant filtering we used control genome sequences from nine wolves and 726 dogs. These genomes were either publicly available [33] or produced during other previous projects (S2 Table). To predict the functional effects of the called variants, SnpEff [34] software together with the CanFam3.1 reference genome assembly and NCBI annotation release 105 was used.

Allele specific PCR and genotyping

We designed an allele-specific PCR with 3 primers for the targeted genotyping of the Chr4:66,946,539_66,963,863del17,325 variant. PCR was performed for 30 cycles using the Qiagen Multiplex PCR kit (Qiagen, Hilden, Germany) in a 10 μl reaction containing 10 ng genomic DNA, 5 pmol primer F1 5'-TGG CAA ATT AAG ATC ACC AGA A-3', and 2.5 pmol each of primers R1 5'- TGA TGA ATT TTT CCC TGA GAC A-3' and R2 5'- CCA CAT TTG GTC AAT TAT GCA C-3'. Product sizes were analyzed on a 5200 Fragment Analyzer (Agilent, Basel, Switzerland). The wildtype allele yielded an amplicon of 455 bp (F1-R1), whereas the deletion allele gave rise to a product of 353 bp (F1-R2).

Sanger sequencing

After treatment with exonuclease I and alkaline phosphatase, PCR 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).

Selenium measurements

Frozen EDTA blood samples were thawed and centrifuged at 2000 x g for 10 min. Selenium concentrations were measured in the supernatant using atomic absorption spectroscopy (AAS) (ZEEnit 650P, Analytic Jena, Jena, Germany). The intra- and interassay coefficients of variation in dog samples were 1.04–4.58% and 4.74–5.12%, respectively. One-way analysis of variance (ANOVA) was used to test for significant differences between dogs with different genotypes. The significance threshold was set to $p = 0.05$.

Supporting information

S1 File. SNV microarray genotypes of 10 Belgian Shepherd dogs (ped- and map-file).
(ZIP)

S1 Fig. Pedigree of Belgian Shepherd dogs with ataxia.
(PDF)

S1 Table. Linkage and homozygosity data.
(XLSX)

S2 Table. Whole genome sequence accessions of 727 dogs and 9 wolves.
(XLSX)

S3 Table. Private variants in the genome of the sequenced affected puppy.
(XLSX)

S4 Table. Results of the selenium measurements.
(XLSX)

S1 Video. Affected puppy showing truncal wobbling and intention tremor.
(MOV)

S2 Video. Affected puppy unable to stand, showing mild tremor and severely uncoordinated motion.
(MOV)

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MYO5A frameshift variant in a miniature Dachshund with coat color dilution and neurological defects resembling human Griscelli syndrome type 1

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MYO5A Frameshift Variant in a Miniature Dachshund with Coat Color Dilution and Neurological Defects Resembling Human Griscelli Syndrome Type 1

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Abstract: A 1-month-old, female, smooth-haired miniature Dachshund with dilute color and neurological defects was investigated. The aim of this study was to characterize the clinical signs, histopathological changes and underlying genetic defect. The puppy had visible coat color dilution and was unable to hold its head on its own or to remain in a stable prone position for an extended period. Histopathological examination revealed an accumulation of clumped melanin and deposition of accumulated keratin within the hair follicles, accompanied by dermal pigmentary incontinence. These dermatological changes were compatible with the histopathology described in dogs with an *MLPH*-related dilute coat color. We sequenced the genome of the affected dog and compared the data to 795 control genomes. *MYO5A*, coding for myosin VA, was investigated as the top functional candidate gene. This search revealed a private homozygous frameshift variant in *MYO5A*, XM_022412522.1:c.4973_4974insA, predicted to truncate 269 amino acids (13.8%) of the wild type myosin VA protein, XP_022268230.1:p.(Asn1658Lysfs*28). The genotypes of the index family showed the expected co-segregation with the phenotype and the mutant allele was absent from 142 additionally genotyped, unrelated Dachshund dogs. *MYO5A* loss of function variants cause Griscelli type 1 syndrome in humans, lavender foal in horses and the phenotype of the *dilute* mouse mutant. Based on the available data, together with current knowledge on other species, we propose the identified *MYO5A* frameshift insertion as a candidate causative variant for the observed dermatological and neurological signs in the investigated dog.

Keywords: *Canis lupus familiaris*; animal model; neurology; dermatology; precision medicine

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1. Introduction

Griscelli syndrome (GS, OMIM #214450) represents a group of rare diseases in humans with a monogenic autosomal recessive mode of inheritance. Griscelli et al. reported the first patients in 1978 [1]. The authors described two patients with partial albinism, frequent pyogenic infections and acute episodes of fever, neutropenia and thrombocytopenia. Three distinct types of GS, with the common feature of silvery grey hair, pigmentary dilution of the skin and melanin clumps within hair shafts, have been described in human medicine. In GS type 1, variants in the *MYO5A* gene, coding for myosin VA, cause severe primary neurologic impairment in addition to the aforementioned dermatological changes [2–4] (OMIM #214450). GS type 2 is caused by variants in *RAB27A* and characterized by pigment dilution in combination with immunological abnormalities [5] (OMIM #607624). GS type 3 is due to variants in *MLPH* and comprises an isolated pigment dilution without any defects in the nervous or immune systems [6] (OMIM #609227).

The pigment dilution in GS is the result of impaired melanosome transport within melanocytes [7]. Perinuclear melanosomes are transported along actin filaments to the

periphery and tethered via myosin VA to the dendritic tips of the cell [8]. In the absence of wild type myosin VA in GS type I patients, this binding step cannot occur and melanosomes are not correctly recruited to the cell periphery. As a result, the transfer of melanosomes from melanocytes to keratinocytes and into growing hair shafts is hindered [9].

Furthermore, myosin VA-mediated transport is also required in neurons and especially in the cerebellum. There, the motor protein myosin VA pulls the endoplasmic reticulum into the dendritic spines of Purkinje neurons [10]. The endoplasmic reticulum is important for Ca^{2+} storage and essential for intracellular Ca^{2+} signaling [11,12]. Consequently, missing myosin VA-mediated transport reduces synaptic plasticity and leads to a neurological phenotype [10] (OMIA 001501-9796).

Analogous to the human phenotype, *Myo5a* loss-of-function variants are responsible for the *dilute* mouse mutant [13] and the *dilute-lethal* rat mutant [14]. Moreover, a single base pair deletion in exon 30 of the *MYO5A* gene causes lavender foal syndrome in horses, which is phenotypically similar to human GS type 1 [15].

This study was initiated after a smooth-haired miniature Dachshund with a striking coat color dilution and neurological deficits was reported. The goal of the study was to characterize the phenotype of the puppy and to investigate a possible underlying causative genetic defect.

2. Materials and Methods

2.1. Clinical Examination

One affected 1-month-old smooth-haired miniature Dachshund puppy was investigated. Clinical examination was carried out by a veterinary clinician. EDTA blood samples from the patient, five unaffected full siblings and their parents were collected for genomic DNA isolation.

2.2. Necropsy, Histopathology, and Immunohistochemistry

A full necropsy of the affected puppy was performed and routinely collected organ samples were fixed in 10% neutral-buffered formalin. Tissue samples were trimmed and embedded in paraffin. For histological examination, organ samples were cut into 4–5 µm thick sections and subsequently stained with hematoxylin and eosin (HE). Immunohistochemistry was performed to visualize axonal damage by using an antibody directed against amyloid precursor protein. After deparaffinization, blocking of endogenous peroxidase and antigen retrieval, signal detection was subsequently achieved to incubation with a primary and a secondary antibody by using the avidin-biotin-peroxidase complex and 3′-diaminobenzidintetrahydrochlorid. Afterwards, sections were counterstained with Mayer's hemalum.

2.3. Control Samples for Genetic Analyses

In addition to the investigated family, 142 blood samples from Dachshunds, which had been donated to the Vetsuisse Biobank, were used. They represented unrelated population controls without reports of a similar phenotype.

2.4. DNA Extraction

Genomic DNA was isolated from the 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 was prepared. We collected 296 million 2 × 150 bp paired-end reads on a NovaSeq 6000 instrument (32.8 × overage). Mapping to the CanFam3.1 reference genome assembly was performed as described [16]. A graphical overview of the bioinformatics pipeline is shown in Figure S1. The sequence data were deposited under study accession PRJEB16012 and sample accession SAMEA8157168 at the European Nucleotide Archive.

2.6. Variant Calling

Variant calling was performed using GATK HaplotypeCaller [17] in gVCF mode as described [16]. For private variant filtering, we used control genome sequences from 786 dogs from genetically diverse breeds and 9 wolves. These genomes either were publicly available [18] or produced during other previous projects (Table S1). To predict the functional effects of the called variants, SnpEff [19] software, together with the CanFam3.1 reference genome assembly and NCBI Annotation Release 105, was used (Figure S1).

2.7. Gene Analysis

Numbering within the canine *MYO5A* gene corresponds to the NCBI RefSeq accession numbers XM_022412522.1 (mRNA) and XP_022268230.1 (protein).

2.8. PCR and Sanger Sequencing

The *MYO5A*:c.4973_4974insA variant was genotyped by direct Sanger sequencing of PCR amplicons. The PCR product was amplified from genomic DNA using AmpliTaq-Gold360Mastermix (Thermo Fisher Scientific, Waltham, MA, USA), together with primers 5′-AGA GAA GTG GGC CTT CTG GT-3′ (Primer F) and 5′-GAG CTT CCA AGC CAC TTC TG-3′ (Primer R). After treatment with exonuclease I and alkaline phosphatase, PCR 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 (Gene Codes, Ann Arbor, MI, USA).

3. Results

3.1. Clinical Examination

In a litter of smooth-haired miniature Dachshunds with two male and four female offspring, one of the female puppies had a striking dilute coat color. This female puppy was presented at four weeks of age to the veterinarian; it had to be kept separate from its siblings for a few days prior to presentation because its siblings had started to nibble at it. The mother, however, was still taking care of the puppy as usual. The owner reported that the puppy did not show any urge to seek the proximity of its siblings and did not whimper when separated. When it was put in the run with its siblings, it fell directly on its side and then rowed with the upper front leg. It managed to roll over to the other side but did not manage to hold a normal prone position. It hardly reacted to environmental stimuli.

On examination, the puppy weighed 0.82 kg and its external features were normally developed; no abnormalities compared to its siblings were found, except for the visible coat color dilution (Figure 1). Upon handling, the puppy could not maintain an upright head position compared to her littermates. The head had to be held at all times, even during feeding. Palpatory tension in the neck and shoulder area was present, but not enough to support the head or coordinate its movement. The puppy was euthanized due to the severity of the clinical phenotype.

3.2. Gross, Histopathological, and Immunohistochemical Findings

Grossly, the fur of the puppy was diffusely light red in color. Other macroscopical changes included a V-shaped bend, 45 degrees dorsocaudally, of the distal portion of the spleen and minor agonal changes in different organs. Histopathological examination revealed multifocal accumulation of melanin and deposition of clumped keratin in the follicular epithelium of haired skin (Figure 2). Furthermore, a mild, multifocal, dermal pigmentary incontinence was observed (Figure 2). Immunohistochemical investigation for the presence of amyloid precursor protein as a marker for axonal damage did not reveal alterations within the central nervous system.

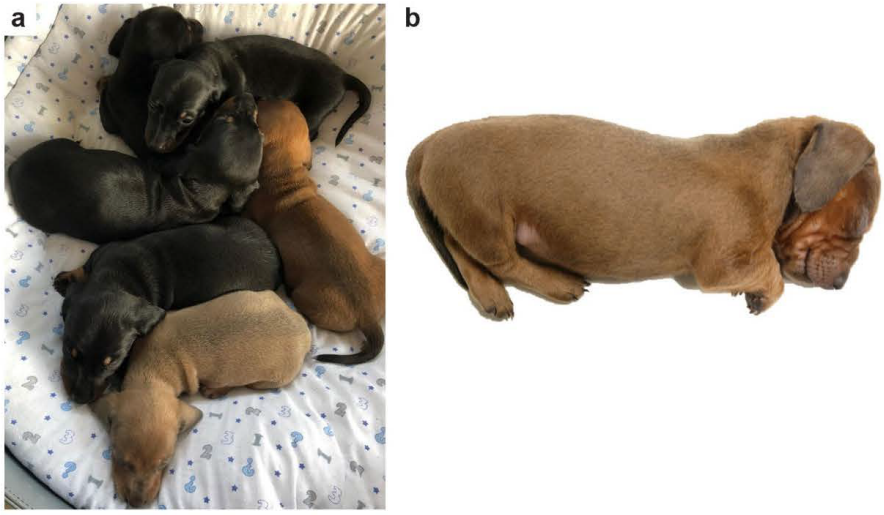

Figure 1. Coat color dilution phenotype. (**a**) The photo shows the entire litter consisting of six puppies. Four of the non-affected puppies had black and tan coat colors. The affected puppy at the bottom of the photo had a dilute red color, which is much lighter than the standard red color of its non-affected sibling on the right. (**b**) Larger photo from the affected puppy alone.

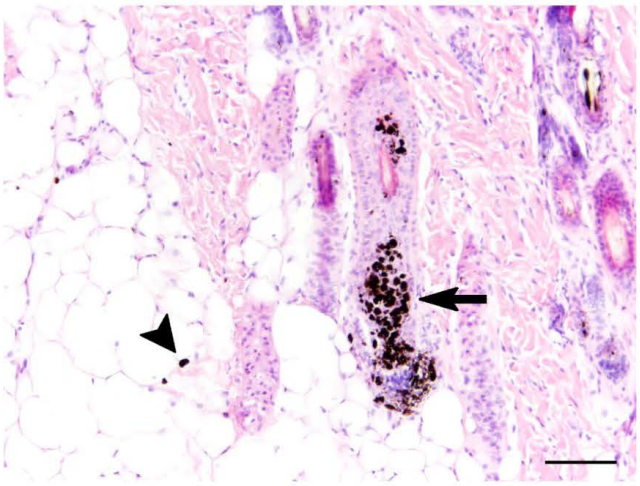

Figure 2. Histopathology of the skin. Multifocally, epithelial cells of hair follicles displayed an accumulation of clumped melanin (arrow) and deposition of accumulated keratin. In the dermis, a mild, multifocal pigmentary incontinence (arrowhead) was present. Hematoxylin and eosin stain (bar = 100 µm).

3.3. Genetic Analysis

We sequenced the genome of the affected dog and searched for homozygous variants in the candidate gene *MYO5A* that were not present in the genome sequences of the 786 control dogs and nine wolves (Table 1 and Supplementary Tables S1 and S2).

Table 1. Results of variant filtering in the affected miniature Dachshund dog against 795 control genomes.

Filtering Step	Homozygous Variants
All variants in the affected miniature Dachshund	2,698,983
Private variants	1688
Protein-changing [1] private variants	12
Protein-changing [1] private variants in *MYO5A*	1

[1] "Protein-changing" variants have a SnpEff predicted moderate or high impact [19]. These include missense, nonsense, frameshift, and splice site variants among others.

This analysis identified a single homozygous private protein-changing variant in the investigated candidate gene. The variant, an insertion of an adenine within the coding sequence, can be designated as Chr30:18,004,551_18;004,552insT (CanFam3.1 assembly). It is a frameshift variant, XM_022412522.1:c.4973_4974insA, predicted to truncate 269 codons encoding the C-terminus of the wild type myosin VA protein, XP_022268230.1:p.(Asn1658Lysfs*28). We did not investigate whether any mutant protein is expressed or whether the premature stop codon caused by the frameshift variant leads to nonsense-mediated decay of the transcript. We confirmed the presence of the frameshift variant in *MYO5A* by Sanger sequencing and genotyped the index family, as well as the 142 control Dachshund dogs (Figure 3).

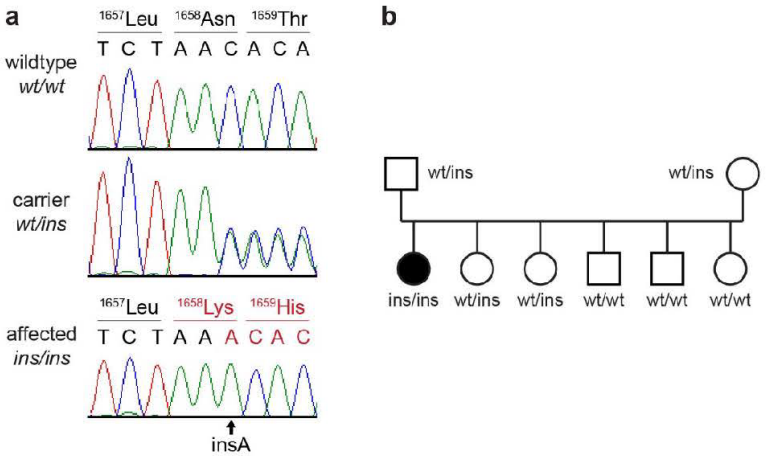

Figure 3. Details of the *MYO5A*:c.4973_4974insA variant. (**a**) Representative Sanger sequencing chromatograms of dogs with the three different genotypes. A homozygous insertion of a single adenine is visible in the affected dog. (**b**) The genotypes in the Dachshund family showed the expected co-segregation with the phenotype in the index family.

The case was homozygous for the mutant allele, while none of the 142 unrelated control dogs carried this allele. The genotypes at the insertion co-segregated with the investigated phenotype in the family as expected for a monogenic autosomal recessive mode of inheritance (Figure 3b). The parents, as well as two healthy siblings of the affected dog, carried the variant allele in a heterozygous state.

4. Discussion

In dogs, coat color dilution that affects the distribution of both eumelanin and pheomelanin has so far only been explained by variants in the *MLPH* gene [20–22]. Initially, the presented case herein caught the breeders' attention only because of the dilution of the coat color. Neurological signs subsequently became apparent. The puppy resembled two previously described cases of Rhodesian Ridgebacks that also showed pigment dilution in

combination with neurological signs [23]. However, the genetic basis for the phenotype in the Rhodesian Ridgebacks was not investigated.

The clinical phenotype of the described smooth-haired miniature Dachshund showed a striking resemblance to previously-reported cases of GS type 1 in humans and lavender foal syndrome in horses [4,15].

Histopathological alterations of the skin matched what has been described in dogs with *MLPH*-related dilute coat color [24]. Loss of function of *MLPH* leads to isolated coat color dilution without neurological signs. However, *MLPH* mutant dogs are predisposed to developing color dilution alopecia [24,25]. Alopecia was not observed in the present case; however, color dilution alopecia typically manifests between 4 months and 3 years of age.

The neurological impairment was not associated with morphologic changes at the gross or histopathological level. However, molecular changes such as impairment of Ca^{2+} storage are not necessarily accompanied by morphologic alterations.

The misshaped spleen probably represents an autonomous congenital malformation with minor clinical relevance. However, a correlation with the observed *MYO5A* variant cannot be excluded with certainty.

The *MYO5A* gene encodes for myosin VA, an intracellular organelle transport protein with important functions in the dendritic spines of melanocytes and Purkinje cells [7,10]. Our analysis revealed a private homozygous frameshift variant, *MYO5A*:c.4973_4974insA, in the studied Dachshund. The frameshift led to a premature termination codon and was predicted to truncate the coding sequence for the C-terminus of the wild type myosin VA protein, including parts of the highly conserved globular tail domain. This domain represents the cargo-binding domain of myosin VA [26]. Therefore, the identified *MYO5A* frameshift variant may be assumed to cause a complete loss-of-function allele. Together with the knowledge of the effects of *MYO5A* variants in other species, these data suggest *MYO5A*:c.4973_4974insA as a candidate causative genetic variant for the phenotype in the investigated puppy.

The phenotype of the affected dog closely resembles the phenotype of human patients with Griscelli syndrome type I. The identification of a candidate causative variant enables genetic testing and the detection of heterozygous carriers so that further unintentional breeding of affected dogs can be prevented. If similarly affected Dachshunds should appear, the genetic test can also be used to quickly confirm the suspected diagnosis.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/genes12101479/s1, Figure S1: Mapping and variant calling pipeline; Table S1: Public Genomes; Table S2: Private homozygous variants.

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

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SLC25A12 missense variant in Nova Scotia Duck tolling Retrievers affected by cerebellar degeneration - myositis complex (CDMC)

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SLC25A12 Missense Variant in Nova Scotia Duck Tolling Retrievers Affected by Cerebellar Degeneration—Myositis Complex (CDMC)

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Abstract: We investigated two litters of distantly related Nova Scotia Duck Tolling Retrievers (NSDTR), of which four puppies were affected by cerebellar signs with or without neuromuscular weakness. The phenotype was termed cerebellar degeneration—myositis complex (CDMC). We suspected a heritable condition and initiated a genetic analysis. The genome of one affected dog was sequenced and compared to 565 control genomes. This search yielded a private protein-changing *SLC25A12* variant in the affected dog, XM_038584842.1:c.1337C>T, predicted to result in the amino acid change XP_038440770.1:(p.Pro446Leu). The genotypes at the variant co-segregated with the phenotype as expected for a monogenic autosomal recessive mode of inheritance in both litters. Genotyping of 533 additional NSDTR revealed variant allele frequencies of 3.6% and 1.3% in a European and a North American cohort, respectively. The available clinical and biochemical data, together with current knowledge about *SLC25A12* variants and their functional impact in humans, mice, and dogs, suggest the p.Pro446Leu variant is a candidate causative defect for the observed phenotype in the affected dogs.

Keywords: *Canis lupus familiaris*; neurology; seizure; N-acetyl aspartic acid; aralar; precision medicine; animal model

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1. Introduction

In dogs, advances in genetic research have led to the discovery of diverse genetic variants causing neurologic phenotypes [1]. Many of these inherited neurologic diseases are restricted to one of approximately 300 recognized dog breeds. In the Nova Scotia Duck Tolling Retriever (NSDTR) breed, a degenerative encephalopathy with presumed autosomal recessive inheritance has previously been described (OMIA 002055-9615) [2]. Affected dogs show episodes of marked movements during sleep, increased anxiety, noise phobia, and gait abnormalities. MRI and post mortem examination showed symmetrical changes in the cerebellar caudate nuclei of affected dogs [2]. The causal genetic variant for the described disease has not been reported so far.

The current investigation was initiated after the presentation of an NSDTR litter, in which a single puppy showed a combination of cerebellar and neuromuscular signs that were not consistent with the previously published degenerative encephalopathy in the NSDTR [2]. Hence, a new inherited disease was suspected. During our study, a second, distantly related litter with three additional affected dogs was identified and included in the analysis. The goal of this project was to grossly characterize the clinical phenotype and to investigate a possible genetic cause.

2. Materials and Methods

2.1. Clinical and Pathological Examination

A clinical and neurological examination, complete blood count, and serum biochemistry was performed in all 4 affected dogs. Electromyography (EMG), MRI of the head, cervical cerebrospinal fluid (CSF) exam, PCR analyses on infectious diseases, and muscle and nerve biopsies were performed in one dog with cerebellar signs combined with neuromuscular weakness. One dog was euthanized, and a full necropsy was performed, including investigation of skeletal muscles and the central and peripheral nervous system.

2.2. Animal Selection for Genetic Analysis

The study was conducted with a total of 563 NSDTR samples. Thirty dogs belonged to two extended families of European origin with a total of four affected puppies. The remaining 533 dogs originated from sample donations to the Vetsuisse biobank (380 dogs of European origin) and the UC Davis Bannasch biobank (153 dogs of mostly North American origin). These 533 dogs were designated as population controls without reports of a similar specific neurologic disease.

2.3. DNA Extraction

Genomic DNA was extracted from EDTA blood samples according to standard methods using the Maxwell RSX Whole Blood DNA kit in combination with the Maxwell RSC instrument (Promega, Dübendorf, Switzerland) or using the Gentra Puregene DNA purification extraction kit (Qiagen, Valencia, CA, USA).

2.4. Whole Genome Sequencing

An Illumina TruSeq PCR-free DNA library with ~420 bp insert size was prepared from one affected dog. We collected 260 million 2 × 150 bp paired end reads on a NovaSeq 6000 instrument (29.1× coverage). Mapping and alignment to the UU_Cfam_GSD_1.0 reference genome assembly were performed as described [3]. The sequence data were deposited under the study accession PRJEB16012 and the sample accession SAMEA10644737 at the European Nucleotide Archive.

2.5. Variant Calling

Variant calling was performed using GATK HaplotypeCaller [4] in gVCF mode as described [3]. To predict the functional effects of the called variants, SnpEff [5] software together with NCBI annotation release 106 for the UU_Cfam_GSD_1.0 genome reference assembly was used. For variant filtering, we used 565 control genomes from dogs of different breeds (Table S1).

2.6. Gene Analysis

We used the UU_Cfam_GSD_1.0 dog reference genome assembly and NCBI annotation release 106. Numbering within the canine *SLC25A12* gene corresponds to the NCBI RefSeq accession numbers XM_038584842.1 (mRNA) and XP_038440770.1 (protein).

2.7. Database Searches and In Silico Functional Predictions

The Genome Aggregation Database (gnomAD) [6] and Online Mendelian Inheritance in Man and Animals databases [7,8] were searched for corresponding variants in the

human and domestic animal *SLC25A12* genes. Additionally, the Mouse Genome Informatics Web Site was screened for corresponding phenotypes in mice [9]. PredictSNP [10], PROVEAN [11], and MutPred2 [12] were used to predict biological consequences of the discovered candidate protein variant. The human and canine SLC25A12 proteins both comprise 678 amino acids, of which 663 (98%) are identical between dogs and humans.

2.8. PCR and Sanger Sequencing

Primers 5′-TCA TCC CTG TGA GCT CCT CT-3′ (Primer F) and 5′-GAA GCC TGG TTT CCA CAT TC-3′ (Primer R) were used for the generation of an amplicon containing the *SLC25A12*:c.1337C > T variant. PCR products were amplified from genomic DNA using AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific, Reinach, Switzerland). Direct Sanger sequencing of the PCR amplicons on an ABI 3730 DNA Analyzer (Thermo Fisher Scientific, Reinach, Switzerland) was performed after treatment with exonuclease I and alkaline phosphatase. Sanger sequences were analyzed using the Sequencher 5.1 software (Gene Codes, Ann Arbor, MI, USA).

3. Results

3.1. Clinical History and Examination

Age of onset of neurological signs was between 10 weeks and 6 months. Clinical abnormalities were restricted to the neuromuscular system in all four dogs. Neurological examination showed generalized ataxia and hypermetria, which was more pronounced in the pelvic limbs in all four dogs. Intentional head tremor was present in one dog. In two dogs, generalized neuromuscular weakness became apparent after 1 month, characterized by exercise intolerance, episodic collapse, stiff gait, and bunny hopping. Hopping was delayed in four limbs in three dogs, menace responses were absent in one dog, and decreased withdrawal reflexes were found in four limbs of three dogs.

3.2. Ancillary Diagnostic Investigations

Blood examination showed increases in serum creatine kinase concentrations in all four dogs (between 3 and 25 times greater than the upper reference limit). An EMG showed mild spontaneous activity in peripheral limb muscles. MRI of the brain in the same dog showed bilateral symmetrical lesions in the cerebellum and multifocal lesions in the masticatory muscles. CSF analysis and PCR analyses (Toxoplasma, Neospora, Distemper virus, Bartonella, and Tick-borne encephalitis) were all within normal limits. Muscle and nerve biopsies showed a fiber-invasive lymphohistiocytic myositis without evidence of intracellular infectious agents on histology and tissue PCR. The only other abnormality seen on postmortem examination was severe cerebellar nuclear degeneration.

Based on the available clinical and diagnostic findings, we tentatively termed the phenotype of the four affected dogs cerebellar degeneration—myositis complex (CDMC).

3.3. Genetic Analysis

At the beginning of the genetic analysis, we only had access to a single affected puppy and its close relatives. We obtained blood samples from the affected puppy, five unaffected littermates, and both parents. Pedigree analysis revealed a common founder of both parents and was thus suggestive of a monogenic autosomal recessive mode of inheritance of the investigated trait (Figure S1).

The genome of the affected dog was sequenced, and we searched for private homozygous variants that were not present in the genome sequences of 565 control dogs of diverse breeds (Tables 1, S1 and S2).

Table 1. Results of variant filtering in the affected dog against 565 control genomes.

Filtering Step	Homozygous Variants	Heterozygous Variants
All variants in the affected dog	2,535,158	3,669,142
Private variants	2057	10,901
Protein-changing private variants	6	88

We prioritized the resulting variants according to functional knowledge of the altered genes in the OMIM database. This process revealed a single homozygous candidate variant for the observed neurologic/neuromuscular phenotype, located in *SLC25A12*, coding for the solute carrier family 25-member 12 protein. The detected missense variant, XM_038584842.1:c.1337C>T, is predicted to result in an amino acid substitution in the protein, XP_038440770.1:(p.Pro446Leu). On the genomic level, the variant can be described as Chr36:16,504,064G>A (UU_Cfam_GSD_1.0 assembly). The other five homozygous private protein-changing variants were located in genes that are not known to have specific functions in the neuromuscular system (Table S2).

The proline to leucine substitution at position 446 of the SLC25A12 protein was predicted to be pathogenic and deleterious by several in silico prediction tools (PredictSNP probability for pathogenicity: 87%; MutPred2 score: 0.911; PROVEAN score: −9.639). The other five private homozygous missense variants were located in genes not known to cause phenotypes similar to our case in humans, mice, or domestic animals.

We confirmed the presence of the *SLC25A12* variant in a homozygous state in the affected dog via Sanger Sequencing (Figure 1).

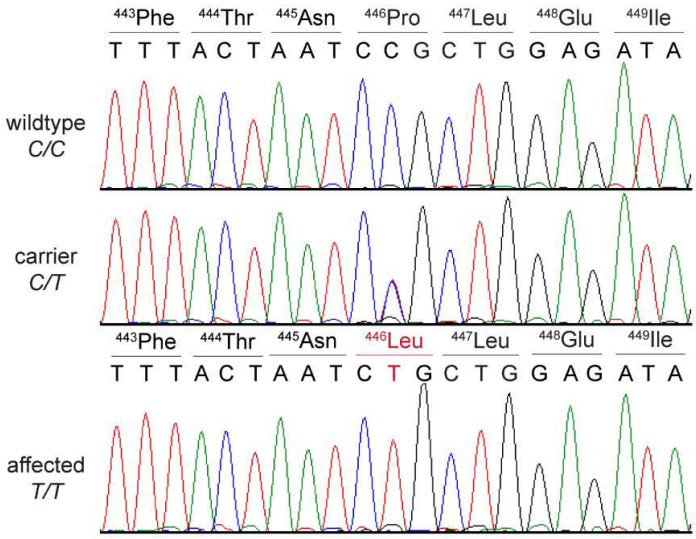

Figure 1. Details of the *SLC25A12*:c.1337C>T variant (p.Pro446Leu). Representative electropherograms of dogs with the three different genotypes are shown. The amino acid translations of the wild type and mutant alleles are indicated.

At this point of the genetic analysis, we became aware of the second litter of NSDTRs, in which three dogs showed a phenotype comparable with the index case. We obtained DNA samples of the second family and genotyped the variant in both families. The genotypes at the variant co-segregated with the CDMC phenotype, as expected for a monogenic autosomal recessive mode of inheritance in both families. Common ancestors were identified for three of four parents of the affected litters (Figure S1).

We genotyped 533 additional NSDTR population controls that were sampled independently of our investigation into CDMC. None of these dogs carried the mutant T-allele in a homozygous state. The carrier frequencies were 7.1% in the European and 2.7% in the North American cohort. The corresponding mutant allele frequencies were 3.6% and 1.3%, respectively (Table 2).

Table 2. Association of the genotypes at *SLC25A12*:c.1337C>T variant with cerebellar degeneration—myositis complex (CDMC) in 563 NSDTR dogs.

Phenotype	C/C	C/T	T/T
CDMC cases (n = 4)	-	-	4
Non-affected family members (n = 26)	12	14	-
Control dogs from Europe (n = 380)	353	27	-
Control dogs from North America (n = 153)	149	4	-

4. Discussion

In this study, we provide an initial clinical characterization of a new disease with monogenic autosomal recessive inheritance in NSDTR dogs. We tentatively termed the disease cerebellar degeneration—myositis complex (CDMC). Further investigations to characterize the phenotype in more detail, including its progression over time, are ongoing.

Whole-genome sequencing of an affected dog identified a homozygous private protein-changing variant, *SLC25A12*:c.1337C>T. Genotypes at this variant co-segregated with the phenotype in two families, and no other homozygous dog with a similar phenotype was identified in whole-genome sequence data of 565 control dogs of different breeds. Additionally, two different cohorts of breed-matched control dogs were genotyped and found to be free of additional homozygous dogs. Thus, the total number of investigated controls is in excess of 1000 dogs.

The *SLC25A12* gene encodes solute carrier family 25 member 12, which has also been termed mitochondrial aspartate–glutamate carrier 1 or aralar. The SLC25A12 protein is located in the inner mitochondrial membrane [13,14]. There, it has the function of a Ca^{2+} activated aspartate–glutamate carrier and is part of the malate–aspartate shuttle, a major NADH redox unit [15].

Slc25a12$^{-/-}$ mice showed growth defects, generalized tremors, postnatal lethality, impaired motor coordination, and CNS dysmyelination [16]. The clinical signs in knockout mice were associated with decreased myelin lipid synthesis and significant reduction in aspartate and NAA levels in the brain. Similar to *Slc25a12*$^{-/-}$ mice, variants in the human *SLC25A12* cause the rare developmental and epileptic encephalopathy 39 (OMIM #612949) [17]. This disease is characterized by global developmental delay, seizures, hypotonia with poor motor function, and hypomyelination on brain imaging. Analogous to the process in knockout mice, the myelination defect is speculated to reflect the impaired supply of NAA to oligodendrocytes caused by SLC25A12 deficiency [18].

In dogs, an *SLC25A12*:p.Leu349Pro missense variant with experimentally verified impairment of transport activity and documented changes in the skeletal muscle metabolome leading to a proinflammatory milieu and increased oxidative stress has been reported in Dutch Shepherd dogs with inflammatory myopathy (OMIA 002294-9615) [19]. The reported inflammatory myopathy was comparable between the affected Dutch Shepherd dogs (L349P) [19] and our findings in affected NDSTRs (P446L). In the Dutch Shepherd dogs, generalized progressive weakness was reported as the main clinical finding. Most of the detailed histological and biochemical analyses in the Dutch Shepherd study were performed on muscle biopsies. A detailed clinical neurological examination was not performed [19]. It is therefore not clear whether there are true differences in the neurological phenotype between the two dog breeds that might be due to the different missense variants and/or additional breed-specific modifiers. Given that the clinical phenotype in affected NDSTRs was quite variable, with some dogs showing more pronounced muscular weakness and other dogs showing generalized ataxia and hypermetria, we think that the existing data do

not yet allow conclusive definition of the genotype–phenotype correlation in dogs. This underscores the need for more comprehensive clinical and histopathological investigations of a sufficiently large number of affected dogs.

5. Conclusions

The clinical, radiological and pathological presentation, together with genetic findings and existing knowledge of *SLC25A12* variants in humans, dogs, and mice, suggest the *SLC25A12*:p.Pro446Leu as a candidate causative variant for CDMC in NSDTR dogs. The identification of a candidate causative variant enables genetic testing for early diagnosis and the detection of heterozygous carriers, thus preventing the further unintentional breeding of affected dogs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes13071223/s1, Table S1: Whole-genome sequence accessions of 566 dogs; Table S2: Private homozygous variants in the genome of the sequenced affected dog; Figure S1: Pedigree of NSDTR dogs with CDMC.

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Informed Consent Statement: Written informed consent was obtained from the owners of the dogs participating in this study.

Data Availability Statement: The accessions for the sequence data 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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Mitochondrial fission factor (*MFF*) frameshift variant in Bullmastiffs with mitochondrial fission encephalopathy

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RESEARCH ARTICLE

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Mitochondrial fission factor (MFF) frameshift variant in Bullmastiffs with mitochondrial fission encephalopathy

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Abstract

Familial cerebellar ataxia with hydrocephalus in Bullmastiffs was described almost 40 years ago as a monogenic autosomal recessive trait. We investigated two young Bullmastiffs showing similar clinical signs. They developed progressive gait and behavioural abnormalities with an onset at around 6 months of age. Neurological assessment was consistent with a multifocal brain disease. Magnetic resonance imaging of the brain showed intra-axial bilateral symmetrical focal lesions localised to the cerebellar nuclei. Based on the juvenile age, nature of neurological deficits and imaging findings, an inherited disorder of the brain was suspected. We sequenced the genome of one affected Bullmastiff. The data were compared with 782 control genomes of dogs from diverse breeds. This search revealed a private homozygous frameshift variant in the *MFF* gene in the affected dog, XM_038574000.1:c.471_475delinsCGCTCT, that is predicted to truncate 55% of the wild type MFF open reading frame, XP_038429928.1: p.(Glu158Alafs*14). Human patients with pathogenic *MFF* variants suffer from 'encephalopathy due to defective mitochondrial and peroxisomal fission 2'. Archived samples from two additional affected Bullmastiffs related to the originally described cases were obtained. Genotypes in a cohort of four affected and 70 unaffected Bullmastiffs showed perfect segregation with the disease phenotype. The available data together with information from previous disease reports allow classification of the investigated *MFF* frameshift variant as pathogenic and probably causative defect of the observed neurological phenotype. In analogy to the human phenotype, we propose to rename this disease 'mitochondrial fission encephalopathy (MFE)'.

KEYWORDS
animal model, *Canis lupus familiaris*, dog, mitochondrion, neurology, precision medicine, veterinary medicine

INTRODUCTION

Familial cerebellar ataxia with hydrocephalus in Bullmastiff dogs was initially described in 1983 (OMIA 002551-9615; Carmichael et al., 1983). Affected puppies of one litter showed ataxia, impaired vision and behavioural abnormalities consisting of hysterical behaviour, difficulty to train, backing compulsively when called and lifting a foreleg while eating. The clinical signs were macroscopically associated with symmetrical hydrocephalus and histologically with cerebellar vacuolisation, gliosis and axonal degeneration. It was hypothesised that the observed phenotype was due to a genetically determined metabolic disturbance with an autosomal recessive mode of inheritance (Carmichael et al., 1983). Four years later, the same author described a total of six closely related Bullmastiff dogs with similar clinical signs (Carmichael, 1987). Additionally, abnormally shaped mitochondria in axons of affected brain regions were found in electron micrographs, leading to the conclusion that the dogs suffered from a neuronal abiotrophy caused by an intrinsic metabolic effect (Carmichael, 1987). Magnetic resonance imaging (MRI) demonstrated changes within the deep cerebellar nuclei in additional affected dogs (Johnson et al., 2001).

The current study was prompted by reports of two cases of Bullmastiffs from different litters showing behavioural and gait abnormalities. We hypothesised that the dogs might be affected by the same disease that was first described almost 40 years ago and initiated a genetic investigation.

MATERIALS AND METHODS

Clinical examinations

An 8-month-old, female entire Bullmastiff (case no. 1) and a 6-month-old, male entire Bullmastiff (case no. 2) underwent physical and neurological examinations. Additional laboratory investigations included A complete blood cell count, serum biochemistry (including electrolyte levels) and serology for canine distemper virus (case no. 2 only), *Toxoplasma gondii* and *Neospora caninum*. Additionally, a cisternal cerebrospinal fluid sample (case no. 2) was submitted for protein levels and total and differential cell counts, and for this dog also urine was submitted to an external human testing laboratory for organic acid analysis.

Magnetic resonance imaging examination

Magnetic resonance imaging of the brain was performed on case no. 2 with a 1.5 Tesla machine (1.5T Magnetom, Siemens) and included T_2-weighted sagittal, dorsal and transverse views, with the following transverse views: fluid attenuated inversion recovery and gradient echo T_2*-, T_1-weighted pre- and post-contrast sequences (gadopentate dimeglumine; Magnevist, Bayer Schering Pharma AG).

Post-mortem examination

Owing to clinical progression, case no. 2 was euthanised and a post-mortem examination was performed. Representative samples of heart, lungs, liver and brain were stained with hematoxylin and eosin and examined histologically.

Genetic analyses

Animal samples

The study included samples from 74 Bullmastiffs. We used EDTA blood samples from the two contemporary clinical cases (case nos 1 and 2) and formalin-fixed paraffin-embedded (FFPE) tissue samples from two additional affected dogs seen in 1996 (case nos 3 and 4) that were reportedly siblings and closely related to the previously reported cases (Carmichael, 1987). The FFPE samples of the historical cases were kindly provided by the pathology service of the School of Veterinary Medicine of the University of Glasgow. EDTA blood samples of 70 additional Bullmastiffs that had been donated to the Vetsuisse Biobank were used as controls. They consisted of five unaffected close relatives of case no. 2 and 65 population controls without known relationships to any of the cases.

DNA extraction

Genomic DNA was isolated from EDTA blood with the Maxwell RSC Whole Blood Kit using a Maxwell RSC instrument (Promega). The same instrument was used for DNA isolation from FFPE tissue samples with the Maxwell RSC DNA FFPE kit.

Whole-genome sequencing

An Illumina TruSeq PCR-free DNA library with a ~390 bp insert size of case no. 1 was prepared. We collected 241 million 2×150 bp paired-end reads on a NovaSeq 6000 instrument (27.5×coverage). Mapping and alignment were performed as described in Jagannathan et al. (2019). The sequence data were deposited under the study accession no. PRJEB16012 and sample accession no. SAMEA8157163 at the European Nucleotide Archive.

Variant calling and filtering

Variant calling was performed using GATK HaplotypeCaller (McKenna et al., 2010) in gVCF mode as described by Jagannathan et al. (2019). To predict the functional effects of the called variants, snpeff software (Cingolani et al., 2012) together with NCBI annotation release 106 for the UU_Cfam_GSD_1.0 genome reference

assembly was used. For variant filtering, we used 782 genetically diverse control dog genomes representing 175 different dog breeds and 21 mixed-breed dogs (Table S1).

Private variants in the case were identified by a hard filtering approach of the vcf-file using a Python script. Private variants were required to have a homozygous alternate genotype in the case (1/1) and a homozygous reference (0/0) or missing genotype (./.) in the controls. The control cohort did not contain any Bullmastiffs; therefore the probability of having a heterozygous carrier in the control group was estimated as negligible.

Variants with SnpEff-predicted 'high' or 'moderate' impact were classified as protein-changing variants. These include missense, nonsense, frameshift and splice site variants.

Gene analysis

We used the UU_Cfam_GSD_1.0 dog reference genome assembly and NCBI annotation release 106. Numbering within the canine *MFF* gene corresponds to the NCBI RefSeq accession nos XM_038574000.1 (mRNA) and XP_038429928.1 (protein).

PCR and sanger sequencing

The candidate variant *MFF*:c.471_475delinsCGCTCT was genotyped by direct Sanger sequencing of PCR amplicons. A 380 bp (or 381 bp in case of the mutant allele) PCR product was amplified from genomic DNA using AmpliTaqGold360Mastermix (Thermo Fisher Scientific) and the primers 5′-CTCCCTTTCTTTGTGCCTCA-3′ and 5′-CGAGAGGATAATGCTACTGGAAA-3′. A smaller PCR product of 101 bp size (or 102 bp in case of the mutant allele) was amplified from FFPE-derived DNA with the primers 5′-TCCCTTTCTTTGTGCCTCAC-3′ and 5′-CTCTGACCAGCTGTCCGTTT-3′. After treatment with exonuclease I and alkaline phosphatase, amplicons were sequenced on an ABI 3730 DNA Analyzer (Thermo Fisher Scientific). Sanger sequences were analysed using the SEQUENCHER 5.1 software (GeneCodes).

RESULTS

Clinical investigations

An 8-month-old, female entire (case no. 1) and a 6-month-old, male entire (case no. 2) Bullmastiff presented with a chronic and progressive history of uncoordinated gait and abnormal behaviour including barking at imaginary objects and no interaction with other dogs, and decreased vision. The clinical signs were noticed by the owners a couple of months before presentation and had been gradually progressing. Some of the littermates were reported to be showing similar clinical signs. Physical examination was mainly unremarkable, apart from case no. 1 exhibiting scuffing wounds on the dorsal aspect of both pelvic limbs. Neurological examination showed abnormal mentation and a wide-base stance (Video S1). The gait was abnormal with hypermetria affecting all limbs. Menace response was absent or decreased with normal pupillary light reflexes in both cases. Additionally, spontaneous and intermittent horizontal nystagmus was observed in case no. 2. Postural reactions (mainly hopping responses) were delayed in all limbs. No pain was elicited on spinal palpation and segmental spinal reflexes were normal. The neurological examination was consistent with a disease involving the cerebral cortex, leading to an abnormal behaviour and decreased vision, and the vestibulo-cerebellum, leading to cerebellar ataxia and nystagmus.

Complete blood cell count and serum biochemistry including electrolyte levels showed a mild increase in cholesterol and creatinine kinase in case no. 2. Serologies for canine distemper virus, *Toxoplasma gondii* and *Neospora caninum* were negative. Cerebrospinal fluid and urine organic acids analyses for case no. 2 were within normal range. MRI of the brain showed intra-axial bilateral symmetrical focal T_2-weighted hyperintense, T_1-weighted isointense and no contrast-enhancing lesions localised to the cerebellar nuclei. Additionally, the sulci of the cerebral cortex appeared widened with an increased amount of cerebrospinal fluid and the lateral ventricles were bilaterally enlarged (Figure 1).

Case no. 1 died owing to progression of clinical signs. No further information regarding the age of the dog and circumstances of death are available. Case no. 2 was euthanised at 10 months of age owing to progression of the clinical signs. Post-mortem examination confirmed spongy vacuolar changes in the cerebellar nuclei as previously reported (Carmichael et al., 1983). Mild to moderate dilation of the left cardiac ventricle, less so the right ventricle, compatible with a dilated cardiomyopathy, was observed, in turn considered the cause for the mild hydrothorax, ascites and hydropericardium present, as well as pulmonary congestion, hepatic centrilobular fibrosis and mild tunica media hyperplasia of the small arteries in the septal myocardium.

Genetic analysis

At the beginning of the genetic analysis, we only had access to a single affected dog (case no. 1). As the clinical and neurological findings of this case resembled previously described cases in Bullmastiffs, we hypothesised that that the phenotype in the affected dog was due to a genetic defect with a monogenic autosomal recessive mode of inheritance. We sequenced the genome of case no. 1 and identified private homozygous variants that were not present in the genome sequences of 782 control dogs of diverse breeds (Table 1 and Tables S1 and S2).

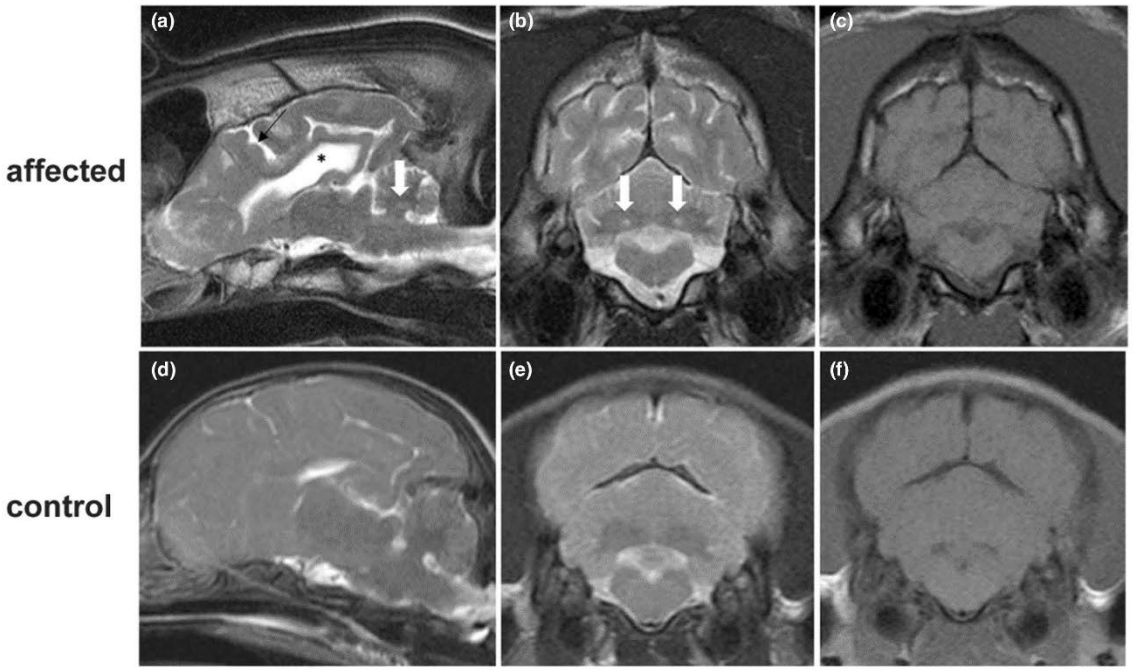

FIGURE 1 Magnetic resonance imaging of case no. 2 and an age-matched control. (a) T_2-weighted parasagittal image of the brain showing widening of the cerebral sulci (black arrow), lateral ventricle enlargement (black asterisk) and hyperintensity of the cerebellar nuclei (white arrow). Transverse *T2*-weighted image (T2WI) (b) and *T1*-weighted image (T1WI) (c) at the level of the cerebellum showing bilateral and symmetric T2WI intra-axial hyperintensities at the level of the cerebellar nulcei (white arrows); these lesions are isointense on T1WI (c). (d) T_2-weighted parasagittal of an age-matched control with no widening of the cerebral sulci or enlargement of the lateral ventricle for comparison. Transverse T2WI (e) and T1WI (f) at the level of the cerebellum of an age-matched control with no lesions.

TABLE 1 Homozygous variants in case no. 1, filtered against 782 control genomes

Filtering step	Variants
All variants in the affected dog	2859546
Private variants	591
Protein-changing private variants	8

The resulting variants were prioritised according to functional knowledge of the affected genes. The automated bioinformatic analysis identified two independent homozygous private protein-changing variants at neighbouring nucleotides in the functional candidate gene *MFF* encoding the mitochondrial fission factor. Visual inspection of the short read alignments in the affected region revealed that the two initially separately called variants actually represented just one single insertion–deletion variant. This variant, XM_038574000.1:c.471_475delinsCGCTCT, leads to a frameshift and is predicted to truncate 209 codons or roughly 55% of the wild type MFF open reading frame, XP_038429928.1:p.(Glu158Alafs*14). On the genomic level, the variant can be described as Chr-25:40,322,999_40,323,003delinsCGCTCT (UU_Cfam_GSD_1.0 assembly). The other six private protein-changing variants were not located in genes known to cause similar phenotypes in humans, mice or domestic animals.

We confirmed the presence of the *MFF* variant in a homozygous state in case no. 1 by Sanger sequencing (Figure 2a). At this point of the genetic analysis, we became aware of the second case with a very similar phenotype to the index case. We also genotyped the second case and five of its unaffected close relatives (Figure 2b).

Furthermore, we identified FFPE archived samples from two additional young Bullmastiff siblings seen in 1996 (cases no. 3 and 4) with similar clinical presentation and closely related to previously reported cases (Carmichael, 1987). We genotyped the *MFF* variant in the additional cases and a cohort of unrelated control Bullmastiffs (Table 2). These analyses confirmed a perfect genotype–phenotype association. All four available cases were homozygous mutant, one obligate carrier was heterozygous and none of the 70 unaffected dogs were homozygous for the mutant allele. The carrier frequency in the unrelated control cohort was 12/65 (18%).

DISCUSSION

We describe two contemporary cases of Bullmastiffs with progressive behavioural and gait abnormalities exhibiting a similar clinical phenotype to dogs reported almost 40 years ago (Carmichael, 1987; Carmichael et al., 1983).

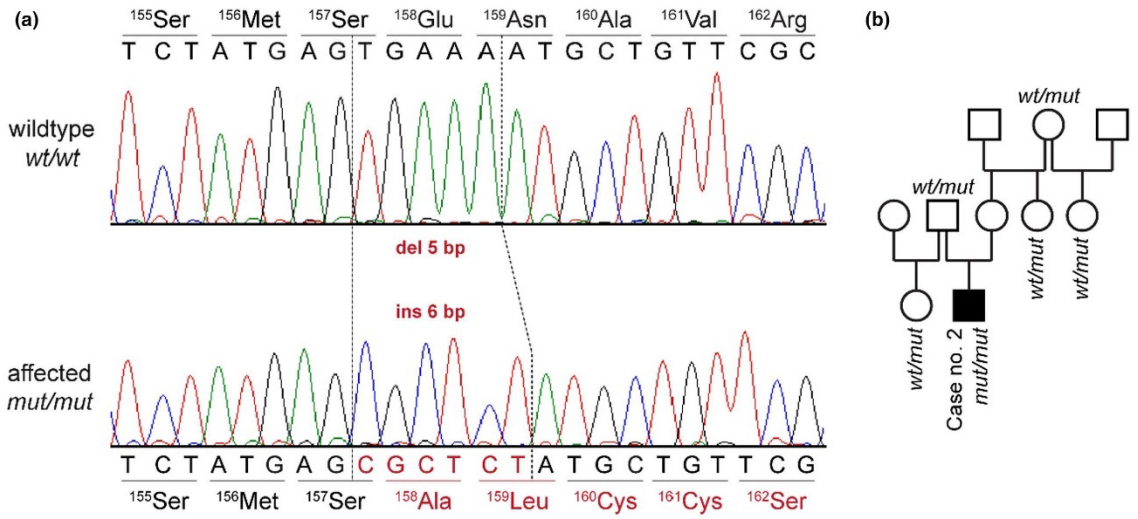

FIGURE 2 Details of the *MFF*:C.471_475delinsCGCTCT variant. (a) Representative electropherograms of a control and an affected dog are shown. The amino acid translations of the wild type and mutant alleles are indicated. (b) Pedigree of case no. 2. The genotypes at the *MFF*:C.471_475delinsCGCTCT variant are indicated for sampled dogs. The sire of case no. 2 was confirmed to be heterozygous as expected for an obligate carrier of a monogenic autosomal recessive trait.

TABLE 2 Association of the genotypes at the *MFF*:C.471_475delinsCGCTCT variant with ataxia and hydrocephalus in 74 bullmastiffs

Phenotype	Wt/Wt	Wt/Mut	Mut/Mut
Affected Bullmastiffs ($n = 4$)	–	–	4
Non-affected relatives of case no. 2 ($n = 5$)	–	5	–
Unrelated control Bullmastiffs ($n = 65$)	53	12	–

In this study, we provide a plausible candidate disease causing variant, *MFF*:c.471_475delinsCGCTCT. The *MFF* gene encodes the mitochondrial fission factor (Gandre-Babbe & van der Bliek, 2008). Mitochondria are subject to a constant series of fusion and fission in order to regulate subcellular processes and their own homeostasis. Thus, mitochondrial morphology is dynamically regulated at all times (Meyer et al., 2017).

Loss of function variants in the *MFF* gene result in a cellular phenotype with elongated tubular mitochondria because the organelles can still fuse while fission is blocked (Gandre-Babbe & van der Bliek, 2008; Otera et al., 2010). The resulting hyperfused mitochondria may be resistant to autophagosomal degradation leading to the accumulation of damaged and abnormally functioning mitochondria as well as reactive oxygen species (Meyer & Bess, 2012; Twig et al., 2008).

These mitochondrial defects result in a specific clinical phenotype in human patients with *MFF* variants, which is termed 'encephalopathy due to defective mitochondrial and peroxisomal fission 2' (OMIM# 617086, Shamseldin et al., 2012; Koch et al., 2016; Nasca et al., 2018; Panda et al., 2020). Changes to mitochondrial morphology with those exhibiting elongated tubular shapes have been observed both in human patients (Koch et al., 2016) and in affected dogs (Carmichael, 1987). Furthermore, bilateral symmetrical MRI changes affecting the basal and cerebellar nuclei have been reported in humans with *MFF* variants (Nasca et al., 2018) sharing some similarities with the MRI changes reported in the present and previously reported dogs (Johnson et al., 2001). Further studies will be necessary to understand the predilection for specific nuclei which appears to vary between species and/or variants.

$Mff^{-/-}$ knockout mice die prematurely with a mean life span of 13 weeks. The murine phenotype is characterised by a severe dilated cardiomyopathy in combination with prominent neuromuscular defects (Chen et al., 2015). Interestingly, the post-mortem examination of one of the affected dogs showed mild to moderate dilated cardiomyopathy. Further studies will be needed to determine the degree and significance of the cardiac changes observed in dogs with this *MFF* variant.

The canine *MFF*:c.471_475delinsCGCTCT variant is predicted to truncate 55% of the open reading frame of the wild type *MFF* transcript, XP_038429928.1:p.(Glu158Alafs*14). We assume that the premature stop codon results in a complete loss of function. The mutant allele was absent in whole genome sequence data of 782 control dogs from genetically diverse breeds. Three additional affected Bullmastiffs with strikingly similar phenotypes carried the same homozygous mutant genotype, while 70 non-affected Bullmastiff dogs were found to be free of the homozygous mutant genotype. The pedigrees

of the affected dogs strongly suggested monogenic autosomal recessive inheritance (Carmichael, 1987). The phenotype with documented changes in mitochondrial morphology (Carmichael, 1987) is highly specific. Extrapolating the established guidelines for the interpretation of sequence variants in human medicine (Richards et al., 2015) to dogs, these arguments allow the *MFF*:c.471_475delinsCGCTCT variant to be classified as pathogenic.

The clinical and pathological features of this disease that closely resemble the human phenotype, together with the genetic findings, suggest MFF:c.471_475delinsCGCTCT as compelling causative variant. To the best of our knowledge, the affected dogs represent the first domestic animals described with an *MFF*-related disease. In analogy to the human phenotype, we tentatively propose to rename the phenotype seen in Bullmastiffs 'mitochondrial fission encephalopathy (MFE)'. Our results enable genetic testing, which can be used to avoid the unintentional breeding of further MFE-affected dogs. In addition, the studied dogs might serve as a spontaneous large animal model to further the understanding of mitochondrial fission dysfunction in human patients.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The accession nos for the sequence data reported in this study are listed in Table S1.

ETHICAL APPROVAL

All animal experiments were performed according to local regulations. The dogs in this study are privately owned and were examined with the consent of the owners. The Cantonal Committee for Animal Experiments approved the collection of blood samples from control dogs that were used in this study (Canton of Bern; permit BE 71/19).

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SUPPORTING INFORMATION

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ACADM frameshift variant in Cavalier King Charles Spaniels with medium-chain acyl-CoA dehydrogenase deficiency

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Article

ACADM Frameshift Variant in Cavalier King Charles Spaniels with Medium-Chain Acyl-CoA Dehydrogenase Deficiency

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Abstract: A 3-year-old, male neutered Cavalier King Charles Spaniel (CKCS) presented with complex focal seizures and prolonged lethargy. The aim of the study was to investigate the clinical signs, metabolic changes and underlying genetic defect. Blood and urine organic acid analysis revealed increased medium-chain fatty acids and together with the clinical findings suggested a diagnosis of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency. We sequenced the genome of the affected dog and compared the data to 923 control genomes of different dog breeds. The *ACADM* gene encoding MCAD was considered the top functional candidate gene. The genetic analysis revealed a single homozygous private protein-changing variant in *ACADM* in the affected dog. This variant, XM_038541645.1:c.444_445delinsGTTAATTCTCAATATTGTCTAAGAATTATG, introduces a premature stop codon and is predicted to result in truncation of ~63% of the wild type MCAD open reading frame, XP_038397573.1:p.(Thr150Ilefs*6). Targeted genotyping of the variant in 162 additional CKCS revealed a variant allele frequency of 23.5% and twelve additional homozygous mutant dogs. The acylcarnitine C8/C12 ratio was elevated ~43.3 fold in homozygous mutant dogs as compared to homozygous wild type dogs. Based on available clinical and biochemical data together with current knowledge in humans, we propose the *ACADM* frameshift variant as causative variant for the MCAD deficiency with likely contribution to the neurological phenotype in the index case. Testing the CKCS breeding population for the identified *ACADM* variant is recommended to prevent the unintentional breeding of dogs with MCAD deficiency. Further prospective studies are warranted to assess the clinical consequences of this enzyme defect.

Keywords: *Canis lupus familiaris*; dog; neurology; metabolism; fatty acid disorder; seizure; precision medicine

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1. Introduction

Medium-chain fatty acids (MCFAs) are monocarboxylic acids with a hydrocarbon chain of six to twelve carbon atoms in length (C6–C12) [1]. They either are taken up in the gastrointestinal tract, or are derived through β-oxidation of long-chain fatty acids, catalyzed by the enzyme very long chain acyl CoA dehydrogenase [2,3]. Through further β-oxidation, now mediated by medium-chain acyl-CoA dehydrogenase (MCAD), MCFAs serve as energy source for the body [4].

In humans, mitochondrial fatty acid β-oxidation disorders are a heterogeneous group of inherited diseases with a wide range of clinical presentation [1]. MCAD deficiency is the most frequently diagnosed disease in this group [5], for which widespread

screening in European newborns has shown that approximately 1/8000–1/20,000 are affected by MCAD deficiency [6–8].

In MCAD deficient patients, unmetabolized MCFAs accumulate in different tissues [9,10]. As a result of the impaired β-oxidation, affected people are not able to produce sufficient energy out of ketone bodies during times of extended fasting or acute stress [5]. They present to the emergency room with an acute crisis of hypoketotic hypoglycemia. Clinically, such a crisis manifests as 'Reye-like symptoms', which consist of vomiting, lethargy, hepatomegaly and liver dysfunction that may eventually result in encephalopathy, seizures and even coma and death [11].

MCAD deficiency is caused by variants in the *ACADM* gene (OMIM #201450) [12]. Many distinct disease-causing variants have been identified in different human populations [8,13]. A targeted mouse model for MCAD deficiency has been developed [14], but otherwise the disease has rarely been observed in animals. In a single Cavalier King Charles Spaniel (CKCS), MCAD deficiency was suspected based on the results of blood and urine organic acid levels [15], but the causative genetic variant was not investigated in this dog.

This study was initiated after the presentation of a CKCS with a history of complex focal seizures and laboratory findings strongly resembling human MCAD deficiency. The goal of the study was to characterize the clinical and metabolic phenotype and to investigate a possible underlying causative genetic defect.

2. Materials and Methods

2.1. Clinical Examination and Investigations

A single, 3-year-old, male neutered CKCS dog originating in the United Kingdom was investigated. Both parents were reportedly healthy, the health status of siblings was unknown. The dog was presented to the Small Animal Hospital of the University of Glasgow for investigations of suspected focal seizures. Blood was taken for hematology and serum biochemistry. Magnetic resonance imaging (MRI) of the brain was performed with a 1.5 Tesla machine (1.5T Magnetom, Siemens, Erlangen, Germany and included T2-weighted sagittal, dorsal and transverse views and the following transverse view: fluid attenuated inversion recovery (FLAIR), Gradient echo (t2*), T1-weighted pre- and post-contrast sequences (gadopentate dimeglumine; Magnevist, Bayer Schering Pharma AG, Berlin, Germany). A cerebrospinal fluid sample was taken for total and differential cell counts, and protein levels. Finally, urine was submitted for organic acid analysis and blood for acylcarnitine levels to an external human laboratory. A control sample of a clinically healthy dog was sent to compare the acylcarnitine levels, as there are no published reference ranges for dogs.

2.2. DNA Extraction

Genomic DNA was isolated from EDTA blood with the Maxwell RSC Whole Blood Kit using a Maxwell RSC instrument (Promega, Dübendorf, Switzerland). In addition to the affected dog, 162 blood samples from CKCS, which had been donated to the Vetsuisse Biobank, were used. Most of these additional samples were obtained during an MRI screening program for syringomyelia in the Swiss and German CKCS population. Potential MCAD deficiency had not been investigated in these dogs.

2.3. Whole-Genome Sequencing

An Illumina TruSeq PCR-free DNA library with ~413 bp insert size of the affected dog was prepared. We collected 280 million 2 × 150 bp paired-end reads corresponding to 30.9 × coverage on a NovaSeq 6000 instrument (Illumina, San Diego, CA, USA). Mapping to the UU_Cfam_GSD_1.0 reference genome assembly was performed as described [16]. The sequence data were deposited under the study accession PRJEB16012 and the sample

accession SAMEA10644719 at the European Nucleotide Archive. Genome sequence data of 923 control dogs of diverse breeds were also included in the analysis (Table S1).

2.4. Variant Calling

Variant calling was performed using GATK HaplotypeCaller [17] in gVCF mode as described [16]. To predict the functional effects of the called variants, SnpEff v 5.0e software [18], together with UU_Cfam_GSD_1.0 reference genome assembly and NCBI annotation release 106, was used.

2.5. Gene Analysis

Numbering within the canine *ACADM* gene corresponds to the NCBI RefSeq accession numbers XM_038541645.1 (mRNA) and XP_038397573.1 (protein).

2.6. Allele Specific PCR and Sanger Sequencing

Primers 5′-GAG TAA AGG CCA GTT CTT TGG A-3′ (Primer F) and 5′-CCT GGT AAC CCA GAA ACA TCA-3′ (Primer R) were used for the generation of an amplicon containing the *ACADM*:c.444_445delinsGTTAATTCTCAATATTGTCTAAGAATTATG variant. PCR products were amplified from genomic DNA using AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific, Reinach, Switzerland). Product sizes were analyzed on a 5200 Fragment Analyzer (Agilent, Basel, Switzerland). Direct Sanger sequencing of the PCR amplicons on an ABI 3730 DNA Analyzer (Thermo Fisher Scientific, Reinach, Switzerland) was performed after treatment with exonuclease I and alkaline phosphatase. Sanger sequences were analyzed using the Sequencher 5.1 software (Gene Codes, Ann Arbor, MI, USA).

2.7. Acylcarnitine Screening

Five animals (3 females and 2 males) were included in each genotype group (homozygous wildtype, heterozygous, homozygous mutant). The initially investigated clinical case was included in the homozygous variant group. All animals were adult (between 2.7 and 9.9 years of age). Whole blood samples of the animals were stored at −20 °C until analysis. Acylcarnitines were analyzed with modification of a previously published protocol [19]. In short, 20 µL of hemolyzed whole blood, 20 µL acetonitrile (ACN), and 360 µL of water containing deuterated acylcarnitines internal standards were pipetted into an Eppendorf tube. The tubes were vortexed, set 5 min into an ultrasound bath and centrifuged at 12,000× *g*. Next, 20 µL of the clear supernatant was transferred into a HPLC vial containing 180 µL of ACN. Then, 2 µL was injected into the liquid chromatography mass spectrometer (HPLC-MS/MS, Waters Xevo TQ-S with Acquity I-Class 2D UPLC). The HPLC was mounted with an ACQUITY UPLC BEH Amide column (2.1 × 100 mm, 1.8 µm, Waters), the eluents were A (water:ACN (1:1) 10 mM ammonium formate with 0.15% formic acid) and B (water:ACN (5:95) 10 mM ammonium formate with 0.15% formic acid acetonitrile) at a flow rate of 0.4 mL/min. The gradient was: 100% B until 1.5 min, 74% B at 6 min, 22% B at 8 until 10 min then back to 100% B. Total run time was 15 min. The acylcarnitines were analyzed with positive electrospray ionization using multiple reaction monitoring (MRM) ion scan mode. Absolute quantification was achieved with a 6-point calibration curve.

3. Results

3.1. Clinical History, Examination and Investigations

A male neutered CKCS, born out of reportedly healthy parents, was presented at the age of 1.5 years, with an acute history of suspected complex focal seizures including prolonged lethargy, being less responsive and proprioceptive ataxia. These episodes initially occurred several times a week, lasting from 20 min to multiple hours during which the dog was mainly lethargic. General physical examination and neurological examination were normal. Complete blood count and serum biochemistry profile were within normal limits. MRI imaging of the brain revealed breed-related changes including occipital malformation with mild cerebellar herniation, medullary kinking and syringohydromyelia, consistent with canine Chiari-like malformation and syringomyelia (CMSM). No other abnormalities of the brain were detected. The results of the cerebrospinal fluid (CSF) analysis collected at the cerebellomedullary cistern showed mild albuminocytological dissociation (total nucleated cell count: 0 cells/μL, RI < 5 cells/μL; protein concentration 40 mg/dL, RI < 25 mg/dL). The dog was prescribed 40 mg/kg levetiracetam three times a day, however this resulted in severe sedation. The levetiracetam dose was therefore lowered to 25 mg/kg three times a day and 3 m/kg phenobarbital twice a day was started, which resulted in a partial response as the seizures decreased in frequency and intensity. The patient remained stable for 3 months before the seizure interval increased again and particularly the lethargy remained present up to 24 h. The dog would return to normal the following morning. An increase in the phenobarbital dose was not accompanied by an improvement. Given the unusual presentation, urine was analyzed for organic acids and revealed significant excretion of hexanoylglycine and a peak of suberic acid, highly suggestive of a fatty acid β-oxidation disorder. A follow-up test consisted of blood spot acylcarnitine analysis and revealed an increase in C6, C8 and C10:1 acylcarnitines, as judged against human adult reference intervals and a clinically normal dog (Table S2). Extrapolating from human patients, and in comparison with the control dog, the acylcarnitine profile was consistent with a diagnosis of medium-chain acyl-CoA dehydrogenase deficiency.

In addition to 25 mg/kg levetiracetam three times a day and 3.75 mg/kg phenobarbital twice a day, the dog was prescribed a low-fat diet and a midnight snack consisting of carbohydrates. Prolonged periods of fasting and formulas that contained medium-chain triglycerides as primary source of fat were also advised to avoid. This management protocol correlated with a complete resolution of clinical signs for the following 6 months. The anticonvulsant medication was therefore reduced to subtherapeutic levels. However, this was reversed as the dose reduction resulted in an increase in seizure frequency. The blood spot acylcarnitines were repeated to test sufficient free carnitine levels and these were found within normal limits (Table S2). At the time of writing, the dog has been stable for 9 months on 25 mg/kg levetiracetam three times a day, 3 mg/kg phenobarbital twice a day and a low-fat diet, with no further major seizures and a repeated normal neurological examination.

3.2. Genetic Analysis

As clinical and laboratory findings resembled human patients and a previously published CKCS with suspected MCAD deficiency [15], we hypothesized that the phenotype in the affected dog was due to a variant in the *ACADM* gene. Hence, *ACADM* was investigated as the top functional candidate gene. We sequenced the genome of the affected dog and searched for private homozygous variants that were not present in the genome sequences of 923 control dogs of diverse breeds (Tables 1 and S3).

Table 1. Results of variant filtering in the affected dog against 923 control genomes.

Filtering Step	Homozygous Variants
All variants in the affected dog	3,063,158
Private variants	1562
Protein-changing private variants	10
Private protein changing variants in *ACADM* candidate gene	3

The automated analysis identified three closely spaced homozygous private protein-changing variants in *ACADM*. Visual inspection of the short read alignments in the region revealed that these three initially separately called variants actually represented just one single insertion-deletion variant. This variant, XM_038541645.1:c.444_445delinsGTTAATTCTCAATATTGTCTAAGAATTATG, leads to a frameshift and is predicted to truncate 267 codons or roughly 63% of the wild type MCAD open reading frame, XP_038397573.1:p.(Thr150Ilefs*6). On the genomic level, the variant can be designated as Chr6:71,401,388_71,401,389delinsCATAATTCTTAGACAATATTGAGAATTAAC (Figure 1).

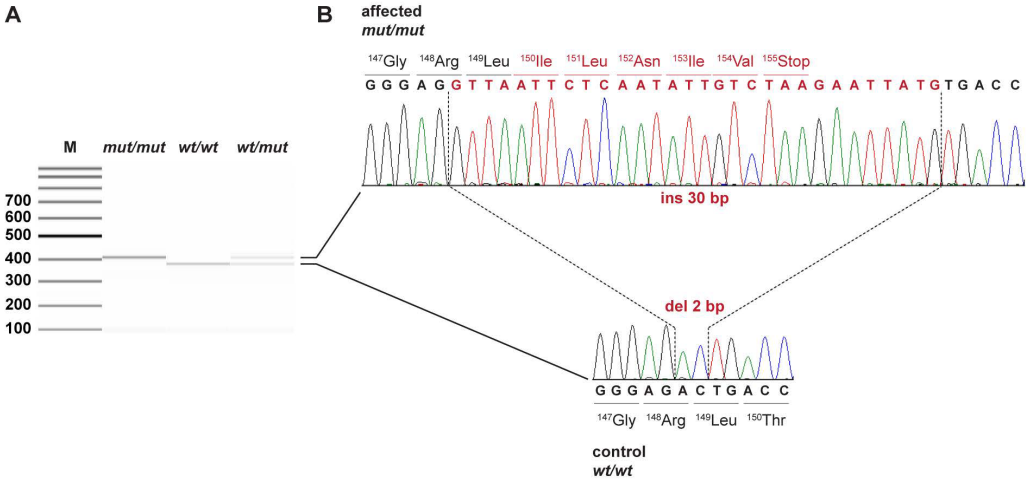

Figure 1. Details of the *ACADM*:c.444_445delinsGTTAATTCTCAATATTGTCTAAGAATTATG variant. (**A**) Fragment Analyzer bands of PCR products from samples of all three genotypes show the expected 28 bp difference in length of the wild type and mutant products. (**B**) Sanger sequencing electropherograms of the dog affected by MCAD deficiency (top) and a control dog (bottom) illustrate the deletion of 2 bp with simultaneous insertion of 30 bp in exon 6 of the *ACADM* gene. Altered nucleotide and amino acid sequences are indicated in red.

We genotyped the variant in a cohort of 162 CKCS that were not closely related to the index case and sampled during an independent study (Table 2). This experiment revealed 52 heterozygous carriers and 12 homozygous mutant dogs. The genotype distribution did not significantly deviate from Hardy–Weinberg equilibrium. The frequency of the putative disease allele was 23.5% in the investigated CKCS population.

Table 2. Genotype distribution at the *ACADM* frameshift variant in 162 CKCS.

Genotype Frequency	*wt/wt*	*wt/mut*	*mut/mut*
Number (Percentage) of dogs	98 (60.5%)	52 (32.1%)	12 (7.4%)

3.3. Acylcarnitine Measurements

To confirm the functional impact of the *ACADM* variant on fatty acid metabolism, acylcarnitines were measured in five dogs of each genotype (Table S2). Biomarkers of MCAD deficiency, C8- and C10:1-carnitines were elevated in all homozygous dogs compared to the five WT dogs. The specific C8/C10 and C8/C12 ratios used for diagnosing MCAD deficiency in humans were elevated 1.3 and 2.9-fold in heterozygous dogs, respectively and 11 and 65-fold in homozygous variant dogs, respectively as compared to wild type dogs (P_{ANOVA} C8/C10= 5.1×10^{-5}; P_{ANOVA} C8/C12= 1.4×10^{-5}, Figure 2).

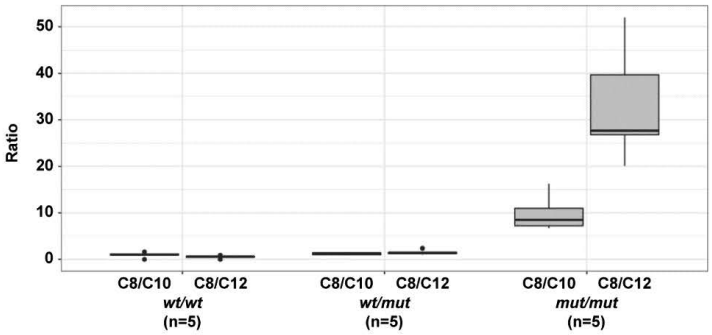

Figure 2. Acylcarnitine C8/C10 and C8/C12 ratios in the blood of dogs with different *ACADM* genotypes.

4. Discussion

In this study, we identified a homozygous *ACADM* frameshift variant in a CKCS with a history of complex focal seizures including lethargy and highly elevated MCFA metabolites in blood and urine metabolic testing. The clinical phenotype of the affected CKCS resembled human patients with MCAD deficiency and variants in the human *ACADM* gene (OMIM #201450) [12]. The investigated dog also showed striking clinical and biochemical similarities to a previously described CKCS with aciduria and elevated levels of urine hexanoylglycine and plasma acylcarnitines [15]. The plasma acylcarnitine C8/C12 ratio in the previously investigated case was at 28, which is comparable to the ratios found in the homozygous mutant dogs of our study (range 20–52, median 28). Typical pathological C8/C10 and C8/C12 ratios in human newborns range between 1.6–18 and 4.4–449, respectively [20]. In humans, the acylcarnitine biomarkers and ratios remain elevated even between decompensation episodes and under appropriate treatment (carnitine supplementation; avoiding prolonged fasting and lipolysis) [21].

Somewhat unexpectedly, the mutant allele was quite common in a representative population of CKCS that were examined for the presence of syringomyelia in a Swiss/German screening program. The CKCS breed is genetically predisposed for the occurrence of Chiari-like malformation and syringomyelia, which may result in phantom scratching, pain, and neurological deficits such as scoliosis, weakness and proprioceptive impairment [22]. An association between Chiari-like malformation and epileptic seizures was hypothesized [23], but could not be confirmed in an experimental investigation [24]. The identified *ACADM* variant now provides a compelling new candidate variant, which might be responsible for a part of the seizure phenotypes that are observed in the CKCS breed. Clinical signs due to Chiari-like malformation and/or syringomyelia and MCAD deficiency are partially overlapping and may be very difficult to disentangle in a clinical setting. The most objective way of differentiating epileptiform seizures would be by recording the electrical activity of the brain using electroencephalography, but this is technically impractical for several reasons in veterinary settings [25]. Further prospective studies are needed to better differentiate between those diseases in CKCS and to evaluate the clinical impact of the observed enzyme deficiency in some dogs of this breed.

MCAD deficiency in dogs seemingly does not clinically manifest as severe as in humans. However, our data show a clear increase in MCFAs in *ACADM* homozygous mutant dogs. This might point to an additional compensatory mechanism in the dog, which prevents or dampens the manifestation of clinical consequences of elevated MCFAs. In humans, phenotypic diversity ranging from sudden neonatal death to asymptomatic status has previously been reported. Human patients with complete loss of MCAD activity can also remain asymptomatic, suggesting that additional genetic or environmental factors may play a role in the phenotypic diversity [26–28].

Additional genetic or environmental factors are also likely to modulate the phenotype in MCAD deficient dogs. The improvement of clinical signs upon changing to a low-fat diet in our index case indicates that the diet has a major influence on the clinical phenotype. At this time, we cannot exclude the possibility that additional genetic factors also modified the clinical phenotype. While our data conclusively demonstrate that the *ACADM* frameshift variant causes MCAD deficiency and the biochemical alterations in the lipid metabolism, it is not yet fully clear whether the MCAD deficiency alone is responsible for the clinical phenotype or whether additional environmental and/or genetic risk factors are required for the expression of clinical signs. The identification of the *ACADM* frameshift variant enables genetic testing for MCAD deficiency and will facilitate future prospective studies to clarify this important question.

In humans, newborn screening programs are now well established, but prior to this, the majority of human MCAD deficiency cases presented at young age (before 2 years) [11]. No newborn screenings are performed in dogs, and it is currently unknown if MCAD deficiency could have an impact in CKCS neonatal mortality. Previous studies have reported a high percentage of perinatal mortality in CKCS as is the case for many purebred dogs [29,30]. We did not observe a significant deviation from Hardy–Weinberg equilibrium in our cohort of 162 dogs. Hence, a possible influence of the *ACADM* variant on neonatal mortality in the breed is presumably low. Nonetheless, further prospective studies might be considered to investigate if MCAD deficiency plays a role in CKCS neonatal mortality.

5. Conclusions

We identified a dog with MCAD deficiency that clinically, biochemically and genetically resembled human patients with variants in the *ACADM* gene. The putative disease allele was common in a representative CKCS cohort and might contribute to seizure phenotypes that are observed in the breed. Our data enable genetic testing to establish a diagnosis in dogs with suspected MCAD deficiency and to prevent the unintentional breeding of further dogs with MCAD deficiency. Further prospective studies are needed to assess the clinical consequences of MCAD deficiency in the CKCS breed.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes13101847/s1. Table S1: Whole genome sequence accessions of 924 dogs; Table S2: Results of the acylcarnitine measurements in the index case and a cohort of 15 CKCS; Table S3: Private homozygous variants in the genome of the sequenced affected dog.

Author Contributions: Conceptualization: D.M., R.G.Q. and T.L.; investigation: M.C., J.B. and D.M.; data curation: V.J.; writing—original draft preparation: M.C., J.B., D.M. and R.G.Q.; writing—review and editing: M.C., J.B., D.M., V.J., R.G.Q. and T.L.; visualization: M.C.; supervision: R.G.Q. and T.L.; All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board 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 ethics committee of the School of Veterinary Medicine of the University of Glasgow approved the use of clinical data for the present study (application reference EA23/22). The Cantonal

Committee for Animal Experiments approved the collection of blood samples from control dogs that were used in this study (Canton of Bern; permit BE 71/19).

Informed Consent Statement: Written informed consent was obtained from the owners of the dogs participating in this study.

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

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SCN9A variant in a family of mixed breed dogs with congenital insensitivity to pain

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Abstract

Background: Congenital insensitivity to pain (CIP) and hereditary sensory and autonomic neuropathies (HSANs) are a rare group of genetic disorders causing inability to feel pain. Three different associated variants have been identified in dogs: 1 in Border Collies, 1 in mixed breed dogs, and 1 in Spaniels and Pointers.

Objectives: To clinically and genetically characterize CIP in a family of mixed breed dogs.

Animals: Two mixed breed dogs from the same litter were independently presented: 1 for evaluation of painless fractures, and the other for chronic thermal skin injuries.

Methods: Physical, neurological, and histopathological evaluations were performed. Whole genome sequencing of 1 affected dog was used to identify homozygous protein-changing variants that were not present in 926 control genomes from diverse dog breeds.

Results: Physical and neurological examinations showed the absence of superficial and deep pain perception in the entire body. Histopathological evaluations of the brain, spinal cord and sensory ganglia were normal. Whole genome sequencing identified a homozygous missense variant in *SCN9A*, XM_038584713.1:c.2761C>T or XP_038440641.1:(p.Arg921Cys). Both affected dogs were homozygous for the mutant allele, which was not detected in 926 dogs of different breeds.

Conclusions and Clinical Importance: We confirmed the diagnosis of CIP in a family of mixed breed dogs and identified a likely pathogenic variant in the *SCN9A* gene. The clinical signs observed in these dogs mimic those reported in humans with pathogenic *SCN9A* variants causing CIP. This report is the first of a spontaneous pathogenic *SCN9A* variant in domestic animals.

KEYWORDS
animal model, *Canis lupus familiaris*, genetics, neurology, precision medicine, sodium channel

Abbreviations: CIP, congenital insensitivity to pain; GATK, genome analysis toolkit; GDNF, glial cell-derived neurotrophic factor; gVCF, genomic variant call format; HSAN, hereditary sensory and autonomic neuropathy; mRNA, messenger ribonucleic acid; NCBI, National Center for Biotechnology Information; OMIA, Online Mendelian Inheritance in Animals; OMIM, Online Mendelian Inheritance in Man; PCR, polymerase chain reaction; RETREG1, reticulophagy regulatory 1; SCN9A, sodium voltage-gated channel alpha subunit 9; WGS, whole genome sequencing.

Rodrigo Gutierrez-Quintana and Matthias Christen contributed equally and shared first authorship.

1 | INTRODUCTION

Pain is a sensory modality used to detect potential and real tissue damage, providing a survival advantage.[1,2] Genetic pain loss disorders are classified as congenital insensitivity to pain (CIP) or hereditary sensory and autonomic neuropathy (HSAN).[2,3] Congenital insensitivity to pain usually is defined by its congenital onset, whereas HSAN tends to develop gradually over time, but occasionally the difference is not clearly specified, and the terms can overlap.[2] In all cases of CIP or HSAN, the consistent feature is decreased pain perception and resulting injuries.[2,3] In humans, seven forms of CIP and eight forms of HSAN have been described based on phenotype, and genetic variants in at least 26 genes have been reported.[2,3]

Congenital insensitivity to pain and HSANs have been described previously in some dog breeds including French Spaniel,[4,5] English Springer Spaniel,[4,5] Pointer,[4,6-8] Border Collie,[9-12] Border Collie cross,[13] Miniature Pincher,[14] Long-haired Dachshund,[15,16] Jack Russell Terrier,[17] Fox Terrier,[18] and a family of mixed breed dogs,[19] but only three causal genetic variants have been identified to date. The first is an inversion disrupting *RETREG1* (*reticulophagy regulator 1*) in Border Collies and Border Collie crosses with HSAN (OMIA 002032-9615).[9,13] The second is a missense variant in the same gene in a family of mixed breed dogs with HSAN (OMIA 002032-9615).[19] The third variant is a regulatory single base substitution in a lincRNA upstream of the *GDNF* (*glial cell-derived neurotrophic factor*) gene encoding glial cell-derived neurotrophic factor in Pointers, English Springer Spaniels and French Spaniels with acral mutilation syndrome (OMIA 001514-9615).[4]

We investigated 2 mixed breed puppies from the same litter. One had tibial and fibular fractures and was weight-bearing with the leg bending and no signs of pain. The other had chronic skin injuries caused by burns from sleeping in contact to the heating radiator. Here we describe the clinical presentation, histopathological features, outcome, and genetic investigations of these cases, in which we found a homozygous missense variant in *SCN9A*, XM_038584713.1: c.2761C>T or XP_038440641.1:(p.Arg921Cys). The *SCN9A* (*sodium voltage-gated channel alpha subunit 9*) gene encodes the alpha subunit of the NaV1.7 voltage-gated sodium channel, which is preferentially expressed in sensory neurons and plays a critical role in the generation and conduction of action potentials.[2] Loss-of-function mutations in this gene have been associated with complete insensitivity to pain in humans.[2]

2 | MATERIALS AND METHODS

2.1 | Animals

Two related female mixed breed dogs were evaluated separately, the first 1 at 2 months of age (Case 1) and the second at 8 months of age (Case 2). They were from the same litter of reportedly healthy parents and some of the littermates also were reported to be healthy. Residual blood samples were retained from Case 2, and buccal swabs were collected from Case 1 for genetic investigations. Samples from the dam, sire or other littermates could not be obtained.

2.2 | Necropsy examination

Owner consent was given for euthanasia and complete necropsy in Case 2. In addition to routine samples taken during necropsy (including brain and cervical, thoracic and lumbar spinal cord), representative samples from dorsal root ganglia (cervical, thoracic, and lumbar) were collected and fixed in 10% buffered formalin. Slices of formalin-fixed samples were embedded in paraffin before staining with hematoxylin and eosin.

2.3 | Sequencing and genotyping

2.3.1 | DNA extraction

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

2.3.2 | Whole-genome sequencing

An Illumina TruSeq PCR-free DNA library with approximately 403 bp insert size from Case 2 was prepared. We collected 200 million 2 × 150 bp paired-end reads on a NovaSeq 6000 instrument (20.8 × coverage). Mapping and alignment to the UU_Cfam_GSD_1.0 genome reference assembly were performed as described.[20] The sequence data were deposited under the study accession PRJEB16012 and sample accession SAMEA110175953 at the European Nucleotide Archive.

2.3.3 | Variant calling

Variant calling was performed using GATK HaplotypeCaller[21] in gVCF mode as described.[20] To predict the functional effects of the called variants, SnpEff software[22] together with NCBI annotation release 106 for the UU_Cfam_GSD_1.0 genome reference assembly was used. For variant filtering, we used 926 genetically diverse control dog genomes of different breeds (Table S1).

2.3.4 | Gene analysis

We used the UU_Cfam_GSD_1.0 dog reference genome assembly and NCBI annotation release 106. Numbering within the canine *SCN9A* gene corresponds to the NCBI RefSeq accession numbers XM_038584713.1 (mRNA) and XP_038440641.1 (protein).

2.3.5 | PCR and Sanger sequencing

The candidate variant *SCN9A*:c.2761C>T was genotyped by direct Sanger sequencing of PCR amplicons. A 325 bp PCR product was amplified from genomic DNA using AmpliTaqGold360Mastermix (Thermo Fisher Scientific, Waltham, MA, USA) and the primers 5′-GAG TAA AGG CCA GTT CTT TGG A-3′ (Primer F) and 5′-CCT GGT AAC CCA GAA ACA TCA-3′ (Primer R). Sanger sequences were analyzed using the Sequencher 5.1 software (GeneCodes, Ann Arbor, MI, USA).

2.3.6 | In silico functional predictions

The protein amino acid change caused by the candidate variant was assessed using multiple in silico prediction tools: PredictSNP, MutPred2 and SNPs & Go.[22-24]

3 | RESULTS

3.1 | Clinical description

Two related mixed breed puppies from the same litter were presented over a 6-month period to the Small Animal Hospital of the University of Glasgow.

Case 1: A 2-month-old intact female puppy was presented with a 4-day history of unusual bending of the right pelvic limb. The owner also mentioned the presence of small, round, superficial ulcers on the digital pads of both pelvic limbs for the last 4 weeks. Examination disclosed small superficial skin ulcers on digital pads IV and V. There was fluctuant swelling of the medial aspect of the right hock with associated instability, but no evidence of pain on palpation and manipulation. Neurological examination showed normal mentation and cranial nerve function. Gait assessment showed weight-bearing lameness with lateral bending of the distal tibia. Postural reactions and segmental spinal reflexes were normal. Superficial and deep pain perception was absent over the entire body, with no evident response despite firm pressure with the forceps. Findings were consistent with a peripheral sensory neuropathy or CIP. Radiographs of the right tibia identified a displaced Salter-Harris fracture type I of the distal tibia and a fibular fracture (Figure 1). Aspiration of the fluctuant swelling yielded purulent material with many neutrophils on microscopic examination. A diagnosis of infected tibial and fibular fractures was made, and the owner elected euthanasia.

Case 2: An 8-month-old intact female dog was presented with a history of chronic skin lesions. The owner reported that since adoption, at 6 weeks of age, the dog already had multiple scars in its skin. During the last 8 weeks, the dog developed large and deep skin lesions in the dorsolateral thoracic region bilaterally. Initial dermatological investigations did not identify a cause for the lesions. Since then, the owner noticed that these lesions were caused by getting burned while sleeping in contact with a heating radiator. Physical examination was unremarkable, except for multiple scars in the skin and the large erosion in the dorsolateral thoracic region. Multiple blood pressure measurements were normal. Neurological examination identified normal mentation, cranial nerve function and gait. Postural reactions and segmental spinal reflexes were normal. Superficial and deep pain perception was absent over the entire body, with no evident response despite firm pressure with the forceps. Findings were consistent with a peripheral sensory neuropathy or CIP. Results of a CBC and serum biochemistry profile were normal. The dog received open wound management (i.e., lavages, debridement, PO antibiotics [cephalexin], an anti-inflammatory drug [meloxicam], and bandaging) and the owners were instructed to avoid any activities that could cause injury and to protect the dog from contact with the heating radiators. Despite these precautions, the dog developed multiple severe skin injuries over the next 2 months, and the owners elected euthanasia.

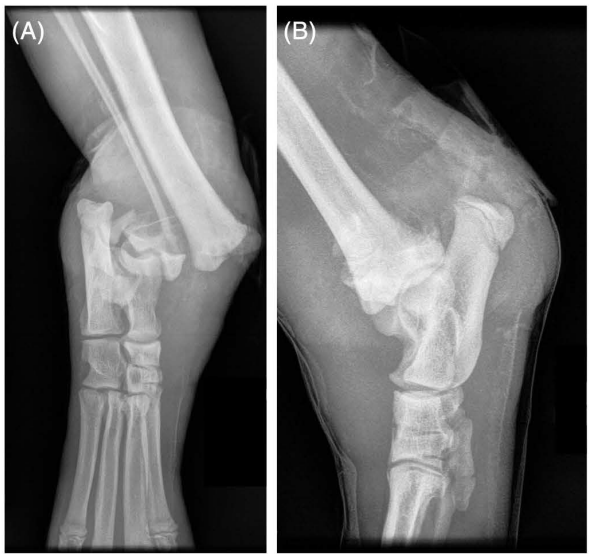

FIGURE 1 Cranio-caudal (A) and lateral (B) radiograph of the right pelvic limb of case 1 showing the tibial and fibular fractures and soft tissue swelling

3.2 | Histopathology

No macroscopic or microscopic abnormalities were detected in any of the tissues examined, except for the skin lesions previously reported.

3.3 | Sequencing, genotyping and protein expression

Because the clinical and neurological findings of these cases resembled CIP previously described in humans, and the parents were

reported to be clinically unaffected, we hypothesized that the phenotype in the affected dogs was caused by monogenic autosomal recessive mode of inheritance. We sequenced the genome of Case 2 and searched for private homozygous variants that were not present in the genome sequences of 926 control dogs of diverse breeds (Table 1 and Tables S1 and S2).

The resulting variants were prioritized according to functional knowledge of the affected genes. The bioinformatics analysis identified a single homozygous private protein-changing variant in a functional candidate gene. The variant was located in the *SCN9A* (*sodium voltage-gated channel alpha subunit 9*) gene. It can be designated chr36:11652662G>A or XM_038584713.1:c.2761C>T and is predicted to result in an amino acid substitution in a highly conserved region of the encoded alpha subunit of the NaV1.7 sodium channel, XP_038440641.1:(p.Arg921Cys) (Figure 2).

The arginine-to-cysteine substitution was predicted to be deleterious by all used prediction algorithms (PredictSNP probability for pathogenicity 87%, MutPred2 score: 0.923, SNP&GO disease probability: 76%). Furthermore, MutPred2 predictions included "altered ordered interface" and "altered transmembrane protein" with probabilities of 0.28 and 0.25, and with *P*-values of 5.9×10^{-3} and 1.4×10^{-3}, respectively.

The other seven private protein-changing variants were not located in genes known to cause similar phenotypes in humans, mice, or domestic animals.

We confirmed the presence of the *SCN9A* variant in a homozygous state in Cases 1 and 2 by Sanger sequencing (Figure 3).

TABLE 1 Homozygous variants in case 2, filtered against 926 control genomes

Filtering step	Variants
All variants in the affected dog	3 036 781
Private variants	589
Protein-changing private variants	8

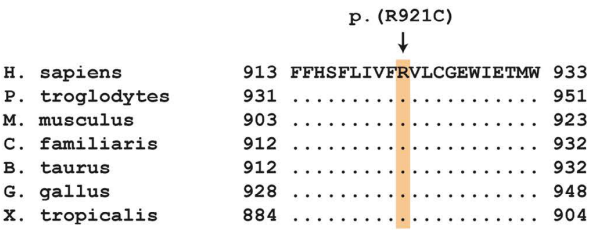

FIGURE 2 Multiple-species alignment of the *SCN9A* amino acid sequences of the second intramembrane pore forming domain harboring the p.(R921C) variant. The variant affects a highly conserved arginine residue. Accession numbers: human (*Homo sapiens*) NP_001352465.1; chimpanzee (*Pan troglodytes*) XP_003309333.2; mouse (*Mus musculus*) NP_061340.2; dog (*Canis familiaris*) XP_038440641.1; domestic cattle (*Bos Taurus*) NP_001104257.1; chicken (*Gallus gallus*) XP_004942840.1; frog (*Xenopus tropicalis*) XP_002939316.2

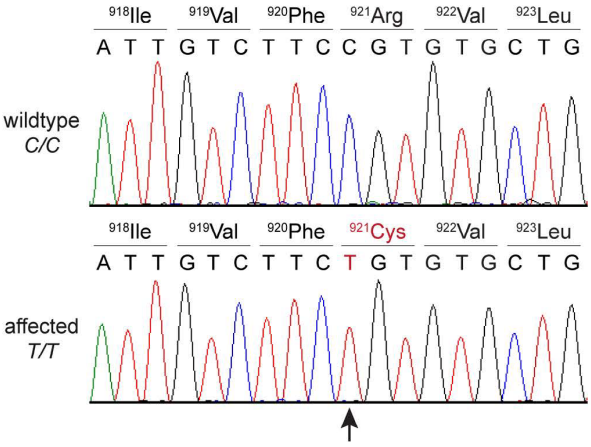

FIGURE 3 Details of the SCN9A:c.2761C>T variant (p.Arg921Cys). Representative electropherograms of a wild type dog and an affected dog are shown. The amino acid translations of the wild type and mutant alleles are indicated

4 | DISCUSSION

We describe two littermate mixed breed dogs with the inability to react to noxious stimuli and secondary injuries, including fractures and burns. We identified a likely candidate disease-causing variant, *SCN9A*:p.(Arg921Cys). Nociception refers to neural encoding of impending or actual tissue damage and pain refers to the subjective experience of actual or impending harm.[1]

The *SCN9A* (*sodium voltage-gated channel alpha subunit 9*) gene encodes the alpha subunit of the NaV1.7 sodium channel. Gain of function variants produce impaired inactivation or enhanced resurgent current of the sodium channel and induce increased excitability in the dorsal root ganglia neurons, resulting in neuropathic pain and variable clinical phenotypes in humans such as primary erythromelalgia (OMIM# 133020), paroxysmal extreme pain disorder (OMIM# 167400) and hereditable small fiber neuropathy (OMIM# 133020). The *SCN9A* loss of function results in inability to feel pain and the human clinical phenotypes are congenital insensitivity to pain (OMIM# 243000) and hereditary sensory autonomic neuropathy type IID (OMIM# 243000).[2,3,25-27] A recent study indicated that nociceptor activity at the level of the dorsal root ganglia is largely unaffected by NaV1.7 and suggested a critical locus of analgesia in the central terminal and not in the periphery as thought previously.[28] The *SCN9A* gene also plays a role in seizures and epilepsy with some variants linked to Dravet Syndrome (OMIM# 607208) and febrile seizures.[29]

The clinical phenotype in humans with CIP caused by variants in *SCN9A* is characterized by anosmia and injuries associated with complete lack of pain sensation. The dogs of our study suffered from painless fractures and burns, which are hallmarks of the phenotype in humans. Anosmia is difficult to identify clinically in dogs, especially if present since birth, and we are not sure if it was present in these cases. When compared with previous reports of HSAN and CIP in dogs, the dogs of our study share more similarities with the Miniature Pinscher,[14] Pointer,[4,6-8] Spaniel,[4,5] and Fox Terrier,[18] that presented

with loss of pain sensation, but no proprioceptive deficits or autonomic signs. In contrast, the HSAN reported in the long-haired Dachshund,[15,16] Jack Russell Terrier,[17] Border Collie,[9-13] and a family of mixed breed dogs[19] was associated with other neurological deficits including proprioceptive deficits and signs of autonomic dysfunction. An important difference in the dogs of our report is that automutilation was not a feature, but severe injuries were caused by external sources. Finally, the young age of presentation in our patients is consistent with CIP.

Several variants in *SCN9A* causing CIP have been identified in humans. Congenital insensitivity to pain causing loss of function variants mostly consist of nonsense, splice site, and frameshift variants, but also include some missense variants.[26] The missense variant identified in the affected dogs of our report changes a highly conserved arginine residue in 1 of the 4 intramembrane pore-forming domains of the NaV1.7 alpha subunit.[30] The change from the positively-charged arginine residue to an uncharged cysteine residue with a reactive thiol group in this region might disrupt sodium channel functionality by altering sodium ion selectivity and conductivity, as was suggested previously.[31]

The XP_038440641.1:(p.Arg921Cys) was predicted to be deleterious by all used in silico prediction tools. An identical amino acid exchange in the corresponding human protein, NP_001352465.1:p.Arg922Cys, has been observed in compound heterozygosity with a known disease-causing variant in a patient with HSAN.[32] The discovery of a homologous change in the dog now provides additional evidence for the pathogenicity of these variants in both species. Finally, the absence of the *SCN9A*: c.2761C>T variant in >900 control genomes and homozygosity in both cases provides additional support and make this variant a compelling candidate disease-causing variant in the 2 affected puppies.

Currently, no cure exists for this condition, and treatment is supportive by early detection, prevention and management of any injuries sustained.[33] Both of the dogs in our study presented with multiple and severe injuries, and owners elected euthanasia at a young age. In humans, many people with CIP do not survive childhood because of recurrent injuries, such as self-injury, burns, repeated fractures, osteomyelitis, and accidental death.[34]

Our study had some limitations. First, because of the lack of pedigree data and inability to obtain samples from relatives, we were not able to confirm the mode of inheritance. Nevertheless, the severity of the phenotype and the reportedly healthy parents make monogenic autosomal recessive the most likely mode of inheritance. Second, no histopathology of the peripheral nerves or autonomic ganglia was performed, and doing so could have helped characterize a form of HSAN or CIP. Finally, no nerve conduction or electromyographic studies were performed.

To our knowledge, our study is the first description of CIP associated with a *SCN9A* variant in domestic animals. The clinical features of this disease closely resemble the human phenotype, suggesting the *SCN9A*:p.Arg921Cys variant as a compelling candidate disease-causing variant. It is not currently known whether the causal variant arose in the recent ancestry of the affected dogs described in our study or whether it arose more distantly, in which case it might be segregating in the wider canine population.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the local ethical committee of the University of Glasgow, School of Veterinary Medicine.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

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

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A *TNR* frameshift variant in Weimaraner dogs with an exercise-induced paroxysmal movement disorder

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A *TNR* Frameshift Variant in Weimaraner Dogs with an Exercise-Induced Paroxysmal Movement Disorder

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ABSTRACT: Background: Some paroxysmal movement disorders remain without an identified genetic cause.
Objectives: The aim was to identify the causal genetic variant for a paroxysmal dystonia–ataxia syndrome in Weimaraner dogs.
Methods: Clinical and diagnostic investigations were performed. Whole genome sequencing of one affected dog was used to identify private homozygous variants against 921 control genomes.
Results: Four Weimaraners were presented for episodes of abnormal gait. Results of examinations and diagnostic investigations were unremarkable. Whole genome sequencing revealed a private frameshift variant in the *TNR* (tenascin-R) gene in an affected dog, XM_038542431.1:c.831dupC, which is predicted to truncate more than 75% of the open read frame. Genotypes in a cohort of 4 affected and 70 unaffected Weimaraners showed perfect association with the disease phenotype.
Conclusions: We report the association of a *TNR* variant with a paroxysmal dystonia–ataxia syndrome in Weimaraners. It might be relevant to include sequencing of this gene in diagnosing humans with unexplained paroxysmal movement disorders. © 2023 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society.

Key Words: canine; neurogenetics; extracellular brain matrix; dystonia; episodic ataxia; precision medicine

Paroxysmal movement disorders are a rare group of diseases characterized by episodic involuntary movements that can include dystonia, dyskinesia, chorea, and ataxia.[1-4] They are divided into paroxysmal dyskinesias (characterized by transient episodes of hyperkinetic movements) and episodic ataxias (characterized by attacks of cerebellar ataxia) depending on the main movement.[1-4] Their cause can be primary (genetic) or secondary (acquired), and advances in next-generation sequencing have allowed the identification of genetic variants responsible for these disorders.[2,3] The large number of genes involved in the pathogenesis of paroxysmal movement disorders reflects a high complexity of molecular causes involved, including synaptic vesicle fusion, postsynaptic intracellular signaling, brain energy metabolism, neurotransmitter synthesis, ion channels, and solute carriers.[1,2] Despite advances in understanding the genetics of these disorders, there is still a number that remains without an identified cause, suggesting that other unidentified genes and disease mechanisms exist.[2-4]

The identification and clinical characterization of spontaneously occurring hereditary paroxysmal movement disorders in different dog breeds provide an opportunity to identify new genes and disease mechanisms involved in these rare diseases.[5-7] The unique population structure of purebred dogs, in which each breed arises from a limited number of founders, and canine reproduction with relatively large litter sizes provide advantages for genetic studies in dogs compared to humans.[8] In recent years, variants in four genes have

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been identified in dogs with paroxysmal movement disorders: juvenile paroxysmal dyskinesia in Markiesje dogs caused by a frameshift variant in *SOD1* (OMIA 002322-9615),[9] a paroxysmal dyskinesia in soft-coated Wheaten terriers with a missense variant in *PIGN* (OMIA 002084-9615),[10,11] and a paroxysmal hypertonicity syndrome in cavalier King Charles spaniels caused by a microdeletion in *BCAN*, encoding the brain-specific extracellular matrix protein brevican (OMIA 001592-9615).[12,13] Furthermore, a *PCK2* missense variant was identified in Shetland sheepdogs with paroxysmal exercise-induced dyskinesia (OMIA 001543-9615). However, the causality of this *PCK2* variant was not proven, and the paroxysmal movement disorder phenotype in these dogs might have been due to other reasons.[14]

Here, we describe a group of Weimaraner dogs with an autosomal recessive paroxysmal dystonia–ataxia syndrome associated with a novel homozygous variant in the tenascin-R (*TNR*) gene. TNR is a member of the tenascin family of extracellular matrix glycoproteins, which are expressed in the nervous system.

Materials and Methods

Animals

Four Weimaraner dogs with a paroxysmal dystonia–ataxia syndrome were included in this study. They were from three litters of different and reportedly healthy parents, with 2 cases being littermates. Residual EDTA (ethylenediaminetetraacetic acid) blood samples were retained from all cases for genetic investigation. Samples from dams, sires, or other littermates could not be obtained. Ethical approval was granted by the ethics committee of the School of Veterinary Medicine of the University of Glasgow.

Clinical Investigations

All cases were examined and investigated by veterinary neurologists. Investigations included blood samples for hematology and biochemistry, fructosamine, lactate, pyruvate, acetylcholine receptor antibodies, enzymatic testing for storage diseases, magnetic resonance imaging (MRI) of the brain and spinal cord, cerebrospinal fluid analysis, electomyography, motor nerve conduction velocities, urine organic acids, muscle and nerve biopsies, and serologies for *Toxoplasma* and *Neospora*.

Sequencing and Genotyping

EDTA blood samples from all 4 cases were collected, and genomic DNA was isolated. The genome of 1 case was sequenced at 21.5× coverage on an Illumina Novaseq 6000 instrument (Illumina, Zurich, Switzerland). Mapping and variant calling were performed with respect to the UU_Cfam_GSD_1.0 reference genome assembly as previously described.[15] The gathered sequence data were compared to 921 genomes of control dogs of different breeds and filtered for homozygous private protein-changing variants (Tables S1 and S2). Identified variants were genotyped in all 4 cases via Sanger sequencing. The candidate variant was additionally genotyped in 70 control Weimaraner dogs.

Results

Clinical Description and Investigations

Four Weimaraner dogs (3 males and 1 female) from three different litters were presented for episodes of abnormal gait characterized by increased muscle contractions (dystonia), ataxia, and hypermetria, leading to occasional collapse. Kyphosis and low head carriage were also consistent features (Video S1; Table 1). Parents and some of the littermates were reported to be clinically normal in all cases. The age of onset was 3 to 7 months. Increased emotional arousal or exercise was reported to trigger the abnormal episodes, which could occur multiple times daily for 5 to 15 minutes. Two dogs displayed intermittent anisocoria associated with the episodes.

Resting physical and neurological examinations were unremarkable in all cases, although the reported abnormalities were elicited by short periods of exercise in 3 dogs. Results of diagnostic investigations, including hematology, biochemistry, urine organic acids, lactate and pyruvate levels, enzymatic testing for storage diseases, acetylcholine receptor antibodies, muscle and nerve biopsies, MRI (brain and spinal cord), cerebrospinal fluid analysis, and electrophysiology, were mainly unremarkable. Treatment with fluoxetine (1 mg/kg once a day) in 2 dogs resulted in a dramatic reduction in episode severity and frequency as soon as it started. Interestingly, when fluoxetine was stopped after a few months in one of the cases, the episodes reoccurred, and frequency increased.

Genetic Analysis

Comparing the sequence data of the affected dog to 921 control dogs revealed 1030 homozygous private variants. Only four of those variants were called with moderate or high impact using SnpEff software[16] and thus predicted to be protein changing. Genotyping of the affected dogs showed that only one of those variants was homozygous in all 4 cases (Table S3). The remaining variant was a single-nucleotide duplication in *TNR*, chr7:23,940,980dupC (UUCfam_GSD_1.0) (Fig. 1), which is a known candidate gene for "neurodevelopmental disorder, nonprogressive, with spasticity and transient opisthotonos" in humans (OMIM 619653). The canine variant XM_038542431.1:

TABLE 1 *Signalment, investigations, episode characteristics, and response to treatment*

Case	Signalment	Investigations	Episode characteristics	Response to treatment
Case 1	Female neutered Age at onset: 7 mo Consanguinity: unknown	Hematology, biochemistry, EMG, MNCV, CSF, urine organic acids, muscle and nerve biopsies, lactate and pyruvate, *Tox.*, *Neo.*, AChR	Duration: 5–10 minutes Frequency: multiple times a day Trigger: exercise and EA Site of onset: pelvic limbs Body distribution: generalized Ataxia: yes Dystonia: yes (pelvic limbs) Hypermetria: yes Collapse: occasionally Other signs: kyphosis, neck down	Diazepam: mild response Phenytoin: mild response Baclofen: moderate response
Case 2	Male entire Age at onset: 3 mo Consanguinity: unknown	Hematology, biochemistry, fructosamine, EMG, MNCV, CSF, MRI (brain and spinal cord)	Duration: 5–10 minutes Frequency: multiple times a day Trigger: exercise and EA Site of onset: pelvic limbs Body distribution: generalized Ataxia: yes Dystonia: yes (pelvic limbs) Hypermetria: yes Collapse: occasionally Other signs: kyphosis, neck down	Diazepam: mild response Baclofen: moderate response
Case 3	Male entire Age at onset: 6 mo Brother of case 4 Consanguinity: yes	Hematology, biochemistry, EMG, MNCV, CSF, enzymatic testing for storage diseases, *Neo.*, MRI (brain)	Duration: 10–15 minutes Frequency: multiple times a day Trigger: exercise and EA Site of onset: pelvic limbs Body distribution: generalized Ataxia: yes Dystonia: yes Hypermetria: yes Collapse: occasionally Other signs: kyphosis, neck down, and anisocoria that persist for up to 12 hours after the episodes	Fluoxetine: excellent response
Case 4	Male entire Age at onset: 6 mo Brother of case 3 Consanguinity: yes	Hematology, biochemistry, EMG, MNCV, urine organic acids, CSF, *Tox.*, *Neo.*, MRI (brain and spinal cord)	Duration: 5–15 minutes Frequency: multiple times a day Trigger: exercise and EA Site of onset: pelvic limbs Body distribution: generalized Ataxia: yes Dystonia: yes Hypermetria: yes Collapse: no Other signs: kyphosis, neck down, and anisocoria that persist for up to 12 hours after the episodes	Fluoxetine: excellent response

Abbreviations: EMG: electromyography; MNCV: motor nerve conduction velocity; CSF: cerebrospinal fluid; *Tox.*: *Toxoplasma gondii* serology; *Neo.*: *Neospora caninum* serology; AChR: acetylcholine receptor antibodies; EA: emotional arousal; MRI: magnetic resonance imaging.

c.831dupC is predicted to result in a frameshift and truncation of about 77% of the wild-type open reading frame of the encoded TNR protein, XP_038398359.1: p.(Asn278Glnfs*38). Genotyping of 70 control Weimaraner dogs showed the expected correlation for an autosomal recessive mode of inheritance (Table 2).

Discussion

We describe 4 young Weimaraner dogs with a paroxysmal dystonia–ataxia syndrome. We identified a frameshift variant in the *TNR* gene (XM_038542431.1: c.831dupC) and demonstrated that the genotypes at this

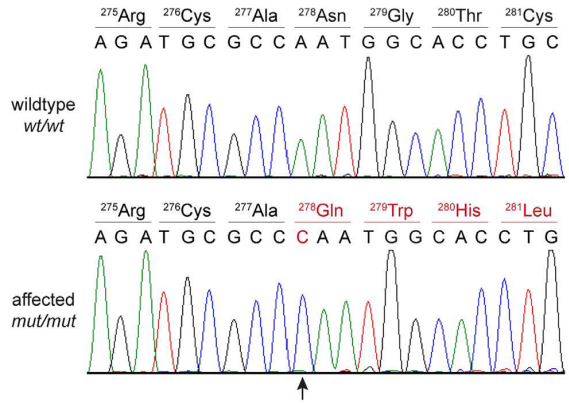

FIG. 1. Details of the *TNR*:c.831dupC variant. Sanger sequencing electropherograms of a wild-type control and an affected dog are shown. The duplicated C in the affected dog is indicated with an arrow. The shifted reading frame with the altered amino acid codons is shown in red. [Color figure can be viewed at wileyonlinelibrary.com]

variant were consistent with the disease phenotype assuming a recessive inheritance pattern.

TNR is a member of the tenascin family of extracellular matrix glycoproteins.[17] It is involved in neurite outgrowth and neural cell adhesion, proliferation and migration, axonal guidance, myelination, and synaptic plasticity.[17,18] It is exclusively expressed in the nervous system, mainly by oligodendrocytes, but also by some neurons in the central nervous system and by Schwann cells in the peripheral nervous system. Its expression increases perinatally, and it represents a major component of perineuronal nets in adults, which are a specialized kind of matrix that ensheathes subtypes of neurons that regulate synaptic plasticity.[17,18] The role of TNR in human pathology is just starting to be elucidated. Variants in *TNR* have recently been reported to cause a nonprogressive neurodevelopmental disorder with spasticity and transient opisthotonos (OMIM 619653).[18-20] Although all human patients shared some common traits affecting motor function, the severity of the phenotype varied.[18-20] Brain MRI of patients showed variable degrees of delayed myelination and abnormalities in the structure of the corpus callosum, something that was not observed in the dogs from this study.[18] In addition, *TNR* has been described as a candidate risk gene for familial Parkinson's disease.[21,22] Our findings in dogs suggest that it might be relevant to include sequencing of this gene in the diagnosis of humans with unexplained paroxysmal movement disorders.

TABLE 2 *Association of the genotypes at the* TNR*:c.831dupC variant with paroxysmal movement disorder in 74 Weimaraner dogs*

Phenotype	*wt/wt*	*wt/mut*	*mut/mut*
Paroxysmal movement disorder (n = 4)	–	–	4
Control dogs (n = 70)	68	2	–

The extracellular matrix plays an important role in neuronal glial interactions, and so far, three genetic causes of inherited dystonia have interactions or contribute to extracellular matrix homeostasis.[23] Interestingly, this is the second gene encoding a brain extracellular matrix protein, causing a paroxysmal movement disorder in dogs, suggesting a role of extracellular matrix in central nervous system motor function. Previously, a paroxysmal hypertonicity syndrome in cavalier King Charles spaniels was found to be associated with a variant in *BCAN*, encoding brevican (OMIA 001592-9615).[12,13] The clinical phenotype had similarities with the Weimaraner dogs from our study, and episodes were also triggered by increased emotional arousal. Something unusual in 2 Weimaraner dogs from this study was the presence of intermittent anisocoria associated with the movement disorder episodes. To our knowledge, this has not been reported before in other paroxysmal movement disorders. The episodes observed in the dogs from this study had some similarities with episodic ataxias in humans, as there was hypermetria and truncal ataxia, but they lacked other inter-ictal signs, such as myokymia or nystagmus. Another important feature of the episodes observed in these dogs was dystonia, with increased muscle tone and kyphosis. Therefore, we decided to classify these episodes in the group of dystonia–ataxia syndromes, which encompass many human genetic disorders.[24] It has been suggested that molecular pathways of ataxia and dystonia are closely related, and the cerebellum seems to play an important role in the control of both.[25]

TNR is an important constituent of the perineuronal nets, and in knockout mice, their distribution, composition, and function are altered.[26,27] $Tnr^{-/-}$ knockout mice display altered levels of excitatory and inhibitory synapses, with an enhancement of excitatory synaptic transmission in some parts of the brain, such as the hippocampus.[28,29] The clinical signs observed in the dogs from this study probably emerge secondary to alterations in the synaptic balance between inhibitory and excitatory neurons at the perineuronal nets. Interestingly, the dogs in the present study responded to fluoxetine treatment. Fluoxetine is a selective serotonin reuptake inhibitor that has been previously used successfully in another movement disorder of dogs affecting Scottish terriers in which altered serotonergic function is suspected.[30-32] Previous studies have shown that fluoxetine promotes structural changes in inhibitory neurons in the cerebral cortex of adult mice, probably through alteration of the extracellular matrix surrounding them, which could also explain the good and sustained response observed in these dogs,

although if this was the main mechanism of action we would have expected to see a slower onset of effect.[33,34]

In humans, no reports of the use of fluoxetine for treating paroxysmal movement disorders could be found, but movement disorders have been reported as side effects of its use.[35,36]

To our knowledge, the affected dogs represent the first domestic animals described with a *TNR*-related disease. Our results enable genetic testing, which can be used to avoid the unintentional breeding of further affected dogs. In addition, the studied dogs might serve as a spontaneous large animal model to further understand the role of *TNR*, the extracellular matrix, and perineuronal nets in movement disorders.

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Data Availability Statement

Data is available

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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Cross-Ethnic Variant Screening and Burden Analysis of *PTPA* in Parkinson's Disease

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ABSTRACT: Background: Recently, homozygous variants in *PTPA* were identified as the disease cause for two pedigrees with early-onset parkinsonism and intellectual disability. Although the initial link between *PTPA* and parkinsonism has been established, further replication was still necessary.

Objectives: To evaluate the genetic role of *PTPA* in Parkinson's disease (PD).

Methods: We analyzed rare variants of *PTPA* in cohorts of Asian and European ancestries ($N_{case} = 2743$, $N_{control} = 8177$) with whole-exome sequencing, and further explored the functional effect of the target variant.

Results: One patient with early-onset PD from a consanguineous family carried the homozygous variant p.Met329Val, while her parents and elder sister with heterozygous p.Met329Val were healthy. This patient developed minor cognitive decline within 1 year, with a Montreal Cognitive Assessment (MoCA) score dropping from 28 to 25. Functional exploration with overexpression studies suggested that this variant was associated with decreased protein phosphatase 2A (PTPA) protein level by affecting protein stability, but not mRNA expression.

Conclusions: These results have broadened the mutation spectrum of *PTPA*, and paved the way for further research into the role of *PTPA* in PD. © 2023 International Parkinson and Movement Disorder Society.

Key Words: Parkinson's disease; *PTPA*; rare variant; protein level

Parkinson's disease (PD) is the second most common neurodegenerative disorder characterized by heterogeneous motor and non-motor manifestations.[1] The disease etiology is complex, and mounting evidence has demonstrated the important role of genetic factors in PD.[2,3] Along with the wide application of next-generation sequencing and burgeoning research into the genetic background of PD, over 90 common risk variants have been identified.[4] However, a number of pedigrees still have no identifiable genetic causes, suggesting that more risk genes require exploration. Rare variants, which might make a major contribution to the missing heritability, could help improve understanding of the disease pathogenesis.[2]

Recently, two homozygous variants (p.Met298Arg, p.Ala171Asp) in *PTPA* (*protein phosphatase 2A*) were identified as the disease cause in two pedigrees of African descent.[5] The patients had early-onset parkinsonism and intellectual disability. The study further demonstrated that *PTPA* ortholog knock-down in *Drosophila* neurons induced a significant impairment of locomotion in the climbing test. Although the initial links between *PTPA* and parkinsonism have been established, further replication from additional cohorts is still necessary, especially in populations of different ancestries.

In this context, we analyzed rare variants of *PTPA* in PD cohorts of Asian and European ancestries, respectively. We identified a novel homozygous rare variant p.Met329Val in a patient with early-onset PD. Further

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Discussion and outlook

Within my PhD thesis, I conducted investigations into the molecular basis of seven types of inherited neurological diseases in dogs. Through a combination of linkage analysis and homozygosity mapping with SNV genotyping arrays, as well as whole-genome sequencing of selected cases, plausible candidate causative variants for all seven investigated phenotypes could be identified. These diseases serve as examples of three different classes of neurological disorders, namely inherited ataxias, HSAN, and inborn errors of metabolism. As the diseases affect distinct biological processes, they cause problems in different parts of the nervous system (Figure 2).

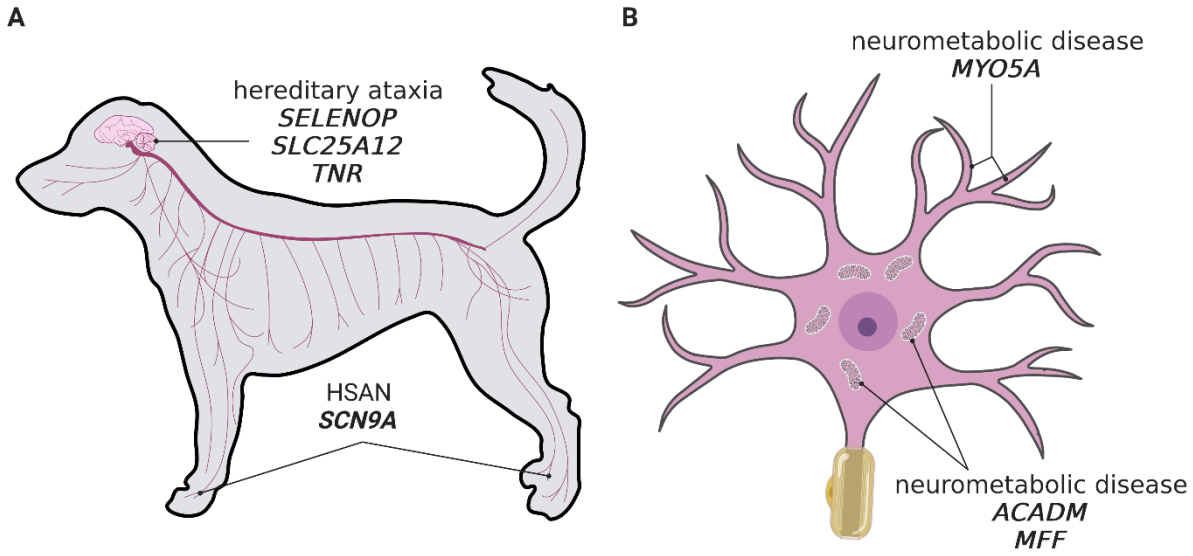

Figure2. Representation of the investigated neurological phenotypes showing the different involved parts of the CNS and PNS (**A**) in a simplified representation of the canine nervous system for hereditary ataxias and HSAN, and (**B**) in the affected parts of nerve cells for neurometabolic diseases. Created with BioRender.com

The first phenotype studied in the thesis was CNS atrophy with cerebellar ataxia in Belgian Shepherd Malinois. In a litter of eight puppies, four displayed truncal wobbling, intention tremor, elevated muscle tone, and short episodic spastic fits. As both parents were healthy, we assumed a recessive mode of inheritance. Employing linkage analysis and homozygosity mapping, we identified a ~52 Mb disease-associated locus. A consequent filtering for short homozygous alterations in the genomic sequence (singe nucleotide alterations or short indel variants) of one sequenced case did however not reveal a fitting candidate variant. A visual inspection of the critical interval in IGV was performed next, revealing a large deletion that encompassed the whole *SELENOP* gene, coding for selenoprotein P, an important protein for selenium transport to the brain [63,64]. As this gene was previously already associated with ataxia in mice [65], we considered the whole gene deletion a highly plausible candidate variant, which segregated perfectly with the phenotype in the family. Further targeted genotyping of ~650 Belgian Shepherd dogs identified an additional ataxic dog that had

reached an age of 10 years, unlike our investigated cases which were euthanised with 5 weeks of age, thus revealing an unexpected phenotypic heterogeneity. We assumed this heterogeneity to be the result of differences in selenium supplementation of the affected dogs, or to be due to different genetic modifiers between single litters. Selenium levels in available residual serum samples of all the puppies of the index litter were measured and we found that dogs with the homozygous deletion indeed only had 30% of the blood selenium value of age matched controls. This confirmed the suspected defect in selenium transport and established *SELENOP* as a new candidate gene for similar human phenotypes. Additionally, the established DNA test enables breeders to avoid mating of two carriers, and additionally might be a helpful tool for diagnosis of selenium deficiency. If further homozygous dogs are discovered, a selenium supplementation trial might be attempted, as this stopped the progression of the neurodegeneration in affected mice [66].

A combination of coat colour dilution (animals) or partial albinism (humans) together with neurological deficits is specific to Griscelli syndrome type 1, which has been linked to homozygous *MYO5A* variants in humans, mice, rats, and horses [67–70]. The encoded myosin VA is required for correct transport of melanosomes and endoplasmic reticulum to the dendritic tips of melanocytes and Purkinje cells, respectively [71,72]. Hence, the absence of this protein results in a unique dermatological and neurological phenotype combination. For the study of this phenotype in a Miniature Dachshund dog, we received samples from a litter of six with one affected dog. *MYO5A* contains over 40 annotated exons in the dog. Hence, a classical candidate gene approach, where all exons are amplified separately by Sanger Sequencing, was less time- and cost efficient compared to whole genome sequencing of the case, which was the approach we chose for further analysis. Our initial hypothesis turned out to be true and I identified an insertion of a single A into the coding sequence of *MYO5A*, presumably leading to a premature stop codon and nonsense-mediated decay of the transcript. DNA samples from 142 unrelated Dachshunds were available for genotyping, but none of them carried the found variant, suggesting a relatively younger mutation event, or a possibly unfitting genotyping cohort, as we did not distinguish between miniature Dachshunds and standard sized dogs for genotyping. However, the phenotype co-segregated as expected with the investigated phenotype in the family, and this phenotype was highly specific for defects in *MYO5A* in other species. Therefore, we concluded that we identified the first candidate disease causing *MYO5A* variant in canines and enabled prevention of unintentional breeding of affected dogs by genotyping via DNA testing.

In contrast to the well characterized Griscelli syndrome, this thesis also gave the opportunity to work on new phenotypes, as was nicely illustrated by the cerebellar degeneration and myositis complex (CDMC) in Nova Scotia Duck Tolling Retrievers (NSDTR). Very similar to

the miniature Dachshunds, we at first received samples of a litter of six with one affected dog, and WGS was performed for the case. A candidate missense variant in *SLC25A12*, a gene that is responsible for a form of developmental and epileptic encephalopathy in humans (OMIM #603667), was subsequently identified. However, as the observed combination of cerebellar ataxia and neuromuscular signs was not specific to the investigated gene, we asked collaborators from the UC Davis to genotype their own NSDTR cohort and to look for additional samples of affected dogs to get a better association. Consequently, a second litter with three additional affected puppies was detected. Pedigree analysis revealed that this litter was distantly related to the index family and the genotype-phenotype association remained as expected for a fully penetrant autosomal recessive mode of inheritance. The variant seems to be more present in the European dog population, as carrier frequencies were at 7.1% in European dogs (N= 380), but only at 2.7% in a North American cohort (N= 153). CDMC is one of the examples, that can be classified according to different systems. On one hand, affected dogs clearly show a form of hereditary ataxia, that belongs to the category of multifocal degeneration with cerebellar component [26]. On the other hand, SLC25A12 encodes the protein aralar, which is a Ca^{2+} activated aspartate-glutamate carrier on the inner mitochondrial membrane [73,74]. As an IEM, such defects belong to the disorders of energy metabolism [15]. Further characterization of the phenotype and its progression are still ongoing and will hopefully reveal if a ketogenic diet might have a similar beneficial effect in dogs, as was seen in a human patient [75].

For the next study, we investigated a phenotype in Bullmastiffs, which had first been described 40 years ago [76,77]. The two contemporary cases of Bullmastiffs with gait and behavioural abnormalities that we received for analysis from two apparently unrelated litters matched the old phenotype description perfectly. Our variant calling pipeline [78] identified one single private protein changing variant, that was located in a gene known to cause a neurological phenotype in humans or other animals in the sequenced case. The variant was located in the *MFF* gene, and loss of function variants in this gene result in a neurological phenotype with elongated tubular mitochondria in humans, as their mitochondria can still fuse, while fission is impaired [79,80]. This fit very well with the abnormal mitochondria that where described in old Bullmastiff cases [77], which is why we obtained samples of two additional cases, which were seen in 1996 and where closely related to the dogs from the second case description [77]. Indeed, those two cases where homozygous for the same *MFF* frameshift variant, thus creating a link to the initial case descriptions. We extrapolated the human guidelines for the interpretation of sequence variants [81] to dogs for the classification of the variant: Firstly, a predicted null variant in a gene where LOF is a known mechanism of disease was identified. This was a very strong argument for pathogenicity. Next, the phenotype involving changes in

mitochondrial morphology together with neurological changes was highly specific for variants in the *MFF* gene. This argument is seen as supporting [81]. Lastly, the absence of the variant in a comparatively large population database of 782 dogs of diverse breeds, and the segregation of the variant with the disease phenotype in four cases and over 70 breed matched controls can be counted as a moderate and supporting argument, respectively. However, these two points have to be evaluated with much care, not only because numbers in human genetic research are generally much larger, but also because the population structures of purebred dogs with their often low diversity within breeds [82,83] and large diversity across breeds [2] cannot be compared so easily to the general human population [84]. Nevertheless, we were able to present one very strong, one moderate, and two supporting arguments for pathogenicity, which is one more argument than would be needed to classify a human LOF variant as pathogenic (one very strong + two supporting as a minimum) [81]. Apart from the developed genetic test, dogs affected by mitochondrial fission encephalopathy might also serve as a spontaneous large animal model to gain more insight into human mitochondrial fission dysfunction.

Apart from fission and fusion processes, which have a direct influence on mitochondrial homeostasis [85], another group of metabolic diseases affecting mitochondrial energy metabolism are defects in fatty acid beta-oxidation [86]. The most frequently diagnosed of these diseases in humans is medium-chain acyl-CoA dehydrogenase (MCAD) deficiency [87]. In dogs, the only genetically resolved fatty acid beta-oxidation disorder thus far had been a defect in *ACADVL* in German Hunting dogs with exercise induced metabolic myopathy [88]. For this project, we initially received the sample of a single Cavalier King Charles Spaniel (CKCS) affected with complex focal seizures, prolonged lethargy, and proprioceptive ataxia. Acylcarnitine profiling was done in the dog and was consistent with MCAD deficiency. Subsequently, WGS of the case with filtering for private protein changing variants revealed a probably causative variant in *ACADM*. Rather surprisingly, genotyping of all CKCS in our biobank thereafter uncovered that twelve additional dogs were homozygous for the same variant. To test if these additional dogs were affected by MCAD deficiency as well, we followed a similar approach that proofed to be useful in the *SELENOP* project and sent blood samples of five dogs per genotype for acylcarnitine measurements. When taking human diagnostic criteria, this indeed revealed highly elevated acylcarnitine biomarkers and diagnostic ratios in all homozygous dogs, as compared to wild types or carriers [89,90]. Even though we were able to conclude that MCAD deficiency in this breed is caused by an *ACADM* variant, we could not define, in what percentage of the CKCS population with MCAD deficiency neurological signs or epilepsy arise. This might be a demanding question, as the breed is also predisposed for the occurrence of Chiari-like malformation and syringomyelia, which can result in similar

neurological signs [91]. We hope that our results will now open the doors to new prospective clinical studies that aim to disentangle the relationship between these phenotypes.

The two mixed breed dogs with inability to feel noxious stimuli, severe secondary injuries, and a missense variant in the HSAN/CIP candidate gene *SCN9A* were a good example of a genetic diagnosis complementing a thorough clinical investigation. Even though the findings in the two cases do not justify widespread genetic testing, it might become a useful tool to test novel cases of impaired pain perception, thus helping in establishing a diagnosis and pointing to a breed origin of the discovered variant.

Lastly, we received four Weimaraner dogs from three different litters, all with reportedly healthy parents, and all suffering from a paroxysmal movement disorder. The clinical signs included episodes of abnormal gait characterized by ataxia, hypermetria and dystonia. None of the additionally used diagnostic tools (e.g., organic acid analysis and MRI) were remarkable. As we did not get all four cases at the same time, we did not consider SNV-Chip genotyping and homozygosity mapping, as would usually be the case with multiple unrelated cases. Instead, all four private protein changing variants that were detected after WGS analysis of one dog were genotyped in all four cases. Under the hypothesis, that the same phenotype in one breed is likely caused by a single recessive variant, three of the four variants could be excluded. The only variant that was homozygous in all four cases, as well as not homozygous in any of the control dogs, was a frameshift in *TNR*. This variant also was the only predicted protein change, which was located in a functional candidate gene for a human neurological disorder (OMIM #619653). As the gene additionally has been implicated in familiar Parkinson's disease in humans [92,93], this spontaneous dog model might provide valuable insights for future human research in this topic. Additionally, this dystonia-ataxia syndrome without visible MRI changes might be the first canine hereditary episodic ataxia model with unravelled genetic background [26]. However, to verify this classification it remains to be seen if the affected dogs show any histopathological changes in the brain.

In summary, I was able to find seven candidate causative variants for diverse neurological phenotypes in different dog breeds (Table 5). All described phenotypes are inherited in a fully penetrant autosomal recessive manner. This finding underlines the described enrichment of recessive alleles that is typical for the reduced genetic diversity in purebred dogs [4,94,95]. In all but one of the herein described diseases, genetic testing has already been introduced or will be introduced by genotyping companies in the near future [96]. Such direct tests are a valuable tool for breeders to improve animal health and welfare in their respective breeds, as they are highly specific [97]. However, they need to be used with caution, as elimination of all carriers for a disease may create further genetic bottlenecks and thus may lead to emergence of new deleterious alleles. Rather, a slow and continual decrease in allele frequency is advised

(mating of a carriers with a wild type animal is still possible) and has already proven to have a positive effect in effectively eliminating affected dogs for certain diseases [95].

Table 5. List of candidate causative variants in the seven as part of this thesis investigated phenotypes and the corresponding new OMIA entries, plus diseases (OMIM#) caused by variants in their human orthologs. Mode of inheritance is left out, as it was autosomal recessive for all investigated cases.

Gene	**VT**[1]	**OMIA#** [30]	**Breed**	**Corresponding Human diseases**	**OMIM#** [31]
Hereditary ataxia – multifocal degenerations with predominant (spino)cerebellar component					
SELENOP	WGD	002367-9615	Belgian Shepherd dog	-	601484
SLC25A12	MS	002294-9615	NSDTR	Developmental and epileptic encephalopathy 39	612949
Hereditary ataxia – episodic ataxia (putative)					
TNR	FS	002663-9615	Weimaraner	Nonprogressive neurodevelopmental disorder with spasticity and transient opisthotonus	619653
HSAN / CIP					
SCN9A	MS	002616-9615	Mixed breed dog	Primary erythermalgia/ Small fiber neuropathy, AD CIP/ HSAN type 2D, AR Paroxysmal extreme pain disorder, AD	133020 243000 167400
IEM – disorders involving energy metabolism					
ACADM	FS	002585-9615	CKCS	MCAD deficiency	201450
MFF	FS	002551-9615	Bullmastiff	Encephalopathy due to defective mitochondrial and peroxisomal fission 2	617086
MYO5A	FS	001501-9615	Miniature Dachshund	Griscelli syndrome type 1	214450

[1]VT: variant type; FS: frameshift, MS: missense, WGD: Whole gene deletion

Before publication of the results, my projects mostly ended with the discovery of candidate causative variants and the genotyping of as big a cohort of breed matched healthy control dogs as possible to strengthen the genetic association. We were able to proof that the variants in *SELENOP* and *ACADM* lead to selenium deficiency and MCAD deficiency, respectively. However, the connection between those deficiencies and the seen neurological phenotypes is still only associative. For fully penetrant recessive alleles, where altered protein function can be predicted with high certainty, this might be enough to go on with the development of a genetic test. However, the prediction of impact for non-coding variants is by far more challenging and additional data on structure and expression of the investigated gene/ protein are needed [8,98]. Experiments in cell lines of affected dogs or with gene edited model organisms are then the last step to obtain effective proof of causality of a genetic variant [11,99].

In conclusion, a combination of NGS based techniques and validation/ genotyping via Sanger Sequencing were used to report the underlying genetic basis of seven rare inherited neurological disorders in dogs. Our results can be used by dog breeders in form of genetic tests that are commercially available for the respective dog breeds. Thus, the described conditions may be eliminated, or at least no more affected dogs are accidentally bred. Additionally, *SELENOP* was identified as a potential candidate gene for yet unsolved genetic movement disorders in human patients. Further research into more canine hereditary phenotypes will remain indispensable in the future, as the canine population structure favours the spontaneous emergence of new recessive diseases.

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Curriculum vitae

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