A dog model for centronuclear myopathy (CNM) carrying the most common DNM2 mutation

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Keywords: Neuromuscular disorder, congenital myopathy, dynamin, large animal model, T-tubules, MTM1

Summary statement: Description of a spontaneous dog model and generation of a cohort carrying the most common DNM2 mutation associated with autosomal dominant centronuclear myopathy (ADCNM) in humans.
ABSTRACT

Mutations in \textit{DNM2} cause autosomal dominant centronuclear myopathy (ADCNM), a rare disease characterized by skeletal muscle weakness and structural anomalies of the myofibres including nuclear centralization and mitochondrial mispositioning. Following the clinical report of a Border Collie male with exercise intolerance and histopathological hallmarks of CNM on the muscle biopsy, we identified the c.1393C>T (R465W) mutation in \textit{DNM2}, corresponding to the most common ADCNM mutation in humans. In order to establish a large animal model for longitudinal and preclinical studies on the muscle disorder, we collected sperm samples from the Border Collie male and generated a dog cohort for subsequent clinical, genetic, and histological investigations. Four of the five offspring carried the \textit{DNM2} mutation and showed muscle atrophy and a mildly impaired gait. Morphological examinations of transverse muscle sections revealed CNM-typical fibres with centralized nuclei and remodelling of the mitochondrial network. Overall, the DNM2-CNМ dog represents a faithful animal model for the human disorder, allows the investigation of ADCNM disease progression, and constitutes a valuable complementary tool to validate innovative therapies established in mice.

INTRODUCTION

Cellular membranes undergo constant shape remodelling through curvature, tubulation, constriction, and fission to enable fundamental biological processes such as cytokinesis, migration, endocytosis, phagocytosis, signalling, intracellular trafficking, recycling, or compartmentalization (McMahon and Gallop, 2005). These dynamic events rely on the concerted interplay of lipids, proteins, and the cytoskeleton, and one of the key factors of membrane remodelling is dynamin 2 (DNM2) (Ferguson and De Camilli, 2012). This ubiquitously expressed mechanochemical enzyme is able to reorganize the microtubule and actin networks, and self-assembles into helical structures at the neck of nascent vesicles to induce vesicle release under GTP hydrolysis (Antonny et al., 2016; Chappie et al., 2009; Gu et al., 2010; Warnock et al., 1997).

Dynamin 2 is composed of an N-terminal GTPase domain, a middle domain (MID), a phospholipid-binding pleckstrin homology domain (PH), a GTPase effector domain (GED) and a C-terminal proline-arginine-rich domain (PRD) implicated in protein-protein...
interactions (Faelber et al., 2011; Ferguson and De Camilli, 2012). Mutations in the DNM2
gene are associated with three distinct autosomal dominant neuromuscular disorders - Charcot-Marie-Tooth neuropathy (CMT1B, MIM# 606482), spastic paraplegia (Sambuughin et al., 2015) and centronuclear myopathy (ADCNM, MIM# 160150), the predominant CNM form in adult patients (Bitoun et al., 2005; Zuchner et al., 2005). ADCNM is characterized by generalized muscle weakness, ptosis, and ophthalmoplegia, and muscle biopsies from affected individuals typically show fibre size heterogeneity, abnormal nuclear centralization, mitochondrial mispositioning, and radial arrangement of sarcoplasmic strands (Bitoun et al., 2005). The age of onset and disease severity ranges from severe neonatal hypotonia and respiratory distress to mild adult-onset muscle weakness, and correlates with the position of the mutation. To date, more than 100 families and about 20 different ADCNM-related DNM2 mutations have been reported, essentially clustering in hot spot regions in exons 8, 11, 14, 15, and 16 (Abath Neto et al., 2015; Bohm et al., 2012; Werlauff et al., 2015). The most common mutation c.1393C>T resides in exon 11, affects approximately 25% of the cases, leads to the amino acid substitution p.Arg465Trp (R465W) in the MID domain, and most often results in a moderate clinical presentation involving childhood-onset and slowly progressive distal muscle weakness (Bohm et al., 2012). In accordance with the clinical and histological presentation of the patients, the Dnm2<sup>R465W/c</sup> knock-in mouse model manifests reduced force associated with abnormal muscle structure and function (Durieux et al., 2010). Here we describe a spontaneous canine model harbouring the DNM2 R465W mutation and the generation of a dog cohort with clinical and histopathological characteristics paralleling the human disorder.

RESULTS

Identification of the DNM2 c. 1393C>T (R465W) mutation in a Border Collie

A 2-year-old Border Collie male presented with a 1-year history of exercise-induced pelvic limb collapse, short-strided and stiff gait, and morphological analysis of a muscle biopsy uncovered histopathological anomalies suggestive of CNM (Eminaga et al., 2012). We therefore Sanger-sequenced the known canine CNM genes HACD1 (previously named PTPLA), MTM1, and BIN1. An insertion of the SINE retrotransposon in HACD1 exon 2 is associated with CNM in Labrador retrievers (Pele et al., 2005), and missense and splice site mutations in MTM1 and BIN1 were respectively shown to cause CNM in Labrador retrievers,
Rottweilers, Boykin spaniels, and Great Danes (Beggs et al., 2010; Bohm et al., 2013; Olby et al., 2020; Shelton et al., 2015). However, no pathogenic DNA variant was found in these genes. Finally, Sanger sequencing of the autosomal gene DNM2 on chromosome 20 disclosed the heterozygous c.1393C>T (R465W) transition in exon 11, corresponding to the most common ADCNM mutation in humans (Fig. 1A).

**Generation of a dog cohort - clinical and histopathological characterization**

To confirm the pathogenicity of the identified DNM2 mutation in dogs and to establish a large and relevant animal model for long-term studies on disease development and the evaluation of innovative therapeutic approaches, sperm was collected from the Border Collie male to inseminate a Beagle female. The resulting litter of two female and three male pups were genotyped and underwent thorough clinical follow-up over 12 months. Biopsies from the tibialis cranialis and biceps femoris muscles, both easily accessible and extensively studied in centronuclear myopathies, were taken at one year of age and used for protein extraction and histological investigations.

From the five offspring, four were found to carry the c.1393C>T (R465W) missense mutation in DNM2 (Fig. 1A). In accordance with human patients harbouring the same R465W substitution (Bitoun et al., 2005), a western blot on muscle extracts detected the dynamin 2 protein in the dogs. Despite variable signal intensities among the samples, quantification revealed comparable dynamin 2 protein levels in affected and control dogs (Fig. 1B), confirming that the identified missense mutation does not impair mRNA or protein stability.

At 12 months of age, all DNM2\textsuperscript{R465W/+} dogs showed general muscle atrophy particularly affecting the masticatory and paraspinal muscles (Fig. 1C). Transcutaneous ultrasound confirmed atrophy of the biceps femoris and sartorius cranialis muscles, and revealed enhanced echo intensities suggesting an alteration of the muscle texture (Fig. 1D-E). The affected dogs had increasing difficulties with jumping and standing on the pelvic limbs (Fig. S1), and gait analysis evidenced subtle anomalies and notably a reduced craniocaudal power pointing to a decreased forward propulsion (Fig. 1F). Complete blood counts and routine biochemistry profiles were within the reference ranges, and serum creatine kinase (CK) levels were normal (85±18 U.I/l compared with 87 U.I/l in the healthy littermate).
Histological and histochemical examinations of transverse sections of *tibialis cranialis* and *biceps femoris* muscles disclosed fibre size variability, endomysial enlargement, and fibres with centralized nuclei in the $DNM2^{R465W/+}$ dogs (Fig. 2A). We also observed major cytoplasmic rearrangements such as central and subsarcolemmal accumulations on H&E, oxidative staining, and COX assay, indicating a remodelled mitochondrial network in 19% to 57% of the myofibers (Fig. 2A-C). In addition, the fibre diameter was significantly reduced in the $DNM2^{R465W/+}$ dogs compared with the control littermate (Fig. 2D). Overall and based on the cumulative anomalies on the muscle sections, the histopathology index was increased by a factor of 3 to 5 in $DNM2^{R465W/+}$ dogs (Fig. 2B).

Taken together, the clinical and histopathological features of the $DNM2^{R465W/+}$ dogs were highly consistent and conformed to the disease signs of the Border Collie male (Eminaga et al., 2012). Our data confirmed dominant disease transmission and the causality and full penetrance of the R465W mutation in the development of a mild and slowly progressive myopathy in dogs.

**DISCUSSION**

The present study describes the first canine model for ADCNM, and the affected dogs carrying the $DNM2$ c.1393C>T (R465W) mutation showed muscle weakness and ADCNM-typical morphological anomalies on muscle sections. Hence, we propose that $DNM2^{R465W/+}$ dogs can be alternatively named DNM2-CNM dogs.

**R465W in humans, mice, and dogs**

In humans, $DNM2$ mutations are the primary cause of ADCNM (Bitoun et al., 2005; Bohm et al., 2012), and investigations in cell and animal models suggest that the mutations involve a gain-of-function (GoF) mechanism. Indeed, ADCNM-related DNM2 mutants were shown to form oligomers with increased stability (Wang et al., 2010), and their exogenous expression in mice compromised skeletal muscle force and structure (Massana Munoz et al., 2019).

R465W is the most common dynamin 2 mutation. It has been reported in more than 30 families so far, and is associated with inter- and intrafamilial variability (Bohm et al., 2012). Although the first signs of muscle weakness usually appear during childhood, patients with
neonatal and adult disease-onset and a varying degree of muscle weakness, facial weakness, and eye movement defects have been described, too (Bohm et al., 2012). A Dnm2<sup>R465W/+</sup> mouse model also exists and exhibits a mildly progressive muscle weakness and atrophy from three weeks of age, and histological anomalies of the sarcoplasmic reticulum and mitochondrial distribution from two months of age (Durieux et al., 2010). Increased nuclear centralization is however not detectable in murine Dnm2<sup>R465W/+</sup> muscles, contrasting the morphological muscle aberrations in DNM2-CNM dogs and patients. This may be related to the dissimilar muscle size and associated mechanical tension in the species, or might reflect physiological differences in muscle fibre development, maturation, or maintenance, and highlights the importance of the DNM2-CNM dogs for further investigations on disease development and the underlying pathomechanisms.

CNM is a genetically heterogenous disease with X-linked, autosomal dominant, and autosomal recessive forms essentially and respectively caused by mutations in MTM1, DNM2, and BIN1 (Bitoun et al., 2005; Bohm et al., 2014; Laporte et al., 1996; Nicot et al., 2007). Spontaneous canine MTM1 and BIN1 models were previously reported, and all recapitulated the human disorders at the clinical and histopathological level with severe muscle atrophy, swallowing difficulties, and a rapidly progressive tetraparesis. These major functional deficits required daily veterinary support and most often necessitated compassionate euthanasia between three and six months of age, preventing longitudinal studies (Beggs et al., 2010; Bohm et al., 2013; Olby et al., 2020; Shelton et al., 2015). Of note, none of the affected Labrador Retrievers, Rottweilers, and Boykin Spaniels carried mutations found in patients. By contrast, the dogs described in the present study harbour the most common ADCNM mutation diagnosed in 25% of the patients, and the slowly progressive disease course enables a large panel of molecular investigations at different time points to decipher the etiopathology and implicated pathways, and to identify relevant therapeutic targets for the prevention or reversal of the muscle phenotype.

The importance of dogs for preclinical trials

Dogs represent valuable tools to complement the continuum of preclinical animal models dedicated to the establishment and evaluation of innovative therapeutic approaches in neuromuscular disorders (Barthelemy et al., 2019; Story et al., 2020). The downregulation of dynamin 2 through antisense oligonucleotides (ASOs) was shown to rescue the DNM2-related
CNM phenotype in mice (Buono et al., 2018), and the application of this and other therapeutic strategies to a larger mammalian model with longer lifespan like the DNM2-CNM dogs would provide important information on drug delivery options, pharmacokinetics, bioavailability, efficacy, durability, or tolerability after sustained administration. In analogy, an AAV-mediated gene therapy for X-linked CNM (XLCNM) was first proven to be efficient on Mtm1 knockout mice (Buj-Bello et al., 2008) and validated on a spontaneous canine XLCNM model (Childers et al., 2014) prior to its usage in clinical trials (NCT03199469). Moreover, dog models served to establish exon skipping, genome editing, and minigene expression strategies for Duchenne muscular dystrophy (DMD) (Amoasii et al., 2018; Koo et al., 2011; Vulin et al., 2012), and have also been used for preclinical proofs-of-concept of disorders affecting other tissues and organs.

In conclusion, the DNM2-CNM dog is a faithful model for the human disorder, allows longitudinal investigations to decipher the sequence of events leading to the muscle dysfunction, and represents an optimal complementary system to assess the safety and efficacy of therapeutic approaches before the translation to humans.

**MATERIALS AND METHODS**

**DNA analysis**

Genomic DNA from the Border Collie male was prepared from peripheral blood by routine procedures, and Sanger-sequenced for HACD1 exon 2 and all coding exons and the adjacent splice elements of MTM1, BIN1, and DNM2. The five offspring were Sanger-sequenced for DNM2 exon 11 for genotyping using forward (TGCTTGTCTCCAGCTGCAG) and reverse (TGGTACCTTGACTGAGGTG) primers. The identified DNM2 mutation was numbered according to GenBank XM_005632882.3 and XP_005632939.1.

**Ethics, animals, and establishment of a colony**

The establishment of the dog colony and animal experimentation were in accordance with the European Community Standards and were performed with approval of the ethics committee of EnvA, the University of Paris Est, and the Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES, 20/12/12-18). The project was
authorized by the Ministère de l'enseignement supérieur, de la recherche et de l'innovation (APAFIS #2018010910531134).

Sperm samples from the Border Collie male were collected in the United Kingdom with consent from the owner, and used to inseminate a Beagle female at Ecole nationale vétérinaire d'Alfort (EnvA, France). The resulting litter of two female and three male pups underwent regular clinical examinations and blood sampling. Muscle biopsies (biceps femoris and tibialis cranialis muscles) were performed under a propofol-induced, isoflurane/morphine-maintained anaesthesia/analgesia.

**Muscle ultrasonography**

The biceps femoris and sartorius cranialis muscles were longitudinally imaged using a linear 12.5-5 MHz transducer. Imaging depth was set at 4 cm, and muscle thickness was assessed with the internal measurement tool of the ultrasound Philips HD7 scanner (Philips, Amsterdam, The Netherlands). Echo intensity was determined in Image J as the mean grey level on the histogram of the muscle after drawing a region of interest. Three images were acquired per muscle and per dog, and the mean values were used for analysis.

**Accelerometry**

Gait analysis was performed using 3D-accelerometry as previously described (Barthelemy et al., 2009). Briefly, a device containing three orthogonally positioned Locometrix® accelerometers (Centaure Metrix, Evry, France) was inserted into a belt tightened around the thorax of the dogs. All dogs were evaluated at a trot. Accelerometric curves were acquired at 50 Hz along the cranio-caudal, dorso-ventral and medio-lateral gait axes, and data were analysed using the Equimetrix® software (Centaure Metrix) to calculate following parameters: stride length, stride frequency, speed, regularity, generated power for each axis, and total power. Generated power of each axis was expressed as percentage of the total power.
**Protein studies**

For western blot, protein extracts from muscle samples were loaded on a 10% SDS PAGE, and membranes were incubated with homemade rabbit anti-dynamin 2 (R2865; diluted 1:700) and rabbit anti-calnexin (C-4731; Sigma-Aldrich, St. Louis, USA; diluted 1:1,000), as well as with peroxidase-coupled goat anti-rabbit antibodies (111-035-144; Jackson ImmunoResearch, West Grove, USA; diluted 1:10,000). The immunoblots were revealed with the Supersignal west pico kit (ThermoFisher Scientific, Waltham, USA), and monitored on the Amersham Imager 600 (GE Healthcare Life Sciences, Chicago, USA). Quantification of band intensity was performed using the Measurement Log plugin of Adobe Photoshop 2022, version 23.1.0. The DNM2/calnexin ratio of the integrated grey level density was calculated for each sample and expressed as the percentage of the average ratio in control dogs. The individual ratio values were plotted using Prism 9 for MacOS, version 9.3.1.

**Muscle morphology**

*Biceps femoris* and *tibialis cranialis* muscle biopsies were taken at 12 months of age. Transverse sections (10 µm) were stained with Hematoxylin & Eosin (H&E), Sirius Red, modified Gomori trichrome, NADH tetrazolium reductase (NADH-TR), and COX, and assessed for fibre morphology, accumulation/infiltrations, and oxidative activity. The myofiber diameter was determined using an Image J-developed macro script (Reyes-Fernandez et al., 2019) and is defined as the minimum Feret on sections immune-stained with a rabbit anti-Caveolin antibody (ab173575; Abcam, Cambridge, UK). Quantitative analyses were performed on H&E staining. Entire muscle sections were analysed using the Visilog 7.0 software (Noesis, Orsay, France). A grid was superimposed onto the image, and muscle morphology was assessed at each of the intercepts and manually annotated (1000 annotations per section) as previously described (Spencer et al., 2001). The histopathology index corresponds to the percentage of pathological features not corresponding to normal myofibers.
ACKNOWLEDGEMENTS

We are grateful to the owner of the Border Collie for his approval to sample the dog and support biomedical research. We thank Carole Drougard and Rémi Régnard for their technical help, and Angelika von Heimendahl (Veterinary Reproduction Service, Cambridge, UK), Dr. Cindy Maenhoudt, Dr. Juliette Roos and Prof. Alain Fontbonne (CERCA, EnvA, Maisons-Alfort, France) for their expertise in reproductive technologies. We also thank Xavier Cauchois, Axelle Lebouvier, Aïchana Islam, Hans Colnot and Lynda Bourguignon (CARE-NMD platform, EnvA, Maisons-Alfort, France) for their daily care of the dogs.

FUNDING

This work was supported by the Fondation Maladies Rares (call "Preclinical Research in rare diseases: translational steps in large animals"), the Association Française contre les Myopathies (AFM-Téléthon; Translamuscle I (# 19507) and Translamuscle II (# 22946)), Inserm, CNRS, University of Strasbourg and the ANR (ANR-10-LABX-0030-INRT under the frame program Investissements d’Avenir ANR-10-IDEX-0002-02).

ETHICAL APPROVAL

Following the acceptance of the project by the local EnvA-UPEC-ANSES ethical committee (approval number 13/02/18-1, ethical committee number 16), the Ministère de l’enseignement supérieur, de la recherche et de l’innovation authorized the project (APAFIS #2018010910531134).

COMPETING INTERESTS

JL is co-founder of Dynacure.

AVAILABILITY OF DATA AND MATERIAL

All data generated or analysed during this study are included in this published article.
AUTHOR CONTRIBUTIONS

IB, SB, JL and LT designed and coordinated the project and obtained funding; JB, IB, CD, CL, NB-G performed the experiments; JB, IB, SB analysed the data; JB drafted the manuscript with help from IB, LT, and JL.

REFERENCES


Figure 1: Molecular, morphological, and functional features of canine DNM2-CNM. (A) Pedigree, segregation, and electropherogram showing the DNM2 mutation. (B) Western blot on tibialis cranialis muscle extracts revealed DNM2 protein in affected dogs (lanes 1-4, black symbols), the healthy littermate (lane 5, open circle) and in an age-matched Golden Retriever control (lane 6, open square). Calnexin served as a loading control. Quantification of the DNM2 protein level, normalised to calnexin, is shown below each lane. (C) Representative pictures of an affected dog and healthy littermate at 12 months of age. Note the marked
atrophy of the masticatory and paraspinal muscles (arrowheads). (D) Ultrasound imaging providing a longitudinal view of the biceps femoris muscle and revealing a reduced muscle thickness and an increased echo intensity in an affected dog. * = skin. (E) Graphs illustrating the reduced thickness and higher echo intensity values of the biceps femoris and sartorius muscles of the affected dogs. (F) Gait analysis through accelerometry at 12 months uncovered a lower relative craniocaudal power in all four affected dogs compared with the healthy littermate. Black squares = affected males, black circle = affected female, white circle = healthy female littermate. Black squares = affected males, black circle = affected female, white circle = healthy female littermate. Dashed horizontal lines represent mean values of the four affected dogs, and the error bars indicate standard deviation.
Figure 2: Histological features of canine DNM2-CNM. (A) Histological and histochemical analyses on transverse *tibialis cranialis* sections at 12 months of age uncovered fibre atrophy and centralized nuclei (arrowhead) on H&E staining, endomysial fibrosis on Sirius red, and prominent central or subsarcolemmal accumulations of mitochondria (arrowheads) on Gomori trichrome, NADH-TR, and COX in the affected dogs. Muscle samples from the healthy littermate served as control. Scale bars of 50 µm apply to entire columns. (B) Histopathology index calculated on transverse sections of the *biceps femoris* and *tibialis cranialis* muscles (C) Percentage of fibres with cytoplasmic rearrangements calculated on transverse sections of the
*biceps femoris* and *tibialis cranialis* muscles (D) Minimum Feret diameter of fibres calculated on transverse sections of the *biceps femoris* and *tibialis cranialis* muscles. Black squares = affected males, black circle = affected female, white circle = healthy female littermate. Symbol position on the graphs correspond to mean, and error bars represent standard deviation.
Movie 1. Movement features of canine DNM2-CNM.

Video captures of two dogs at 12 months of age. The first dog is the healthy female littermate, alert, fast in her U-turns, and able to jump easily upon stimulation. The second dog is an affected male, also alert but with a slightly stiff gait, less ease in the U-turns, and with a greater difficulty standing on his pelvic limbs.