Mitochondrially Inherited Sensory Ataxic Neuropathy in Golden Retriever Dogs

Phenotype, Clinical Course and Genotype of a Novel Neurological Syndrome

Karin Hultin Jäderlund
Faculty of Veterinary Medicine and Animal Science
Department of Clinical Sciences
Uppsala

Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2009

Acta Universitatis agriculturae Sueciae
2009:73

Cover: A 5 year-old Golden Retriever dog affected by mitochondrially inherited sensory ataxic neuropathy.
(photo: M. Ek)

ISSN 1652-6880
ISBN 978-91-576-7420-3

Print: SLU Service/Repro, Uppsala 2009

Mitochondrially Inherited Sensory Ataxic Neuropathy in Golden Retriever Dogs. Phenotype, Clinical Course and Genotype of a Novel Neurological Syndrome

Abstract

A novel neurological syndrome, mitochondrially inherited sensory ataxic neuropathy (SAN), was discovered in Golden Retriever dogs in Sweden. The purpose of the work described in the present thesis was to investigate the phenotype, clinical course and genotype of this syndrome by clinical, neurological and pathological examination of affected dogs, as well as to determine the mode of inheritance and identify the causative mutation.

Mitochondrially inherited SAN in Golden Retriever dogs has an insidious onset during puppyhood. Affected dogs develop ataxia and dysmetria, with abnormal postural reactions and depressed spinal reflexes.

The disease has a chronic, slowly progressive clinical course. Of the affected dogs investigated within the scope of this thesis, none became non-ambulatory or died spontaneously during the study period. However, about half of the affected dogs were euthanized because of neurological impairment before attaining 4 years of age.

Post mortem examinations of affected dogs revealed degenerative changes in both the central and the peripheral nervous system. A chronic active central–peripheral axonopathy, neuroaxonal dystrophy-like alterations in spinal cord and brainstem, and a neuron-sparing encephalopathy with spongiosis in the basal nuclei were the most prominent findings.

A maternal mode of inheritance was concluded from pedigree analysis, indicating a causative mutation in the mitochondrial DNA. Laboratory data confirmed that affected dogs had malfunctioning mitochondria. A single base-pair deletion in the mitochondrial *t*RNA^{Tyr} gene was found and proven to be pathogenic.

In summary, canine SAN is a slowly progressive neurodegenerative disease with onset in puppyhood. The disease is maternally inherited and is caused by a mutation in the mitochondrial *t*RNA^{Tyr} gene.

Keywords: ataxia, axonopathy, dog, encephalomyelopathy, Golden Retriever, hyporeflexia, inherited, mitochondrial, mtDNA-mutation, neurodegeneration.

Author: Karin Hultin Jäderlund, SLU, Department of Clinical Sciences, Uppsala, Sweden. *Current address:* Norwegian School of Veterinary Science, Department of Companion Animal Clinical Sciences, Postboks 8146 Dep, 0033 Oslo, Norway.

E-mail: karinhultin.jaderlund@nvh.no

Contents

List of Publications

This thesis is based on the work contained in the following papers, which are referred to by Roman numerals in the text:

I Hultin Jäderlund, K., Örvind, E., Johnsson, E., Matiasek, K., Hahn, C., Malm, S. & Hedhammar, Å. (2007). A Neurologic Syndrome in Golden Retrievers Presenting as a Sensory Ataxic Neuropathy. *Journal of Veterinary Internal Medicine* 21, 1307-15.

II Baranowska, I., Hultin Jäderlund, K., Nennesmo, I., Holmqvist, E., Heidrich, N., Larsson, N.-G., Andersson, G., Wagner, E. G. H., Hedhammar, Å., Wibom, R. & Andersson, L. (2009). Sensory Ataxic Neuropathy in Golden Retriever Dogs is Caused by a Deletion in the Mitochondrial $tRNA^{Tyr}$ Gene. *PLoS Genetics* 5(5): e1000499. doi:10.1371/journal.pgen.1000499

III Hultin Jäderlund, K., Baranowska, I., Hedhammar, Å. & Matiasek, K. Neuropathological features in Golden Retriever dogs with a mutation in the mitochondrial $tRNA^{Tyr}$ gene. (manuscript)

IV Hultin Jäderlund, K., Baranowska, I., Örvind, E., Matiasek, K., Johnsson, E., Egenvall, A. & Hedhammar, Å. Follow-up study of dogs carrying the mutation for mitochondrially inherited sensory ataxic neuropathy. (manuscript)

Abbreviations

ATP	adenosine-5'-triphosphate
CNS	central nervous system
COX	cytochrome oxidase
CSF	cerebrospinal fluid
Cys	cysteine
DNA	deoxyribonucleic acid
EMG	electromyography
Gln	glutamine
HE	haematoxylin and eosin
MNCS	motor nerve conduction study
mtDNA	mitochondrial DNA
NAD	neuroaxonal dystrophy
PCR	polymerase chain reaction
PNS	peripheral nervous system
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SAN	sensory ataxic neuropathy
SDH	succinate dehydrogenase
SNCV	sensory nerve conduction velocity
SLU	Swedish University of Agricultural Sciences
tRNA	transfer RNA
Tyr	tyrosine
qOLA	quantitative oligonucleotide ligation assay

1 Introduction

Several neurodegenerative diseases with a familial distribution have been described in dogs (Sisó *et al.,* 2006). Typically, such diseases are breed-related with onset of clinical signs in young dogs and clinical features that reflect the nature and distribution of degenerating cells in the nervous system that is unique for each disease. Commonly, rather few cases of each disease have been reported. Even though many diseases are suspected to be inherited, the underlying genetic defects are largely unknown.

A novel canine neurological disease in Golden Retriever dogs was proven to be maternally inherited and so far has been seen only in that breed. For the first time it has been described clinically as well as post mortem. With the features of a mitochondrially inherited syndrome it has implications for health and breeding practice in this breed, and may also serve as a spontaneous model for comparative studies.

1.1 Background

Almost a decade ago a number of Golden Retriever dogs with strikingly similar gait abnormalities were examined by practising veterinarians without attaining either an aetiological or pathological-anatomical diagnosis. The abnormal gait of the affected dogs was described as quite characteristic and with onset during puppyhood. The affected dogs were related to each other on the maternal side. The first clinical case examined by a board-certified neurologist (the author) was admitted to the University Clinic, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden in 2002. The clinical picture of that dog was not in agreement with any other previously described canine disease. Since then, an increasing number of new cases have been admitted for examination. With more dogs examined, the clinical

characteristics of this disease were easier to delineate. The assumption that this disease was inherited maternally was reinforced over time. Studies were initiated of the mitochondrial molecular genetics in the affected families of dogs, and this assumption was later confirmed.

The effects of mutations in mitochondrial deoxyribonucleic acid (DNA) differ in many aspects from those passed on by Mendelian inheritance. A short review of the basic facts of mitochondrial genetics and mitochondrial diseases is therefore presented as an introduction.

1.2 The mitochondrial genome

Mitochondria are cellular organelles, which originated by endosymbiosis of primitive bacteria during the evolution of the eukaryotic cell (Gray, 1993). In the mitochondria, electron transfer and oxidative phosphorylation take place in the respiratory chain, resulting in the production of adenosine-5'-triphosphate (ATP). Mitochondria harbour their own DNA. The sequence of human mitochondrial DNA (mtDNA) was first published in 1981 (Anderson *et al.,* 1981) (Cambridge Reference Sequence), and the first mtDNA mutations causing human diseases were described in 1988 (Holt *et al.,* 1988; Wallace *et al.,* 1988). The complete nucleotide sequence of canine mtDNA was published in 1998 (Kim *et al.,* 1998). Mammalian mtDNA is a circular double-stranded molecule, which contains 37 genes and approximately 16 500 base-pairs. The 37 genes encode 13 protein subunits of the respiratory chain enzymes together with 22 transfer ribonucleic acids (*t*RNAs) and two ribosomal RNAs. All the other subunits of the respiratory chain enzymes, and also the factors involved in replication, transcription and translation of mtDNA, are encoded from the nuclear genome and imported into the mitochondria.

1.3 Mutations in the mitochondrial DNA

Every cell contains hundreds to thousands of mtDNA molecules. The mtDNA replicates irrespective of the cell cycle and replication occurs also in post-mitotic cells. The mutation rate in mtDNA is higher than in nuclear DNA, because of a less effective repair mechanism (Kang & Hamasaki, 2006). Single nucleotide polymorphisms of mtDNA are therefore relatively common, and they are important clues utilized both in phylogenetic research and in forensic medicine. Occasionally, a mutation results in pathogenic effects. Disease-causing mutations in the mtDNA can occur spontaneously in somatic cells and give rise to non-hereditary acquired

diseases. Accumulation of mtDNA mutations has been pointed out in the pathogenesis of the neurodegenerative diseases Alzheimer's disease and Parkinson's disease (Lin & Beal, 2006), as well as being a cause of ageing (Harman, 1956; Harman, 1992) and occurring in neoplastic tissue (Polyak *et al.,* 1998). The mtDNA is transferred maternally from generation to generation. Disease-causing mtDNA mutations can also appear in germ-line cells and thus be inherited.

Mutations of different types occur in the mitochondrial genome. There are, for example, point mutations (a single base-pair substitution), large scale deletions (a loss of a part of the DNA molecule, containing sometimes many thousand base-pairs), and single base-pair deletions associated with clinical disease (http://www.mitomap.org/).

1.4 Heteroplasmy, the threshold effect and the genetic bottleneck

Concerning the role of mtDNA in mitochondrial dysfunction and clinical disease, some important concepts are the phenomena of *heteroplasmy*, the *threshold effect* and the *genetic bottleneck (*Hauswirth & Laipis, 1982; Ashley *et al.,* 1989; Chinnery *et al.,* 1997; Moslemi *et al.,* 1998). Because of the polyploid nature of the mtDNA content in any one cell, both mutant and wild type mtDNA can coexist in the cell, a condition called heteroplasmy. The proportion of mutant mtDNA to total mtDNA is called the mutant load. With just one type of mtDNA present – wild type or mutant – the condition is called homoplasmy. A cell maintains its normal activity of oxidative phosporylation unless a pathogenic mutant mtDNA reaches a certain level, called the threshold effect of heteroplasmy. The number of mitochondria, and hence mtDNA molecules, is decreased dramatically in precursors to primary oocytes (Jansen, 2000), which undergo cell division without mtDNA replication at that stage. By random segregation in primordial germ cells, this phenomenon increases the likelihood of differences in the level of heteroplasmy in mature oocytes from the same heteroplasmic female (Jenuth *et al.,* 1996). Thus, it is possible that a clinically unaffected heteroplasmic mother gives birth to offspring that are severely, mildly or not at all clinically affected. This phenomenon is called the mitochondrial genetic bottleneck.

1.5 Pathogenicity of mutations in the mitochondrial DNA

Because of the relatively large amount of single nucleotide polymorphisms in the mitochondrial genome, a number of criteria should be met before a mutation is determined to be pathogenic. Whenever a disease is associated with a mutation in the mitochondrial genome it is appropriate to consider a) the presence of heteroplasmy for the mutation, b) the correlation between the degree of heteroplasmy and the clinical phenotype, c) histochemical and biochemical evidence of mitochondrial dysfunction in tissue biopsies, and d) the evolutionary conservation of the mutated site in the mtDNA molecule. A pathogenic mutation in a *t*RNA gene is correlated with reduced steady-state levels of the mutated *t*RNA. In addition, a strong indicator that a mutation is pathogenic is evidence for malfunctioning mtDNA in cybrid cells (cell lines depleted of their own mtDNA fused with mitochondria from a patient). (DiMauro & Schon, 2001; McFarland *et al.,* 2004a; DiMauro & Davidzon, 2005).

1.6 Mutations in different types of mitochondrial genes

Mitochondrial dysfunction can develop from mtDNA mutations in *t*RNA genes, ribosomal RNA genes or in protein coding genes. The biochemical expression differs between these mutations. For example, whereas pathogenic mutations in *t*RNA genes influence the majority of complexes in the respiratory chain, mutations in protein coding genes affect primarily single complexes in the chain.

1.7 Diseases from mitochondrial mutations

More than 500 mtDNA mutations and rearrangements associated with diseases in humans have been reported (http://www.mitomap.org/). Mitochondrial disorders comprise a very heterogeneous group of phenotypes. In general, the clinical signs reflect most often a dysfunction of post-mitotic tissues that consume high levels of energy, such as the nervous system and striated muscles. The presenting symptoms include seizures, ataxia, ophthalmoplegia, ptosis, blindness, deafness, muscle weakness and cardiomyopathy, as isolated symptoms or in different syndromic combinations that more or less overlap. The clinical pictures of many diseases associated with mtDNA mutations include also a diversity of other signs, e.g. diabetes mellitus, sideroblastic anaemia, intestinal pseudo-obstruction, ovarian failure and short stature in humans (DiMauro & Davidzon, 2005; Taylor & Turnbull, 2005). Mitochondrial diseases are

among the most common genetic disorders in humans and have been considered a major burden for society (Schaefer *et al.,* 2004).

Several disease phenotypes in humans have been correlated with specific mtDNA mutations, but the relationship between genotype and phenotype for mitochondrial disorders is only partly understood and confusion exists regarding the correlation between disease presentation and genetic background for many cases. One factor partly explaining this relationship is that different levels of heteroplasmy may occur in different parts of the human body (Sciacco *et al.,* 1994; Moslemi *et al.,* 1998; Tanji *et al.,* 2000; Kärppä *et al.,* 2005). Other important contributing factors that determine the phenotype for some mtDNA-related diseases are the nuclear genetic background (Dunbar *et al.,* 1995; Cock *et al.,* 1998; McFarland *et al.,* 2004b; Hudson *et al,* 2005a), interactions with other mitochondrial genes (Fischel-Ghodsian, 1998; Hudson *et al.,* 2007), and environmental factors (Prezant *et al.,* 1993; Estivill *et al.,* 1998; Kirkman *et al.,* 2009).

Mitochondrial dysfunction can also evolve from nuclear DNA mutations. Nuclear DNA encodes many enzymes in the respiratory chain, several factors needed in other biochemical processes in the mitochondria and also factors involved in the replication, transcription, translation and maintenance of mtDNA as well as fission and fusion of the mitochondria. As an example, pathogenic mutations in either of the nuclear genes *POLG*, *C10Orf2* or *ANT1* result in incorrect replication of mtDNA. Secondary to these nuclear DNA mutations, development of pathogenic mutations in the mtDNA may occur because of errors induced by the defective replication process. Human mitochondrial diseases have been correlated with mutations in these nuclear genes (Agostino 2003; Van Goethem *et al.,* 2003; Van Goethem *et al.,* 2004; Hudson *et al.,* 2005b; Gago *et al.,* 2006; Milone *et al.,* 2008).

In animals, only one spontaneous mitochondrially inherited disease has previously been fully revealed genetically: i.e. canine spongiform leucoencephalomyelopathy in Australian Cattle dogs and Shetland Sheepdogs (Li *et al.,* 2006). The underlying genetic defect was a point mutation in the protein-encoding mt-cytochrome *b* gene, which encodes a subunit of the respiratory chain complex III. The associated clinical picture was whole body tremor and inability to ambulate in young puppies, with a progressive clinical course.

1.8 Diagnostics in mitochondrial diseases

In a patient with neurological or neuromuscular clinical signs, the clinical features, family history and/or pathomorphological characteristics may

suggest mitochondrial disease. Besides routine diagnostic work-up of the case, a muscle biopsy for biochemical and microscopic analyses may give valuable information and should be considered before molecular genetic studies are performed.

Preferably, muscle biopsies should be harvested from a muscle rich in oxidative type I fibres and hence rich in mitochondria. In dogs, a high percentage of type I fibres has been found in antigravity muscles (Armstrong *et al.,* 1982). Some of the stains useful for histochemistry are the Gomori's trichrome stain, succinate dehydrogenase (SDH) stain and cytochrome oxidase (COX) stain. Gomori's trichrome highlights mitochondria, giving a picture called "ragged red fibres" when mitochondria have accumulated to a certain point, which is not specific for but may be seen with some human mitochondrial disorders (Bourgeois & Tarnopolsky, 2004). The SDH and COX stain, respectively, reflect the activity of complex II and complex IV of the respiratory chain (Bourgeois & Tarnopolsky, 2004). Complex II is the only complex entirely encoded by the nuclear DNA whereas complex IV contains subunits encoded by the mtDNA. With a combined SDH/COX-stain, fibres coloured blue have SDH activity but lacks COX activity, which is indicative of a mitochondrial disorder. Common findings in muscle biopsies from humans with mtDNA mutations are COX-negative fibres and ragged red fibres (Bourgeois & Tarnopolsky, 2004). Confirmation of such findings in dogs is considered difficult owing to the larger mitochondrial volume in canine muscles compared with those of humans (Wakshlag *et al.,* 2004). Previous reports of COX-negative fibres (Paciello *et al.,* 2003; Tauro *et al.,* 2008) and ragged red fibres (Vijayasarathy *et al.,* 1994; Olby *et al.,* 1997; Paciello *et al.,* 2003) in dogs are rare and only concern single dogs. Electron microscopically, findings of paracrystalline inclusions in mitochondria are characteristic for human adult mitochondrial muscle pathology (Bourgeois & Tarnopolsky, 2004). Moreover, a biochemical diagnosis of a mitochondrial disorder may be established by measurements of the ATP production rate and activity of respiratory chain complexes in muscle mitochondria (Wibom *et al.,* 2002).

2 Aims of the thesis

The overall aim of the present thesis was to investigate a novel neurological syndrome which presented clinically as sensory ataxic neuropathy in the Golden Retriever breed, thereby also establishing an animal model for a spontaneous mitochondrially inherited disease.

The specific aims were:

- To describe and define clinical and post mortem features to serve as inclusion criteria
- To evaluate the clinical course and survival
- To determine the mode of inheritance, to identify the causative mutation in the mitochondrial genome of affected dogs if maternally inherited, and to provide advice on how to reduce its incidence
- To relate the neurological signs to neuropathological changes and molecular mechanisms

3 Materials and methods

The materials and methods used in this study are summarized below. For further details, see papers I–IV.

3.1 Animals

3.1.1 Affected dogs

A total of 27 privately owned Golden Retriever dogs diagnosed with mitochondrially inherited sensory ataxic neuropathy (SAN) serve as case material in this thesis. Of these, 15 were females and 12 males, and at presentation the median age was 16 months (range 6–77 months). The diagnosis was based on the clinical features but was studied further according to the methods below. Twenty of the dogs were examined by the author at the SLU in Uppsala, Sweden, while the remaining seven were included by scrutinizing records from other veterinarians (Table 1, Figure 1).

3.1.2 Maternally related dogs

Thirty-three Golden Retriever dogs (28 females and five males) maternally related to the affected dogs were included in the studies (papers II and IV). Samples from all these dogs were used in molecular genetic analyses. Eleven of these dogs, at a median age of 60 months (range 7–131 months), were examined in a clinical setting similar to that of the affected dogs (Table 1, Figure 1).

3.1.3 Controls

A total of 78 Golden Retriever dogs, 48 females and 30 males, served as controls in different parts of the studies (papers I, II and IV). These dogs were maternally unrelated to affected dogs as far as their pedigrees and

information from the Swedish Kennel Club registry could reveal (http://www.skk.se/). Samples from all these dogs were used in the molecular genetic analyses. Thirteen of those Golden Retriever dogs were admitted to SLU for clinical signs of gait disorder but did not fulfil the clinical diagnostic criteria for SAN. The remainder were clinically healthy dogs with regard to neurological gait disorders. Healthy control dogs used for reference purposes in electrophysiology studies (n=11, papers I and IV) were age-matched to the affected dogs. The control dogs used for biochemical and microscopic analyses of muscle tissue (n=5, paper II) were pair-wise age-matched to the affected dogs. In addition, samples from 86 dogs of 18 other breeds and 6 wolves (see paper II) were used in the molecular genetic analyses (Table 1).

Table 1. *The number of Golden Retriever dogs used for each procedure in this thesis.*

	Affected	Maternally related	Controls
Evaluation because of gait disorder	27	0	13
Neurological exam 1'	27	11	34
Neurological exam 2'	10	1	1
Neurological exam 3'	3	0	0
Electrophysiology 1'	18	4	12
Electrophysiology 2'	6	1	0
Molecular genetics	22	33	78
Post mortem	9	1	0
Muscle biopsy	5	0	5
Video tape	15	0	0
Total	27	33	78

The local Animal Ethics Committee in Uppsala (no. C110/4, C161/5 and C138/6) and the Swedish Animal Welfare Agency (no. 31-1437/04 and 2006-0561) approved the experimental designs. The owners of privately owned dogs all provided written consent to participation in the study. All the 27 affected dogs belonged to 27 different owners. Some owners of control dogs and/or maternally related dogs owned more than one included dog each.

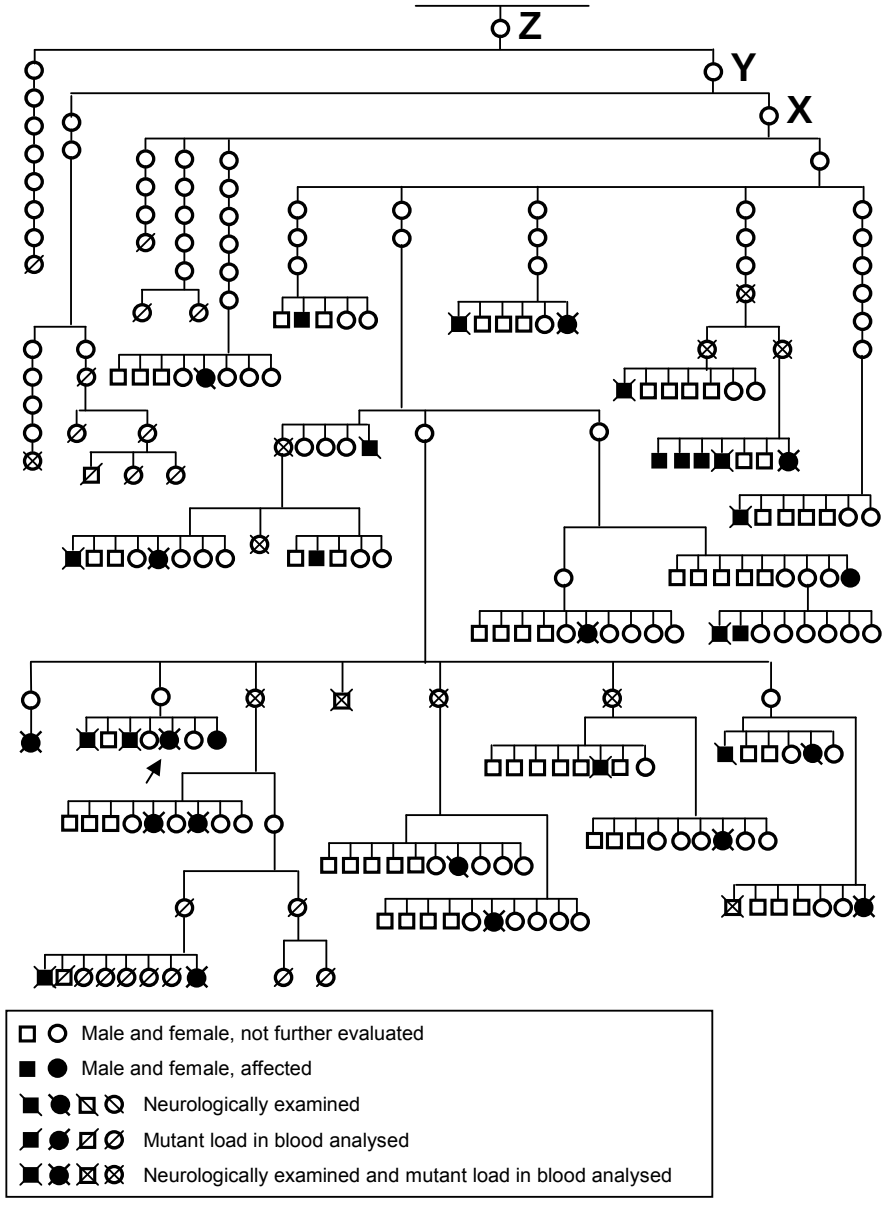

Figure 1. A schematic pedigree of Golden Retriever dogs affected by mitochondrially inherited sensory ataxic neuropathy (SAN) and their relatives. Only maternal lineages with affected or neurologically examined dogs or dogs analysed for mutant load are depicted. Entire litters are included only for affected dogs. Arrow points at index case.

3.2 Clinical procedures

3.2.1 Clinical history

For all affected dogs, information about the presenting clinical signs, age at onset and progression of signs were retrieved from owners and/or breeders and/or referring veterinarians. Also, if the dog had died, the age at and reason for death were documented.

3.2.2 Clinical and neurological examination

The primary investigator performed a full clinical and neurological examination of 20 affected dogs, 11 maternally related Golden Retriever dogs, 13 maternally unrelated Golden Retriever dogs with gait disorders, and 21 maternally unrelated healthy Golden Retriever controls (papers I, II and IV) (Table 1). In the neurological examination, assessment of consciousness, behaviour, posture, gait, cranial nerve functions, postural reactions, spinal reflexes and pain perception were included. Parts of the neurological examination of 15 affected Golden Retriever dogs were video taped to allow simultaneous comparisons between patients (Table 1). Five of those 15 affected dogs were also video taped at a later neurological examination, to allow simultaneous comparisons between occasions. The neurological examination was repeated ≥6 months (range 6–51 months) after the primary examination in 10 affected dogs (Figure 1 in paper IV) (Table 1). The neurological status of these 10 dogs was scored in such a way that a comparison between neurological examinations at different occasions was possible (see paper IV). In the affected cases not examined in person by the author (n=7), the results from clinical and neurological examinations were provided by clinical records from other veterinarians. In addition, serum chemistry, blood lactate analysis, serum antinuclear antibodies, serology for *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum* and *Neospora caninum*, polymerase chain reaction (PCR) for *Anaplasma phagocytophilum* DNA, radiology and cerebrospinal fluid (CSF) analysis were performed in some dogs (Tables 2 and 3). Some of these examinations had already been performed by the referring veterinarians prior to admittance to SLU.

Table 2. *An overview of serum chemistry examinations in dogs affected by mitochondrially inherited sensory ataxic neuropathy and in 1st and 2nd degree relatives.*

Examination	Number of affected dogs examined	Number of samples from affected dogs examined	Number of values below reference ranges	Number of values above reference ranges	Number of 1st and 2nd degree relatives examined	Number of values outside of reference ranges
S-alanine aminotransferase	11	14	n. a.	1	1	0
S-albumin	8	9	4	0	0	–
S-alkaline phosphatase	10	13	n. a.	1	0	–
S-amylase	7	7	n. a.	0	0	–
S-aspartate aminotransferase	19	27	n. a.	3	9	0
S-bile acids	11	13	n. a.	0	0	–
S-blood urea nitrogen	10	12	0	1	1	0
S-calcium	10	11	0	1	0	–
S-cobalamin	1	1	0	0	0	–
S-creatine kinase	19	27	n. a.	3	9	0
S-creatinine	11	14	0	1	0	–
S-chloride	7	7	0	0	1	0
S-copper	7	7	1	0	0	–
S-folic acid	1	1	0	0	0	–
S-fructosamine	13	16	1	1	9	0
S-globulins	9	9	0	0	0	–
S-glucose	5	6	0	2	1	0
S-iron	1	1	0	0	0	–
S-lipase	7	7	n. a.	0	0	–
S-magnesium	7	7	0	1	0	–
S-phosphorus	7	7	1	3	0	–
S-potassium	11	12	0	0	1	0
S-protein electrophoresis	8	8	1	1	0	–
S-sodium	10	11	0	2	1	0
S-total protein	8	9	0	0	0	–
S-troponin	14	15	n. a.	3	9	1, above
S-thyroid stimulating hormone	17	21	n. a.	0	0	–
S-thyroxine (free and total)	17	21	0	0	0	–
S-zinc	8	8	0	3	0	–

n. a. = not applicable

Table 3. *An overview of miscellaneous examinations in dogs affected by mitochondrially inherited sensory ataxic neuropathy.*

Examination	Number of affected dogs examined	Number of results outside of reference range
Venous blood lactate	8	0
Antinuclear antibodies in serum	7	0
Serology *Neospora caninum*	9	0
Serology *Anaplasma phagocytophilum*	12	2
Serology *Borrelia burgdorferi* sensu lato	12	2
PCR *Anaplasma phagocytophilum*	5	0
Myelography	3	0
Plain spinal radiographs	9	0
CSF cytology, cell count, protein	4	0

CSF = cerebrospinal fluid

3.2.3 Electrophysiology

Electrophysiological examinations were undertaken in 18 affected dogs (8 months to 6 years old), four maternally related dogs (2–10 years old), one maternally unrelated control dog with a non-SAN gait disorder, and 11 healthy control dogs (5 months to 8 years old) using the neurophysiology equipment Counterpoint MK2 (Medtronic A/S, Skovlunde, Denmark). Six affected dogs were re-examined electrophysiologically ≥2 years later (when 3–8 years old) (papers I and IV) (Table 1). Dogs were sedated with a combination of medetomidine (Domitor vet, 1 mg/ml, 0.01 ml/kg, Orion, Sollentuna, Sweden) and butorphanol (Torbugesic, 10 mg/ml, 0.01 ml/kg, ScanVet, Animal Health A/S, Fredensborg, Denmark) intramuscularly pre-examination. Electromyography (EMG) of proximal and distal muscles of the thoracic and pelvic limbs was conducted using concentric needle electrodes (Myoline 40 mm, Judex, Aalborg, Denmark). Motor nerve conduction studies (MNCS) of ulnar, peroneal and tibial nerves were performed using surface electrodes (Medelec Gold Disc Electrodes part. no. 54426T, Cephalon, Norresundby, Denmark), while disposable sensory needle electrodes (15mm*0.70mm (22G), Medtronic A/S, Skovlunde, Denmark) were used for sensory nerve conduction studies (SNCS) of superficial radial, ulnar, peroneal and tibial nerves. The same examiner (DVM Eva Örvind) performed all the electrophysiological examinations.

3.3 Post mortem examinations

A total of nine affected dogs and one mother of two affected dogs were necropsied (Tables 1 and 4). Samples from the nervous system and muscle tissue were either frozen or fixed in formalin or glutaraldehyde. Formalin- and glutaraldehyde-fixed samples from the necropsied dogs were studied by the same examiner, Dr. Kaspar Matiasek. For paper I, post mortems of four affected dogs aged 11–30 months at euthanasia were performed (dogs 1 to 4, Table 4). In paper II, degrees of heteroplasmy were determined from frozen samples of different body tissues from three of the necropsied dogs (dogs 3, 5 and 7, Table 4). For paper III, in-depth post mortem examinations of five affected dogs aged 4–10 years at euthanasia were conducted (dogs 5 to 9, Table 4). The post mortem of a mother of two SAN-dogs (dog 10, Table 4) was studied in paper IV.

Table 4. *An overview of age and gender of the necropsied dogs by paper in studies of mitochondrially inherited sensory ataxic neuropathy in the Golden Retriever breed.*

Dog	Age	Gender	Paper I	Paper II	Paper III	Paper IV
1	11 months	male	X			
2	15 months	female	X			
3	23 months	female	X	X		
4	30 months	female	X			
5	4 years	male		X	X	
6	4 years	male			X	
7	5 years	male		X	X	
8	5 years	female			X	
9	9 years	female			X	
10	10 years	female				X

Frozen muscle biopsies were processed and stained using haematoxylin and eosin (HE), Gomori's trichrome, periodic acid Schiff (PAS), PAS/diastase, oil red O, acid phosphatase, alkaline phosphatase, SDH, COX and ATPase techniques. Formalin-fixed material was also processed using HE, PAS, PAS/diastase and Masson's trichrome stained sections, as well as Luxol fast blue, cresyl echt violet, Woelcke-Spielmeyer-Schröder, Bodian, glial fibrillary acidic protein (GFAP) and neurofilament (NF) staining for the central nervous system. Nerve biopsies were processed according to standard protocols: in short, all samples underwent immersion in 2.5% glutaraldehyde followed by osmium tetroxide (OsO_4) postfixation, repeated buffer rinses and a graded alcohol series before being embedded in epoxy resin. For routine histological inspection, semithin sections (0.5 µm) were mounted on triethoxysilane-coated glass slides and stained with azur II–methylene blue–

safranin. Additional OsO_4 stained probes were subjected to nerve fibre teasing. Thereby, 300 single nerve fibres with at least five internodes were considered representative.

3.4 Pedigree analyses

The pedigrees of affected dogs and their families were retrieved from the dog registry of the Swedish Kennel Club (http://www.skk.se/). Inbreeding coefficients for five-generation pedigrees of affected dogs and for all age-matched Golden Retriever dogs in the same registry were calculated using the software package Pedig (Boichard, 2002). Both common ancestors and matrilineal relatives born in 2001–2005 were searched for in this registry. Information about the total number of Golden Retriever litters born in Sweden during the years 2001–2005 was retrieved from the stud book. The maternal transmission of SAN was evaluated statistically by comparing the prevalence of affected dogs in two groups of offspring. First, we retrieved information about the identity of all dogs in litters (n=12) where the mother of one of our affected dogs was one of the litter mates. Then we divided all dogs (n=97) in those litters into one group of males (n=39) and one group of females (n=58). The next step was to count the number of affected offspring relative to the total number of offspring from the male group and the female group, respectively.

3.5 Molecular genetics

The complete mtDNA was re-sequenced in four affected dogs, one unaffected maternally related dog and two unrelated control Golden Retriever dogs. The mutant load in samples from 22 affected Golden Retriever dogs, 33 maternal relatives and all control Golden Retriever dogs (n=78) was estimated by a quantitative oligonucleotide ligation assay (qOLA) method. A northern blot analysis was applied to measure the steady-state levels of three mitochondrially encoded *t*RNAs (the *t*RNA for tyrosine (*Tyr*), the *t*RNA for glutamine (*Gln*) and the *t*RNA for cysteine (*Cys*)) in muscle tissue from three affected dogs and two unrelated, unaffected control dogs (one Golden Retriever and one Dachshund). All the laboratory work in molecular genetics was performed by the same examiner, PhD student Izabella Baranowska. For further details about the analytical methods used, see paper II.

3.6 Studies of mitochondria

Fresh muscle biopsies were harvested from *musculus quadriceps vastus medialis* of five affected dogs (1–6 years of age) and five age-matched control Golden Retriever dogs (Table 1). Prior to surgery, the dogs were sedated with a combination of medetomidine (Domitor vet, 1 mg/ml, 0.01 ml/kg, Orion, Sollentuna, Sweden) and butorphanol (Torbugesic, 10 mg/ml, 0.01 ml/kg, ScanVet, Animal Health A/S, Fredensborg, Denmark), both administered intramuscularly. The skin and subcutaneous tissues at the incision site were anaesthetized locally with xylocaine (Xylocain 5 mg/ml, AstraZeneca, Södertälje, Sweden).

Mitochondria were prepared from the excised muscle tissue according to the method of Wibom *et al.* (2002). Respiratory chain enzyme activities and rates of mitochondrial ATP production (Wibom *et al.,* 2002) were determined pair-wise (one case and one control) in a blinded fashion. In addition, citrate synthase activity was determined in muscle tissue and in isolated mitochondria (Wibom *et al.,* 2002).

Freshly frozen muscle biopsies from the same animals were used for histochemistry. Sections were stained with HE and Gomori's trichrome and for oxidative enzymes (reduced nicotinamide adenine dinucleotide (NADH)-tetrazolidium reductase, SDH, COX and the combined reaction for SDH and COX). Pairs of biopsies (one case and one control) were stained on the same occasion and evaluated blindly. Muscle samples were also fixed in 2.5% glutaraldehyde for electron microscopy. For further details about the analytical methods used, see paper II.

The presence of heteroplasmy in muscle homogenate and single muscle fibres was studied by restriction fragment length polymorphism, RFLP, in one case and one control dog (hitherto unpublished) as described previously (Moslemi *et al.,* 1998). DNA from muscle homogenates of each dog and 10 muscle fibres with reduced COX activity and five fibres with apparently normal COX activity (COX-positive) from the affected dog were subjected to PCR using a 6-carboxyfluorescein (6-FAM)-labelled forward primer (L5191-5210) and a reverse primer (H5383-5359). The amplified DNA-fragments were digested with restriction enzyme *BccI*, which cleaves the mutant DNA, and the proportion of mutated DNA was calculated by GenScan software (Applied Biosystems, CA). The resolution of this method allows detection of wild type levels of about $\geq$ 2%.

3.7 Statistics

The survival of affected dogs from birth to euthanasia was demonstrated with a Kaplan–Meier diagram, using the procedure LIFETEST in SAS (SAS Institute Inc., Cary, NC, 27513, US). Electrophysiologic parameters from different groups of dogs were compared using the two-sample t-test, and comparisons between different occasions of examination of the same dogs were compared by the paired t-test (Minitab 12.23, Minitab Inc.). Statistical significance was set to $p < .05$.

The maternal transmission of SAN was tested by applying Fischer's exact test to the prevalence calculated in different groups of offspring (see 3.4). Hybridization intensity for the different *t*RNAs of affected dogs in the northern blot analysis was compared with that of control dogs using the Student's t-test. Differences in the rate of ATP production and enzyme activities between the group of affected dogs and the group of control dogs were tested for significance by the two-sample t-test.

4 Results

The results from these studies are summarized below. For further details, see papers I–IV.

4.1 Onset of gait disturbances in puppies

The clinical signs in the affected dogs (n=27) were reported to have been noticed first at 2–8 months of age (n=26), and in one case the age at onset was reported as "less than one year". It was reported that the first impression of any abnormalities was often rejected by the owners based on the assumption that the dogs were "just being clumsy and puppyish".

The first signs observed by the owners were in most cases referred to as unstable or uncoordinated pelvic limbs (n=9) or a strange gait in the pelvic limbs (n=8). However, one owner had instead first noticed a weakness in the carpi. In another case, difficulties in getting up from a sitting position were reported as the presenting sign. All the other owners (n=8) made remarks about a generalized gait disturbance as the presenting sign.

With time, all affected dogs developed further clinical signs that were noticed by the owners. The signs also became more severe. Besides the obvious gait disturbances, a majority of the owners reported that the dogs were reluctant to walk on slippery floors and on stairs, had difficulties in entering cars and that the pelvic limb claws were worn. In addition, a majority (5/8) of the adult (≥1-year-old) male dogs were reported to maintain a body posture like that of a bitch at urination. More uncommon signs included in the clinical history reported by the owners were development of urinary incontinence in adolescence (n=3), inability to swim (n=3), wagging of the hind part at urination/defecation (n=2), nibbling the skin of pelvic limbs without exhibiting dermatological changes (n=2), not being ticklish in the pelvic limb paws (n=2), showing signs of

discomfort when touched in the caudal spinal area (n=2), or in the face (n=1), intolerance of hot weather (n=2), rarely coming into heat (n=2), cow-hocks (n=1) and strabismus on excitement (n=1).

4.2 Neurological deficits

The general physical examination of affected dogs did not identify any clinically relevant findings except signs associated with neurological dysfunction, nor did serum chemistry, blood lactate analysis, serology or screening for infectious diseases with PCR, diagnostic imaging or cerebrospinal fluid analysis (Tables 2 and 3). Occasional values were found below or above the reference ranges: in all instances these were close to the reference values, and thus not paid further attention.

At neurological examination, the gaits of all affected dogs were ataxic and conspicuously dysmetric (Figure 2). Especially in the pelvic limbs, the movements altered intermittently between hypermetria and hypometria. An inability to bear weight on the pelvic limbs with knees extended was seen – but not at every step. Truncal swaying was apparent in a few cases (n=3). Postural reactions and spinal reflexes were decreased in all affected dogs. The most prominent finding was absent (n=20) or obviously decreased (n=7) patellar reflexes. The pelvic limbs were more affected by neurological deficits than the thoracic limbs. The most obvious abnormality in the thoracic limbs was hyperextension of the carpi, which was present in a majority of the dogs examined in person by the author (13/20). Among the dogs examined neurologically by other veterinarians (n=7), for only two dogs was the thoracic limb posture mentioned, and then as hyperextended carpi. The only cranial nerve deficit observed was a bilaterally decreased menace reaction in two cases. Pain perception was within normal limits in all dogs. No dog seemed to be in pain. The neuroanatomical diagnosis in SAN-dogs referred to the sensory compartment of the spinal reflex arcs in both pelvic and thoracic limbs, and to afferent proprioceptive pathways in sensory nerves and spinal cord.

The constellation of signs was similar from dog to dog but the severity of signs differed between dogs, apparently irrespective of age or duration from onset of signs. Two cases examined at 8 months of age were already severely ataxic whereas one case examined at 8 years of age was only mildly ataxic.

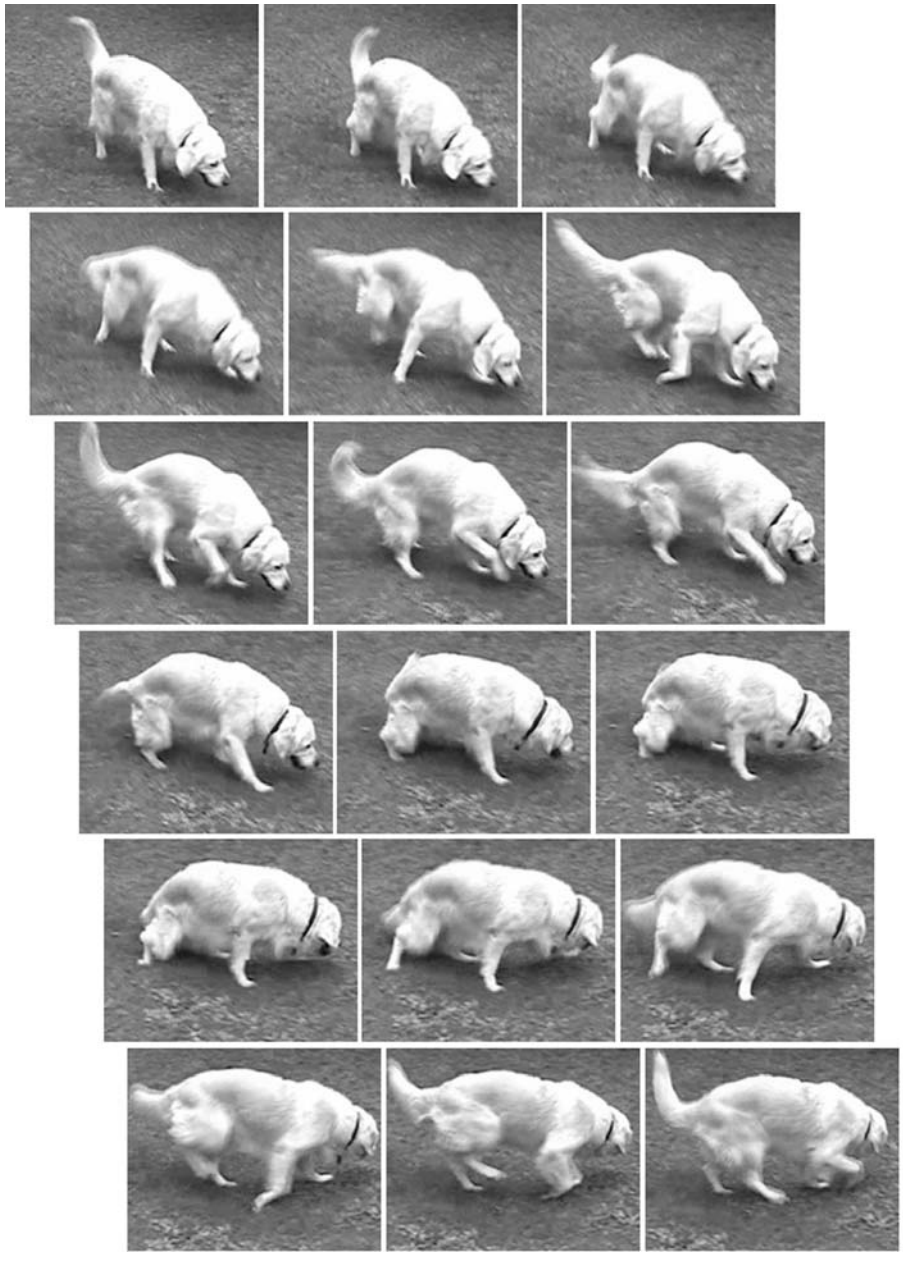

Figure 2. Serial images showing the gait of a severely ataxic Golden Retriever dog affected by mitochondrially inherited sensory ataxic neuropathy.

4.3 Slowly progressive clinical course

Dogs that were re-examined neurologically had more severe deficits at re-examination (paper IV). This was also in accordance with the impressions of owners/breeders and information from referring veterinarians. The neurological signs reported waxed and waned to some degree, and in general this did not seem correlated with any factors observed by the owners. However, there were two exceptions; one dog was said to deteriorate every winter and one dog was worse repeatedly when abnormally thin. Some owners also reported that their dogs improved during adolescence in handling stairs, slippery floors or entering cars. However, no dog was ever free of signs after onset, and over time all the dogs deteriorated.

Eighteen affected dogs were deceased prior to the last follow up (June 2009). All of them had been euthanized. Sixteen dogs had been euthanized because of neurological impairment and two dogs (9 and 10 years old) were euthanized owing to unrelated incapacitating diseases. About half of all the affected dogs included were euthanized before 4 years of age, and yet some dogs (n=3) were euthanized at an older age, owing to neurological impairment (Figure 3 in paper IV). Nine dogs were still alive; six of them were more than 6 years old at the time of last follow up (June 2009). So far, no dog has become non-ambulatory as a result of SAN.

4.4 Related dogs that were affected subclinically

The 11 maternally related dogs that were examined neurologically were considered to be unaffected by gait disturbances by their owners, with two exceptions: one dog had had surgery for a ruptured cruciate ligament several years before and one had been diagnosed with hip dysplasia. None of the dogs showed ataxia at examination, but three of these dogs (all three were mothers, of two, three and five affected dogs respectively) had other neurological deficits in agreement with deficits seen in dogs affected by SAN. For example, all of them had decreased or absent patellar reflexes. These three neurologically affected related dogs were considered to be subclinical cases of SAN.

4.5 Low sensory nerve conduction velocities

Dogs affected by SAN had significantly lower sensory nerve conduction velocities (SNCVs) than control dogs in all sensory nerves tested. The SNCVs were not dramatically low, and despite statistical significance, there was an overlap of individual values between the groups. Also, affected dogs with severe ataxia had lower mean SNCVs than affected dogs with mild ataxia in all four sensory nerves tested, but this was not statistically significant for any nerve. Concerning amplitudes in sensory nerve testing, MNCS and EMG, no conclusive differences from control dogs were found. One mother of three SAN-dogs had neurological deficits on neurological examination and the lowest SNCVs of all the dogs examined, but the MNCS were in accordance with published reference ranges (van Nes, 1986) and no abnormal discharge was found on EMG examination.

4.6 Degenerative changes throughout the nervous system

In the first four affected dogs (11–30 months old) that underwent necropsy, the neuropathological findings consisted of degeneration and loss of large myelinated Ia/b-afferents extending into the dorsal nerve roots and associated spinal white matter tracts. Further degenerative changes were seen in the ventral horns and descending motor pathways of the spinal cord. One of these dogs displayed presynaptic buttons and axonal spheroids throughout the ventral column of the spinal cord intumescences, identical to those seen with neuroaxonal dystrophy (NAD). The same dog had some eosinophilic spheroids close to nerve cell perikarya in the cerebellar roof nuclei, otherwise no conspicuous brain abnormalities were found at that time in any dog. Taken together, the changes in the central nervous system (CNS) and peripheral nervous system (PNS) were consistent with a chronic progressive central and peripheral sensorimotor axonopathy affecting in particular the proprioceptive pathways with very mild involvement of peripheral motor axons.

In the five other affected dogs (4–9 years old) that were examined post mortem, more profound and widespread neurodegenerative changes were found. A typical symmetrical spatial distribution of different types of neurodegenerative changes was seen, consisting of spongiosis in the basal nuclei, most prominent in the caudate nuclei, with relative sparing of neurons and NAD-type alterations in spinal cord and brainstem nuclei in addition to the central–peripheral axonopathy.

In conclusion, the dogs diagnosed with mitochondrially inherited SAN had progressive neurodegenerative changes of certain cell populations, especially in the extrapyramidal motor system in the brain and the general proprioceptive system. Two distinct patterns of pathology had developed: neuron-sparing encephalopathy in the basal nuclei and NAD-type alterations. The neurodegenerative alterations were more prominent in older dogs than in younger ones.

A 10-year-old mother of two affected dogs was also necropsied. Post mortem examination revealed neurodegenerative changes in both CNS and PNS at histopathology. The findings consisted of a chronic, marked, predominantly axonal polyradiculoneuropathy and advanced stage neurodegeneration of vestibular and cuneate nuclei in the brainstem.

4.7 Non–neurological manifestations in necropsied dogs

Non-neurological manifestations at necropsy were found in four dogs. A 4-year-old affected dog had enteritis, one 5-year-old affected dog had hypoplastic ovaries, and a 9-year-old affected dog had a metastasizing cardiac haemangiosarcoma and a mammary complex adenoma. One 10-year-old mother of two affected dogs had a mammary carcinoma with lung metastases. Another affected dog, 10 years old, was euthanized because of a tumour in the spleen, but this dog was not necropsied.

4.8 Mitochondrial inheritance

A common ancestor born in 1976 (female X) was in the maternal lineages of all affected dogs, for at most eight generations back in time (Figure 1). Her grandmother (female Z), born in 1971, was the oldest dog with descendants still alive potentially carrying the same mitochondrial mutation, which it was possible to find through the Swedish Kennel Club registry. During 2001–2005, a total of 2180 Golden Retriever litters were registered in the stud book. Female Z had offspring through maternal lineages in 94 litters registered in the Swedish Kennel Club database during the same period, which constituted 4.3% of the registered litters in the breed. Dogs in the same mitochondrial lineage are also registered in Norway, Denmark, Finland and Estonia, and anecdotally exist in other countries e.g. Russia, France and the Netherlands.

In those groups of offspring for which the prevalence of SAN was calculated, in the female group there were 25 affected offspring out of a total of 272 born. No affected dog had been diagnosed among 177 offspring in

the male group. This excludes a nuclear inheritance, and hence transmission was proven to be mitochondrial ($p < .000001$). The inbreeding coefficient for affected dogs born in 1997–2004 was very similar to the coefficient for the entire Golden Retriever population registered by the Swedish Kennel Club born during the same period (1.6% compared to 1.5%).

4.9 Deletion in mtDNA

A single base-pair deletion was identified in the mtDNA from affected dogs and maternal relatives. A "T" was deleted at position 5304 (ΔT5304) in the mitochondrial genome, in the $t\text{RNA}^{Tyr}$ gene. The disease was unambiguously associated with this sequence variant. This site in the mitochondrial genome is conserved in evolution and the deletion has never been reported in any other mammal. The mutation resulted in a deleted base in the TΨC stem of the $t\text{RNA}^{Tyr}$ molecule.

4.10 High mutant load

All the affected dogs and all their tested clinically unaffected 1st, 2nd, 3rd and 4th degree maternal relatives had a very low level of the wild type sequence in blood, ranging from 0 to 11.2%. Concerning the degree of heteroplasmy, the group of affected dogs was not possible to distinguish from these close maternal relatives. One entire litter with two affected dogs and six, according to the owners, unaffected dogs and their unaffected mother was included in the analyses. No differences in mutant load could be detected between affected and unaffected dogs in that family (unpublished data). All control dogs and wolves had 100% wild type sequence. Also two dogs traced back to female X, in a lineage spanning six generations from her in which no affected dogs were known hitherto, had 100% wild type sequence (unpublished data). In even more distant relatives (tracing back to female Z but not to female X) the degree of heteroplasmy varied from 5 to 60% wild type among dogs. These results indicated that female Z or some ancestor to her was the founder (Figure 1).

The mutant load in body tissues (frontal lobe, spinal cord with dorsal root ganglia (7th thoracic and 5th lumbar), optic nerve, recurrent laryngeal nerve, pancreas, thyroid gland, and skeletal muscle of pelvic and thoracic limbs) was estimated in three affected dogs. All analysed tissues had a higher mutant load, close to 0% wild type, compared with the degree of heteroplasmy in blood cells from the same dogs. In RFLP analysis of COX-positive single muscle fibres and fibres with reduced COX activity of one affected case, no

detectable level of wild type sequence was revealed (unpublished data). By the same molecular genetic method, the muscle homogenate from the control dog showed 100% wild type sequence whereas the homogenate from the affected dog had no detectable wild type sequence (unpublished data).

4.11 Reduced steady-state levels of *t*RNA^{Tyr}

By northern blotting, all three affected dogs showed significantly reduced steady-state levels of *t*RNA^{Tyr} compared with the two control dogs. Concerning the other mitochondrial *t*RNA species assessed, *t*RNA^{Cys} and *t*RNA^{Gln}, no differences were found in hybridization intensities between affected dogs and controls. These data indicated an increased degradation and hence an impaired stability of mutated *t*RNA^{Tyr}.

4.12 Dysfunction of the respiratory chain

In muscle biopsies from four out of the five affected dogs that were sampled, the ATP production rate and the enzyme activities in the respiratory chain complexes were reduced in a mode that indicated dysfunction of the respiratory chain units encoded by mtDNA. Moreover, the reduced activities of multiple enzymes with subunits encoded by mtDNA were in accordance with mutations in *t*RNA genes. One affected dog (dog 3, paper II) was not possible to distinguish from the controls with these assays.

4.13 Bluish staining of muscle biopsies

On combined staining for SDH/COX activity, four of the five sampled affected dogs had a bluish staining of the muscle fibres compared with their matched controls. This indicated a shift towards a lower COX activity compared with the SDH activity in the muscles of affected dogs. However, no fibres that showed a total lack of COX activity were detected and no ragged red fibres were found. One affected dog (dog 3, paper II) did not display any muscle pathology on histochemistry, in agreement with the non-pathological biochemistry for the same dog.

4.14 Inclusions in mitochondria

On electron microscopy, one affected dog (dog 3, paper II) had paracrystalline inclusions in the mitochondria. This was the dog that was

indistinguishable from controls regarding biochemistry and histochemistry. No other dog had any clear signs of structural mitochondrial pathology.

5 Discussion

5.1 The novelty of this neurological syndrome

The clinical picture and pathomorphological changes of Golden Retriever dogs affected by the neurodegenerative disease SAN are unique. Breed-related neurodegenerative diseases in dogs with clinical signs reflecting dysfunction of predominantly sensory parts of the nervous system, thus sharing some similarities with SAN, have been described. In some diseases, nociceptive loss is included as a clinical sign, e.g. for Border Collies and long-haired Dachshunds with sensory neuropathy (Duncan & Griffiths, 1982; Wheeler, 1987; Vermeersch *et al.,* 2005): this is a sign not detected in Golden Retriever dogs with SAN. In others, e.g. neuroaxonal dystrophy in Rottweiler dogs and Papillon dogs (Cork *et al.,* 1983; Chrisman *et al.,* 1984; Franklin *et al.,* 1995), vestibulocerebellar signs are part of the clinical picture, in contrast to SAN.

In addition, diseases that are partially similar to SAN in pathomorphological features have been described. In Rottweiler dogs and Papillon dogs (Cork *et al.,* 1983; Chrisman *et al.,* 1984; Franklin *et al.,* 1995), breed-related neuroaxonal dystrophies have been described without concomitant basal nuclei degeneration or peripheral nerve pathology, a picture not similar to that of SAN in Golden Retriever dogs. Bilaterally symmetric degeneration of extrapyramidal nuclei has been reported in an English Springer Spaniel (Brenner *et al.,* 1997), Kuvasz dogs (Hazlett *et al.,* 2005), Alaskan Husky dogs (Brenner *et al.,* 2000), Yorkshire Terriers (Baiker *et al.,* 2009) and Kerry Blue Terriers (de Lahunta & Averill, 1976). Only the Kuvasz dogs and Kerry Blue Terriers had involvement of the caudate nuclei and in no case were concomitant NAD-changes or peripheral nerve affection reported, distinguishing these diseases from canine SAN. In

progressive axonopathy in Boxer dogs, both the clinical picture and the observed central–peripheral axonopathy with axonal spheroids and presynaptic buttons in the CNS have similarities to SAN: however, the degenerative changes in the optic pathways but absence of degeneration of basal nuclei noted in Boxer dogs discern these two diseases from each other (Griffiths *et al.,* 1980; Griffiths *et al.,* 1985). A central–peripheral axonopathy in New Zealand Huntaway dogs has also been described, but no involvement of the basal nuclei or NAD-like changes were reported for these dogs (Jolly *et al.,* 2000).

Familial neurological diseases with onset in puppyhood have occurred previously in the Golden Retriever breed (Braund *et al.,* 1989; Matz *et al.,* 1990; da Costa *et al.,* 2009), but these are unlike canine SAN regarding both clinical and pathological features. Braund *et al.* (1989) and Matz *et al.* (1990) described a hypomyelinating polyneuropathy with onset in puppies of 6–7 weeks old. These dogs had markedly diminished motor nerve conduction velocities suggesting involvement of peripheral motor nerve fibres to a larger extent than in SAN-dogs. da Costa *et al.* (2009) presented a multisystem axonopathy and neuronopathy with onset in puppies of 6–15 weeks old. The predominating signs were tetraparesis and severe muscle atrophy but not ataxia, in contrast to the features of SAN.

There are some other reports of encephalopathies in dogs that have been proposed (Gruber *et al.,* 2002) or proven (Li *et al.,* 2006; Wood & Patterson, 2001) to be mitochondrial disorders, of which neither the clinical picture nor the pathomorphological features resemble those of canine SAN. Also, it seems that in comparison with neurodegenerative diseases in humans or other mammals, no counterpart has been described.

To summarize, in all these reports the combination of age at onset, clinical course, clinical and neurological signs, and the nature and distribution of neuropathological changes are not in agreement with the features of SAN; thus SAN in Golden Retriever dogs is considered to be a novel neurological syndrome.

5.2 Characterization as a sensory ataxic neuropathy

The disease in Golden Retriever dogs reported here was characterized as a sensory ataxic neuropathy owing to the neurological signs, in agreement with a similar clinical syndrome in humans. Sensory ataxic neuropathies present with loss of proprioceptive sensations and tendon reflexes, with preservation of muscle strength (Illa *et al.,* 2001). This syndrome is associated with a number of aetiologies (Illa *et al.,* 2001) and mitochondrial disorders

are recognized among them (Fadic *et al.,* 1997; Van Goethem *et al.,* 2003; Okun & Bhatti, 2004; Van Goethem *et al.,* 2004; Hudson *et al.,* 2005b; Gago *et al.,* 2006; Milone *et al.,* 2008).

5.3 Description and definition of criteria for clinical features

A tentative diagnosis of SAN in Golden Retriever dogs can be made on clinical grounds, the basis being a combination of signalment, age at onset, history, clinical signs, neurological signs and clinical course. The diagnosis should be anticipated in a Golden Retriever dog of either gender, affected by ataxia as a pup with a progressive clinical course and with absent or decreased patellar reflexes besides unaffected general appearance. There are often affected dogs that are not-too-distant maternal relatives, but such information is often unavailable to owners.

Besides the ataxia, all affected dogs have had hyporeflexia (7/27 dogs) or bilateral absence (20/27 dogs) of patellar reflexes as a consistent finding at neurological examination. This feature is considered very typical for SAN, even in the early stages. The three youngest cases at examination, 6 and 7 months old, all had bilaterally absent patellar reflexes. The patellar reflex is the most reliable tendon reflex in the dog and should always be checked in neurological examination of dogs (de Lahunta & Glass, 2009a). Patellar reflex responses have been studied previously in normal dogs and abnormalities are not frequent; two of 72 dogs of <10 years old and four out of 14 dogs of ≥10 years old had absent reflexes (Levine *et al.,* 2002). To define the patellar reflexes as "lost", as in paper I, is probably not accurate if it is not known whether the reflexes were present previously. They should be considered rather as just "absent" if they are not possible to elicit.

The remainder of the clinical and neurological examination should be in agreement with the results described in section 4.2 and papers I and IV. To check for possible differential diagnoses, blood work, serological tests, diagnostic imaging, CSF analysis and electrophysiology may be indicated. At electrophysiology, the SNCV in peripheral nerves may be below reference ranges in cases of SAN, whereas MNCS and EMG are within reference ranges. These results are compatible with neuropathic diseases in humans that involve the peripheral portion of the sensory nerves (Tankisi *et al.,* 2005). Lactic acidaemia is present in many human patients with mitochondrial disorders (Tatuch *et al.,* 1992; Fadic *et al.,* 1997; Pulkes et al., 2000; Sahashi *et al.,* 2001; Liolitsa *et al.,* 2003; Van Goethem *et al.,* 2003; Kirby *et al.,* 2004; Kärppä *et al.,* 2005). Lactate measurements in SAN-dogs at rest were inconspicuous.

Given that there are subclinical cases among maternal relatives, an onset where neurological signs are first observed in older dogs cannot be excluded. However, in the case material studied here there is no suggestion for a late-onset form of SAN.

A genetic test for the causative deletion is now available commercially (http://www.hgen.slu.se/), and should test positive in an affected dog. It should also be noted that a positive genetic test means "carrier" and not automatically "affected". This genetic test does not separate carriers from clinically affected dogs.

5.4 Description and definition of criteria for post mortem features

In young affected dogs, at 1–2 years of age, neuropathological changes consist predominantly of mild central–peripheral axonopathy. In the spinal cord, axonopathy is most pronounced in the fasciculus gracilis and the dorsal part of the lateral funiculus, followed by the fasciculus cuneatus and the ventral descending motor pathways. In peripheral nerves, ongoing Wallerian degeneration, particularly of large myelinated fibres, is seen, some fascicles being affected more severely than others. Histopathology of muscle tissue display subtle variation in fibre size, type II angular fibres and small group atrophy.

In older dogs, axonal spheroids and presynaptic buttons in the ventral columns of the spinal cord, most prominent in the intumescences, are added to the histopathological appearance. The same kind of change is also seen in some brainstem nuclei (reticular nuclei, cuneate and accessory cuneate nuclei, nuclei of the spinal tract of cranial nerve V and olivary nuclei) and in association with the grey matter of the ponto- and spinocerebellum. In older dogs with a fully developed pathomorphological picture, degenerative changes of the extrapyramidal system are also seen, including a neuron-sparing encephalopathy in the basal nuclei with spongiosis, which is most prominent in the caudate nuclei.

In younger dogs, histological lesions can be overlooked easily unless the specific regions outlined above are carefully examined. The lesions can also be difficult to differentiate from post mortem changes if necropsy is delayed. So far, very young affected puppies have not been examined post mortem, but in these cases the lesions are expected to be even milder and more difficult to detect.

Neoplastic diseases were diagnosed in three dogs, but were considered to be unrelated to the neurological syndrome. Even though there are

indications of a general association between mitochondrial malfunction and neoplasia (Polyak *et al.,* 1998), no such conclusions were drawn in these cases because neoplastic diseases are common in the ageing Swedish dog population (Egenvall *et al.,* 2000) and all three dogs with neoplasia were old. An association between the hypogonadism diagnosed post mortem in one affected dog and the mitochondrial disorder is not excluded, because a similar association has been seen in humans (Taylor & Turnbull, 2005).

5.5 Clinical course and therapy

The clinical course of SAN is chronic and slowly progressive. This statement is underlined by information from owners, breeders, referring veterinarians and repeated neurological examinations. The assessment of the degree of neurological deficit is subjective. Because of this, a single examiner performed all the primary and subsequent neurological examinations in the re-examined dogs, and in addition an effort to reduce biases by use of a scoring system and review of video tapes was introduced in paper IV.

The disease onset is insidious, with the first signs in puppyhood. To date, it is not known whether affected dogs have any clinical signs already from birth. Their gait disorder is easily confused with "puppyish movements" at the beginning. An impression was obtained that owners who were used to observing the gait of normal puppies detected the gait disturbances earliest.

No dog has become non-ambulatory during the disease course. At the last check-up, three dogs of 9–10 years old were still ambulatory. However, the main clinical problem in all dogs is limited activity associated with the abnormal gait. The most severe cases completely refused to walk on floors indoors and some owners had carpets all over their house to facilitate for their dog.

The clinical course observed is considered to be the natural course of SAN. Non-steroidal anti-inflammatory drugs (NSAIDs) and/or corticosteroids had been used for short or longer periods in some dogs, but no clinical response was reported. Some dogs (n=3) were exercised assiduously by their owners and apparently overcame the reluctance to walk e.g. on stairs and on slippery floors over time. However, their neurological status deteriorated over time.

No therapeutic trials have so far been initiated, owing to a lack of knowledge about the natural course of SAN and natural variations in this course. To evaluate treatment regimens, such knowledge is fundamental. The clinical management of humans affected by primary mtDNA disorders is mainly symptomatic, including for example anticonvulsant therapy for

patients with seizures, pacemakers for cardiac conduction defects, and specific agents against lactic acidosis. Supportive therapy with antioxidants, respiratory chain co-factors, carnitine, creatine, hormones or a high-fat diet aimed at improving the function of the respiratory chain are used commonly, and anecdotally some patients respond, though not all. A few controlled trials have resulted in contradictory results (Chinnery *et al.,* 2006). Improvement as a result of physical training has been documented in patients with myopathy (Taivassalo *et al.,* 1998) but the baseline capacity returned after the training period was over (Taivassalo *et al.,* 2006).

5.6 Survival

Survival analysis suggests a guarded prognosis for dogs affected by SAN with respect to the expected canine lifespan, bearing in mind that "a full-length dog life" is a somewhat hypothetical concept in Sweden where most dogs are euthanized sooner or later. However, about half part of all affected dogs had been euthanized before 4 years-old. In a study of mortality in over 350,000 insured Swedish dogs, the probability of death by 5 years for Golden Retriever dogs was 7% (Egenvall *et al.,* 2005). The natural course until spontaneous death is not known in any SAN case studied, because all the contributing deceased dogs were euthanized. The time point for euthanasia was at the owners' request. The time point elected for euthanasia due to neurological impairment is not always correlated with the relative severity of neurological signs but instead is thought to represent the respective owner's tolerance of the gait disturbances of their dog. Degrees of ataxia in dogs examined by the author (n=20) are presented in Table 5.

Table 5. *Number of euthanized dogs and dogs still alive at last follow-up (June-09) with different degrees of ataxia at the last or the only neurological examination. Included are also the ages of the different dogs at these neurological examinations. All these dogs (n=20) were diagnosed with mitochondrially inherited sensory ataxic neuropathy and were examined neurologically by the author.*

	Mild	Mild–moderate	Moderate	Moderate–severe	Severe
Euthanized dogs	n = 2	n = 3	n = 1	n = 2	n = 4
	1 y, 8 y	1 y, 2 y, 4 y	8 m	1 y, 3 y	8 m, 10 m, 5 y, 5 y
Dogs still alive	n = 1	n = 1	n = 3	n = 1	n = 2
	5 y	5 y	5 y, 6 y, 9 y	3 y	1 y, 3 y

m = months old, y = years old

5.7 Determination of the mode of inheritance

A hereditary pattern for this disease was indicated at an early stage because the affected dogs were related to each other and cases were clustered in some litters. A common ancestor in the maternal lineage of all the affected dogs was found, and maternal inheritance was eventually proven. Further analyses of the Swedish Kennel Club registry traced this maternal lineage back for 10 generations (Figure 1). This registry is detailed and contains reliable pedigree data, computerized from 1976. There are mandatory rules for the identification by tattoo or microchip for dogs registered in the database since 1997. Knowing the mode of inheritance, it is possible to reduce the incidence of this disease simply by not mating females from this matrilineal family.

The basis for this thesis comprises investigations of 27 diagnosed clinical cases, of which 20 were examined by the author and seven dogs were examined neurologically by other veterinarians. All these dogs had both the clinical course and the pattern of neurological deficits in common, and were therefore included as affected dogs. In addition, some undiagnosed siblings and other maternal relatives with similar gait disturbances (n=8), according to breeders familiar with the diagnosed dogs, are depicted as affected dogs in Figure 1. Anecdotally, there are and have been additional cases in the Swedish Golden Retriever population. This is anticipated and does not challenge the conclusions about SAN. Also anecdotally, the first clinical case in Sweden suspected in retrospect to have had this disease was born in the late 1980s.

The described pedigree of SAN-dogs is large compared with the pedigrees reported for almost all other maternally inherited diseases, the vast majority of them being human diseases. Extensive pedigrees of somewhat comparable sizes have been described for maternally inherited sensorineural deafness in a six-generation Israeli-Arab kindred (Jaber *et al.,* 1992) and for Leber's hereditary optic neuropathy in seven generations of Brazilians (Sadun *et al.,* 2002) and an Australian six-generation pedigree (Sudoyo *et al.,* 1992).

For dogs, only one other maternally inherited disease has been reported previously: canine spongiform leucoencephalomyelopathy in Australian Cattle dogs and Shetland Sheepdogs (Li *et al.,* 2006). For both these breeds, the published pedigrees consisted of one healthy female dog and their affected offspring in three different litters each. The mutation was the same in both breeds (a point mutation in the cytochrome *b* gene), and they had the same nucleotide sequence in the D-loop of the mtDNA molecule,

indicating that these two families, through maternal lineages, may have originated from the same ancestor before the breeds were separated.

5.8 Identification of the causative mutation

A deletion was found at base-pair 5304 in the *t*RNA^{Tyr} gene in the mitochondrial genome of affected dogs. This finding is in itself not sufficient to claim the mutation pathogenic. McFarland *et al.* (2004a) applied scoring criteria to mutations in mitochondrial *t*RNA genes, thereby categorizing the mutations as (i) definitely pathogenic; (ii) probably pathogenic; (iii) possibly pathogenic; (iv) neutral variants. This scoring system assesses 1) the evolutionary conservation of the base, 2) the number of independent reports about the association between disease and mutation, 3) the presence of heteroplasmy, 4) histochemical evidence of mitochondrial disease, 5) biochemical defects in complex I, III or IV, 6) segregation of the mutation with disease, 7) single-fibre studies, demonstrating higher levels of mutation in COX-negative muscle fibres, 8) steady-state levels of mutated mitochondrial *t*RNA, and 9) evidence of pathogenicity from cybrid cells. In this system, the SAN mutation falls into the category "definitely pathogenic" because the deletion is in an evolutionarily highly conserved site, heteroplasmy is present, there is weak histochemical evidence of mitochondrial disease, there are biochemical defects in complexes I, III and IV, the mutation segregates with disease within the family and there is a reduction in the steady-state level of the particular mitochondrial *t*RNA, which altogether suffice.

According to the four canonical rules of DiMauro and Davidzon, (2005) a novel mtDNA mutation should be considered pathogenic if 1) the mutation is not present in normal individuals of the same ethnic group, 2) the mutation alters an evolutionarily conserved site, 3) the mutation causes respiratory chain enzyme deficiencies in affected tissues, and 4) there is a correlation between the degree of heteroplasmy and clinical phenotype. These criteria are more laborious to apply to the SAN-mutation. The first criterion is not fulfilled for dogs carrying the ΔT5304 mutation in the *t*RNA^{Tyr} gene, because maternally related but clinically unaffected dogs carry the same mutation. However, it is not clarified whether or not DiMauro and Davidzon (2005) included clinically unaffected maternal relatives as "normal individuals of the same ethnic group". Most logically, in cases of mitochondrially inherited pathogenic mutations, there should be maternally related individuals carrying the same mutation. However, the SAN deletion is not present in any unrelated dog of the same breed. The second criterion,

alteration of an evolutionarily conserved site, is fulfilled for the SAN deletion. For the third criterion, "affected tissues" are not defined further. For SAN, clinical signs of muscle dysfunction were not obvious but biochemical evidence of respiratory chain enzyme deficiencies was shown in muscle tissue, and the respiratory chain enzyme activities in malfunctioning nervous tissue were not analysed. For the fourth criterion, there was a correlation between the degree of heteroplasmy in blood and the clinical phenotype because all cases occurred in the part of the pedigree with the highest mutant load (Figure 1), but the mutant load in blood from affected dogs was not distinguishable from that in closely maternally related unaffected dogs. In humans, there are situations resembling SAN, e.g. documented pathogenic mutation in mtDNA with both affected and asymptomatic homoplasmic carriers of the same maternal lineage in the family (McFarland *et al.,* 2004b).

About 5% of dogs in the Swedish Golden Retriever population born during 2001–2005 was estimated to be carriers of this mutation (paper II). This figure presumed that the mean size of litters born to carrier bitches was the same as that of litters from unrelated bitches during this period. The mean litter size in the Golden Retriever breed in 2008 was 6.3 puppies per litter (http://www.rasdata.nu/). Looking at the sizes of the litters with affected dogs in the pedigree (Figure 1), the presumption seems reasonable, but no mathematical calculation was done. Moreover, all dogs descended from female Z (Figure 1) through maternal lineages that had been tested for the mutation in paper II were positive, suggesting that all her maternal descendants were carriers. In fact, two Golden Retriever dogs tested since then in this matrilineal family harboured 100% wild type mtDNA, and hence the proportion of carriers in the population was probably somewhat lower than assumed initially.

A pathogenic single base-pair deletion in a mitochondrial *t*RNA gene seems rare and has previously been reported just a few times (Shoffner *et al.,* 1995; Raffelsberger *et al.,* 2001), in humans. One of these reports concerns a deletion in the mitochondrial $tRNA^{Tyr}$ gene (Raffelsberger *et al.,* 2001). Much more frequently reported mutations of mtDNA are pathogenic point mutations or large scale deletions (http://www.mitomap.org/).

5.9 Molecular mechanisms - neurological signs

The clinical features of dogs affected by SAN are surprisingly uniform in comparison with many human mitochondrial disorders. In addition, no

indications of manifestations in other tissues than the nervous and neuromuscular systems have been found.

The correlation between genotype and clinical presentation of a mitochondrial disorder is not always easy to understand. For example, the same mutation can result in different human phenotypes, which is seen for example in the A3243G mutation in one of the mitochondrial *t*RNA genes for leucine. This affects different tissues with differing clinical signs in the three syndromes mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), chronic progressive external ophthalmoplegia (CPEO), and maternally inherited diabetes and deafness (MIDD) (Goto *et al.,* 1990; Fang *et al.,* 1993; van den Ouweland *et al.,* 1994). One mutation can also result in different disease courses and different ages at onset between affected humans, as for the T14709C-mutation in the mitochondrial *t*RNA gene for glutamic acid with congenital and late-onset forms of myopathy (Mancuso *et al.,* 2005). A common phenotype can also develop from different mutations, e.g. the MELAS syndrome caused by mutations in either the protein encoding gene for *MTND1* or that for *MTND5,* or the mitochondrial *t*RNA gene for leucine (Goto *et al.,* 1990; Liolitsa *et al.,* 2003; Kirby *et al.,* 2004). A specific mutation can also give different human syndromes at different levels of heteroplasmy, e.g. the T8993G mutation in the *MTATP6* gene that causes Leigh's disease, with lactic acidosis, hypotonia and neurodegenerative disease at a high mutant load or ataxia and retinitis pigmentosa at a lower degree of heteroplasmy (Holt *et al.,* 1990; Tatuch *et al.,* 1992).

The often confusing clinical signs and symptoms are, at least in part, correlated with different levels of heteroplasmy in different parts of the human body. This can be seen for example in different compartments of the same cell (Kärppä *et al.,* 2005), or in different cells, for example different muscle cells (Sciacco *et al.,* 1994; Moslemi *et al.,* 1998). There can also be a discrepancy in mutant load between different tissues and organs in the body (Tanji *et al.,* 2000). However, to fully explain the penetrance of mtDNA mutations in human medicine, further factors are needed. In many instances these factors are unknown, although for some mutations an important role for the nuclear genetic background has been shown (Dunbar *et al.,* 1995; Cock *et al.,* 1998; McFarland *et al.,* 2004b; Hudson *et al.,* 2005a). Interactions with other mitochondrial genes also seem to play a role for some phenotypes (Fischel-Ghodsian, 1998; Hudson *et al.,* 2007). In addition, environmental factors may contribute to the disease presentation. For example, treatment with aminoglycoside antibiotics (Prezant *et al.,* 1993; Estivill *et al.,* 1998) or smoking (Kirkman *et al.,* 2009) have been shown to

be associated with disease onset of different mitochondrial diseases in humans.

The mutant load in blood from SAN-dogs was high (>88%), and overlapped considerably with the mutant load in blood from dogs affected subclinically and unaffected 1st to 4th degree maternal relatives. The mutant load in tissues from affected dogs was even higher than in blood. No difference in the level of heteroplasmy has been found between neurological and non-neurological tissues, between different parts of nervous system or between single muscle fibres. So far, these figures have not been compared with the mutant load in tissues of any maternal relative. The penetrance of clinical disease may possibly be explained by this observation.

The mutant load in blood was analysed by qOLA, as were tissues sampled post mortem from affected dogs. This quantification method was evaluated in paper II, where cloned plasmids containing wild type or mutant sequences were purified, diluted to the same concentration and mixed into dilution series. The method gave accurate estimates of values of heteroplasmy, even for extreme values (close to 0% and 100%). The resolution was not as good in the RFLP method applied for comparing heteroplasmy levels in single muscle fibres, and it is possible that variations of the degree of heteroplasmy within the range 0–2% wild type mtDNA between different fibres was present but remained undetected.

The biochemical method used had not been evaluated on dogs, therefore the muscle samples from affected and age-matched control dogs were pair-wise examined at the same occasion. One affected dog of the five included in the studies of mitochondria (dog 3, paper II) diverged from the other four. On histochemistry, no indication for a lowered COX activity was seen and by biochemistry no affection on the rate of ATP production or the activities of the respiratory chain enzymes could be found in comparison with the age-matched control dog of this affected dog. The mtDNA genetic content of these samples was double-checked afterwards (Table S1 in paper II). This excluded the possibility that the samples had been mixed up, and the outcome was instead considered to be a result of a mosaic pattern of heteroplasmy (Dufour *et al.,* 2008). On the other hand, this dog was the only one that showed clear signs of mitochondrial pathology (paracrystalline inclusions) on the muscle biopsy section analysed by electron microscopy. The inference from this finding was that this dog also had muscle fibres that were affected by the mtDNA deletion.

Histochemistry was also performed on muscle tissue from dogs undergoing post mortem examinations. No ragged red fibres were found, nor were any completely COX-negative fibres detected. The lack of control

muscle tissue stained on the same occasion is suggested to explain why no indications for a lowered COX activity could be detected by increased bluish staining compared with controls.

Assuming that the mutant load in blood shows some correlation with the mutant load in nervous tissue in both affected and related animals, the phenotypic expression of canine SAN requires a high mutant load of the pathogenic deletion and penetrates at a low frequency. Based on the studies performed, the factors that lead to penetrance are undetermined and involvement from the nuclear genetic background cannot be excluded. There are no indications for interacting mitochondrial genes, because the affected and related dogs were of the same mitochondrial haplotype.

No conspicuous differences in environmental factors between affected dogs and their unaffected maternal relatives were suggested from the history. However, no systematic epidemiological study was conducted and the owners were not asked about, for example, the presence of smoking in the home.

A few mutations suspected or proven to be pathogenic have been reported in humans in the mitochondrial $tRNA^{Tyr}$ gene, albeit at other positions in the gene. One common neuromuscular symptom is chronic progressive external ophthalmoplegia (Pulkes *et al.,* 2000; Raffelsberger *et al.,* 2001; Sahashi *et al.,* 2001; Scaglia *et al.,* 2003). Thus, a mutated $tRNA^{Tyr}$ gene per se does not give a uniform phenotype in humans and dogs. However, one of the dogs, in addition to the gait abnormalities, showed strabismus on excitement, according to the owner. This may well be correlated with a mitochondrial myopathy of the extraocular muscles of the same kind as seen in chronic progressive external ophthalmoplegia. Chronic progressive external ophthalmoplegia is a common feature in many mitochondrial disorders of humans, both syndromic and non-syndromic, and thus is not unique for mitochondrial $tRNA^{Tyr}$ mutations.

Sensory ataxic neuropathy as the presenting feature of a mitochondrial disease in humans is often combined with dysarthria and ophthalmoplegia (SANDO) (Fadic *et al.,* 1997; Van Goethem *et al.,* 2003; Okun & Bhatti, 2004; Gago *et al.,* 2006; Milone *et al.,* 2008). Sensory ataxic neuropathy syndromes have been seen with mutations in the genes for *POLG* and *Twinkle* (Van Goethem *et al.,* 2003; Van Goethem *et al.,* 2004; Hudson *et al.,* 2005b; Gago *et al.,* 2006; Milone *et al.,* 2008). *Twinkle* and *POLG* are both nuclear encoded enzymes, which are needed in the replication of mtDNA. Acquired spontaneous mutations of the mtDNA may develop secondary to any of these mutations. Most commonly, mitochondriopathies correlated with sensory ataxic neuropathy syndromes in humans have been associated

with large scale deletions in mtDNA. In contrast to canine SAN, a single base-pair deletion (or other single base-pair mutation) in mtDNA has not been reported for any human patient.

5.10 Neuropathological changes - neurological signs

Some but not all of the histopathological lesions observed in the dogs studied can be correlated with neurological deficits. The axonopathy in the PNS and the NAD-type appearance in the CNS, affecting large myelinated Ia/b-afferents and proprioceptive pathways, are both mirrored in the gait disturbance (ataxia and weak knee extension), posture (hyperextended carpi) and decreased spinal reflexes.

The involvement of descending motor pathways seen on histopathology was not reflected clinically by paresis, but a mild motor dysfunction in the gait abnormalities could well overlap with the more evident sensory signs. However, the degenerative changes in ventral horn cells were not indicated by any clinically detectable lower motor neuron dysfunction. The pathomorphological changes observed in the extrapyramidal basal nuclei were not correlated with any clinical or neurological signs. Extrapyramidal nuclear lesions in dogs do not have to be correlated with clinical signs at all (de Lahunta & Glass, 2009b). A few (n=3) of the affected dogs (n=27) displayed truncal swaying on neurological examination, a sign commonly associated with cerebellar lesions. One of the dogs with truncal swaying was necropsied, but displayed no histopathological changes in the cerebellum. One possible explanation could be that the truncal swaying seen in these SAN-dogs is unrelated to the cerebellum, but instead reflects dysfunctioning afferent neurons from the spinal musculature. Changes in the ponto- and spinocerebellum were seen post mortem in some dogs, but the dogs with pathomorphological changes in the cerebellum had not shown truncal swaying or any other classical cerebellar signs. This lack of correlation between lesions in the cerebellum and cerebellar signs is difficult to explain. Possibly, the slow progress of degenerative processes may play a role. Asymptomatic cerebellar degeneration considered to precede cerebellar signs has been documented in humans (Yokota *et al.,* 2006).

Some other clinical signs occurred rarely, and possibly but not unambiguously were signs of a malfunctioning nervous system (i.e. decreased menace response, urinary incontinence, inability to swim, cow-hocks, nibbling of the skin, absent ticklishness or hypersensitivity when touched). These signs could also have been coincidental findings. However, if any or all of them represent dysfunction in the nervous system, they are all

unspecific regarding neuroanatomical diagnosis, and neuropathological changes corresponding to them may be of an unspecific nature and localization. In addition, many affected male dogs were reported to urinate in the same way as bitches. Given that this behaviour is also seen in normal male dogs at an unknown frequency, the clinical relevance of this sign is doubtful.

Post mortem examination of a mother of two affected dogs revealed neurodegenerative changes in some brainstem nuclei and in peripheral nerves, although not exactly the same picture as in affected dogs. Neurologically, this mother of two SAN-dogs had moderately decreased patellar reflexes bilaterally and was considered a subclinical case of SAN. The polyradiculoneuropathy correlates well with her hyporeflexia. The family history and conclusions from other findings and non-findings suggested a mitochondrial disease, but other aetiologies for the neurological alterations were not completely ruled out. For example, the histological phenotype had similarities to the steroid neuropathy seen in canine cases of Cushing's disease, but adrenal glands and liver were without histopathological changes.

To summarize, affected dogs that were necropsied 1-2 years-old had distinct neurological signs but the neuropathological changes were mild. In affected dogs necropsied 4-9 years-old, neurological signs retained the same characteristics as in the younger dogs, but neuropathological changes were more widespread than the corresponding neurological signs indicated. The pathomorphological appearance at end stage of this disease is not known since necropsied dogs were all euthanized.

5.11 Molecular mechanisms - neuropathological changes

The mitochondrial *t*RNA^{Tyr} molecules in dogs with SAN had impaired stability, and affected dogs had a decreased rate of ATP production. Affected dogs also had histopathological changes compatible with a neuron-sparing spongiosis in the basal nuclei, NAD-like changes, and a central–peripheral axonopathy. The typical features of NAD have been associated with disrupted axonal transport, leading to accumulation of synaptic proteins and membranous deposits in the dystrophic axons (Sisó *et al.,* 2001). A decreased rate of ATP production in affected cells in the nervous system of affected dogs is presumed. Given that axonal transport is dependent upon oxidative metabolism (Ochs & Ranish, 1970) and synapses normally are densely packed with mitochondria (Ly & Verstreken, 2006), the appearance of NAD is probably correlated with the malfunctioning mitochondria and ATP

deficiency. The central–peripheral axonopathy and the spongiosis are similarly supposed to be correlated with energy deficiency in affected nerve fibres.

Neuronal cell death was not the most prominent histopathological feature of SAN, but some neuronal populations, in for example the putamen (a basal nucleus) and the ventral horns, were subject to cell death. Whether or not this was secondary to the axonopathy or was a primary event was not elucidated. The energy deficit could lead to cell death through excitotoxicity (Nardin & Johns, 2001). In addition, disruption of electron transport and ATP-production may result in generation of radical oxygen species (ROS), which activate apoptotic cell death (Zhang *et al.,* 1998).

The progressive nature of SAN is reflected by a more widespread distribution and increased severity of pathomorphological changes in older dogs than in younger dogs and there are also indications of ongoing neurodegeneration, i.e. Wallerian-like degeneration, in affected cases. The distribution of degenerative features by age in different nerve cell populations is mainly thought to reflect their general susceptibility to the intracellular metabolic derangement. Known mechanisms that underlie a temporal progression of neuropathological alterations between cells in involved pathways are trans-synaptic neuronal degeneration and retrograde degeneration (Summers *et al.,* 1995), which possibly account for some of the progression seen. However, because there are no direct anatomical connections between the proprioceptive pathways and the basal nuclei, this cannot be the only explanation. Other mechanisms that may lead to disease progression in mitochondrial disorders are induction of trans-neuronal degeneration (Dufour *et al.*, 2008), the "vicious cycle" initiated by the oxidative stress attributable to mitochondrial dysfunction, which leads to even further deterioration of mitochondrial function, or an accumulation of the mutant load over time in nerve cells.

5.12 Molecular mechanisms - neuropathological changes - neurological signs

In canine SAN, one specific mitochondrial mutation gives rise to a uniform clinical and pathomorphological picture in affected dogs, compared with the confusing pattern seen in many mitochondrial disorders in humans (see e.g. section 5.9). The factors that determine the penetrance of clinical signs in SAN are however not understood so far, because many of the dogs carrying a high mutant load (at least in blood) apparently never display any overt

neurological signs, and a number of the carriers that have been studied have been normal on neurological examination.

Perhaps even more puzzling is the selective vulnerability of tissues to this mutation. The mutant load was high in all examined tissues from affected dogs (close to 100%), thus differences in tissue heteroplasmy is not thought to be an explanatory factor for the clinical and pathological involvement of certain specific cell populations. It is more likely, that interplay between mutated mtDNA and some tissue-specific expression of nuclear genes in affected cell populations contributes to the vulnerability. In addition, ATP has other roles than energy storage, where a decreased production rate perhaps can contribute to selective tissue vulnerability. Indeed, ATP acts as a neurotransmitter at purinergic receptors (P2X receptors). Interestingly, subgroups of these receptors are known from sensory neurons in the nociceptive system (Burnstock, 2000). Another contributing factor may be the direct involvement of different respiratory chain enzymes in other cellular metabolic pathways, e.g. cytochrome *c,* albeit nuclear encoded, is also a mediator for apoptosis (Zhivotovsky *et al.,* 1998). Hypotheses that have been proposed to explain selective tissue vulnerability in human mitochondrial disorders include the role of mitochondrial diversity between different cell types (Kunz, 2003), more roles for *t*RNA genes in addition to involvement in mtDNA translation (DiMauro & Schon, 2001), and different mechanisms of action of mutations in different *t*RNA genes (DiMauro & Davidzon, 2005). A hypothesis for the specific vulnerability of retinal ganglion cells to mitochondrial dysfunction in Leber's hereditary optic neuropathy was presented recently by Yu-Wai-Man *et al.* (2009). These authors suggested that the abrupt mitochondrial concentration gradient normally seen at the transition from unmyelinated to myelinated segments of the optic nerve is maintained by active processes that involve the cytoskeletal architecture. They proposed that even subtle mitochondrial energy deficits result in impaired axonal transport, fragmentation of the mitochondrial network and eventually apoptotic cell death. However, this theory does not explain why these transition zones are not affected in other mitochondrial diseases with ubiquitous mitochondrial energy deficits. With respect to SAN in Golden Retriever dogs, optic nerves from three affected dogs (of three examined) were close to homoplasmic for the mutation and hence were considered likely to suffer from a decreased rate of ATP production, but no dog displayed visual deficits or had neuropathological changes detected in the visual pathways.

5.13 Establishment of a new animal model for a spontaneous mitochondrially inherited disease

Only one study of a spontaneous and maternally inherited condition in dogs with a causative mutation in the mtDNA has been reported previously (Li *et al.,* 2006). In that case, the mutation was in a protein encoding gene, whereas the SAN mutation is in a *t*RNA gene. In contrast to Golden Retriever dogs with SAN, the affected dogs reported by Li *et al.* (2006) were all dead when their disease was proposed to be a mitochondrial disorder, preventing all prospective investigations in the light of their disease being a mitochondrial disorder. Another spontaneous mitochondrial disease, pyruvate dehydrogenase phosphatase I deficiency in Clumber Spaniels and Sussex Spaniels, was also reported as a canine model for a mitochondrial disease with a proven genetic background (Cameron *et al.,* 2007). In that case, the mutation was in the nuclear DNA and the inheritance pattern was not maternal.

Documentation of this spontaneous mitochondrially transmitted disease in Golden Retriever dogs makes a contribution to mitochondrial medicine in general. There are some murine models that have been generated with mtDNA mutations (Larsson *et al.,* 1998; Inoue *et al.,* 2000; Sligh *et al.,* 2000; Trifunovic *et al.,* 2004), but owing to difficulties in introducing mtDNA into the mitochondria, there is a lack of good laboratory animal models (DiMauro & Davidzon, 2005; Taylor & Turnbull, 2005). No single model is considered really comparable to authentic mitochondrial diseases (Taylor & Turnbull, 2005; Kang & Hamasaki, 2006).

So called cybrid cells have been produced by fusing human cell lines that are completely devoid of mtDNA with enucleated cells containing functional mitochondria (King & Attardi, 1989). By using enucleated cells from patients that harbour a spontaneously occurring mtDNA mutation, this method can be used in vitro to test for the biochemical and cellular consequences of specific mtDNA mutations and also their interplay with different nuclear genetic backgrounds (Dunbar *et al.,* 1995; Cock *et al.,* 1998; Taylor & Turnbull, 2005). No cybrid cell system has been developed yet to test canine mtDNA mutations, to the author's knowledge. However, cybrid cells can not replace in vivo models in understanding the clinical expression of mtDNA diseases (DiMauro & Davidzon, 2005).

Mitochondrially inherited SAN in Golden Retriever dogs is now well described regarding its phenotype, clinical course and genotype thanks to a successful cooperation between dog owners, dog breeders, veterinarians and geneticists. This disease may thereby serve as an excellent animal model of a naturally occurring mitochondrial disease.

6 Conclusions

Maternally inherited SAN is a novel neurological syndrome in the Golden Retriever breed, which is suitable to serve as a naturally occurring animal model for mitochondrial diseases.

- Affected dogs present with ataxia, reduced postural reactions and reduced spinal reflexes. They develop degenerative changes, i.e. central–peripheral axonopathy, neuroaxonal dystrophy-type changes and basal nuclei encephalopathy, that are distributed in a specific anatomical pattern throughout the CNS and PNS.

- SAN has a chronic, slowly progressive clinical course with onset during puppyhood. The prognosis is guarded with respect to a full-length dog life, with about half of affected dogs being euthanized before 4 years of age, because of neurological impairment.

- SAN is a maternally transmitted mitochondrial disorder caused by a deletion in the mitochondrial *t*RNA^{Tyr} gene. By not using carrier bitches for breeding, the disease incidence can be reduced.

- The phenotypic expression of SAN is associated with a high mutant load, but the disease seems to penetrate only at a low frequency. Therefore, the nuclear genetic background and/or some environmental factor may also be involved in the disease presentation.

7 Future perspectives

Several questions have arisen during this study that may be elucidated in the future. For example, have there been dogs carrying this mutation even before "female Z", and if so, do they have matrilineal descendants anywhere in the world today? Is it possible to detect the degenerative nervous system lesions by magnetic resonance imaging, and if so, at what age? What role does the nuclear genetic background play in the clinical penetrance of this disease? What role does the expression of nuclear genes in different populations of nerve cells play to give the specific tissue vulnerability? Certainly, another perspective for the future is to elucidate the possible role of the mtDNA and the mitochondria in other canine neurodegenerative diseases.

A major challenge with mitochondrial disorders is to find an explanation for the puzzling phenotype/genotype relationship, both regarding the penetrance of clinical disease and regarding the selective tissue vulnerability in affected individuals, canine SAN being no exception. One way to approach this question for SAN is to continue with the analysis of tissues from affected dogs and unaffected carriers, as well as from pathologically affected and unaffected tissues. A comparison between the mutant load in different tissues from maternal relatives and the load in affected dogs is planned. Also, an investigation of the level of heteroplasmy with a high-resolution method such as qOLA in single cells of the nervous system from affected and related dogs would be of interest. An immunohistochemical characterization of degenerating cells in the nervous system of affected dogs is also planned. Studies of markers for neurodegeneration will be of help in understanding the pathogenesis of SAN and possibly also of other mitochondrial disorders, as will an investigation of respiratory chain enzymes in tissues by immunohistochemistry. In addition, a systematic study to search for triggering environmental factors is waiting to be done.

8 References

Agostino, A., Valletta, L., Chinnery, P.F., Ferrari, G., Carrara, F., Taylor, R.W., Schaefer, A.M., Turnbull, D.M., Tiranti, V. & Zeviani, M. (2003). Mutations of ANT1, Twinkle, and POLG1 in sporadic progressive external ophthalmoplegia (PEO). *Neurology* 60, 1354-6.

Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H.L., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R. & Young, I.G. (1981). Sequence and organization of the human mitochondrial genome. *Nature* 290, 457-65.

Armstrong, R.B., Saubert, C.W. 4th, Seeherman, H.J. & Taylor, C.R. (1982). Distribution of fiber types in locomotory muscles of dogs. *The American Journal of Anatomy* 163, 87-98.

Ashley, M.V., Laipis, P.J. & Hauswirth, W.W. (1989). Rapid segregation of heteroplasmic bovine mitochondria. *Nucleic Acids Research* 17, 7325-31.

Baiker, K., Hofmann, S., Fischer, A., Gödde, T., Medl, S., Schmahl, W., Bauer, M.F. & Matiasek, K. (2009). Leigh-like subacute necrotising encephalopathy in Yorkshire terriers: neuropathological characterisation, respiratory chain activities and mitochondrial DNA. *Acta Neuropathologica* 118, 697-709.

Boichard, D. (2002). Pedig: A fortran package for pedigree analysis suited for large populations. *7th World Congress on Genetics Applied to Livestock Production*, Montpellier, France, Communication No. 28-13.

Bourgeois, J.M. & Tarnopolsky, M.A. (2004). Pathology of skeletal muscle in mitochondrial disorders. *Mitochondrion* 4, 441-52.

Braund, K.G., Mehta, J.R., Toivio-Kinnucan, M., Amling, K.A., Shell, L.G. & Matz, M.E. (1989). Congenital hypomyelinating polyneuropathy in two Golden Retriever littermates. *Veterinary Pathology* 26, 202-8.

Brenner, O., de Lahunta, A., Cummings, J.F., Summers, B.A. & Monachelli, M. (1997). A canine encephalomyelopathy with morphological abnormalities in mitochondria. *Acta Neuropathologica* 94, 390-7.

Brenner, O., Wakshlag, J.J., Summers, B.A. & de Lahunta, A. (2000). Alaskan husky encephalopathy – a canine neurodegenerative disorder resembling subacute necrotizing encephalomyelopathy (Leigh syndrome). *Acta Neuropathologica* 100, 50-62.

Burnstock, G. (2000). P2X receptors in sensory neurones. *British Journal of Anaesthesia* 84, 476-88.

Cameron J.M., Maj, M.C., Levandovskiy, V., MacKay, N., Shelton, G.D. & Robinson, B.H. (2007). Identification of a canine model of pyruvate dehydrogenase phosphatase 1 deficiency. *Molecular Genetics and Metabolism* 90, 15-23

Chinnery, P.F., Howell, N., Lightowlers, R.N. & Turnbull, D.M. (1997). Molecular pathology of MELAS and MERFF. The relationship between mutation load and clinical phenotypes. *Brain* 120, 1713-21.

Chinnery, P.F., Majamaa, K., Turnbull, D. & Thorburn, D. (2006). Treatment for mitochondrial disorders. *Cochrane Database of Systematic Reviews* Issue 1, Art. No.: CD004426. DOI: 10.1002/14651858.CD004426.pub2.

Chrisman, C.L., Cork, L.C. & Gamble, D.A. (1984). Neuroaxonal dystrophy of Rottweiler dogs. *Journal of the American Veterinary Medical Association* 184, 464-7.

Cock, H.R., Tabrizi, S.J., Cooper, J.M. & Schapira, A.H.V. (1998). The influence of nuclear background on the biochemical expression of 3460 Leber's hereditary optic neuropathy. *Annals of Neurology* 44, 187-93.

Cork, L.C., Troncoso, J.C., Price, D.L., Stanley, E.F. & Griffin, J.W. (1983). Canine neuroaxonal dystrophy. *Journal of Neuropathology and Experimental Neurology* 42, 286-96.

da Costa, R.C., Parent, J.M., Poma R & de Lahunta, A. (2009). Multisystem axonopathy and neuronopathy in Golden Retriever dogs. *Journal of Veterinary Internal Medicine* 23, 935-9.

de Lahunta, A. & Averill, D.R. Jr (1976). Hereditary cerebellar cortical and extrapyramidal nuclear abiotrophy in Kerry Blue Terriers. *Journal of the American Veterinary Medical Association* 168, 1119-24.

de Lahunta, A. & Glass, E. (2009a). The neurologic examination. In: de Lahunta, A. & Glass, E., eds. *Veterinary Neuroanatomy and Clinical Neurology* 3rd ed, St. Louis, Missouri: Saunders Elsevier. Pp. 487-501.

de Lahunta, A. & Glass, E. (2009b). Upper motor neuron. In: de Lahunta, A. & Glass, E., eds. *Veterinary Neuroanatomy and Clinical Neurology* 3rd ed, St. Louis, Missouri: Saunders Elsevier. Pp. 192-220.

DiMauro, S. & Schon, E.A. (2001). Mitochondrial DNA mutations in human disease. *American Journal of Medical Genetics* 106, 18-26.

DiMauro, S. & Davidzon, G. (2005). Mitochondrial DNA and disease. *Annals of Medicine* 37, 222-32.

Dufour, E., Terzioglu, M., Sterky, F.H., Sörensen, L., Galter, D., Olson, L., Wilbertz, J. & Larsson, N.G. (2008). Age-associated mosaic respiratory chain deficiency causes trans-neuronal degeneration. *Human Molecular Genetics* 17, 1418-26.

Dunbar, D.R., Moonie, P.A., Jacobs, H.T. & Holt, I.J. (1995). Different cellular backgrounds confer a marked advantage to either mutant or wild-type mitochondrial genomes. *Proceedings of the National Academy of Sciences of the United States of America* 92, 6562-6.

Duncan, I.D. & Griffiths, I.R. (1982). A sensory neuropathy affecting long-haired Dachshund dogs. *Journal of Small Animal Practice* 23, 381-90.

Egenvall, A., Bonnett, B.N., Olson, P. & Hedhammar, Å. (2000). Gender, age and breed pattern of diagnoses for veterinary care in insured dogs in Sweden during 1996. *The Veterinary Record* 146, 551-7.

Egenvall, A., Bonnett, B.N., Hedhammar, Å. & Olson, P. (2005). Mortality in over 350,000 insured Swedish dogs from 1995-2000: II. Breed-specific age and survival patterns and relative risk for causes of death. *Acta Veterinaria Scandinavica* 46, 121-36.

Estivill, X., Govea, N., Barceló, A., Perelló, E., Badenas, C., Romero, E., Moral, L., Scozzari, R., D'Urbano, L., Zeviani, M. & Torroni, A. (1998). Familial progressive sensorineural deafness is mainly due to the mtDNA A1555G mutation and is enhanced by treatment with aminoglycosides. *American Journal of Human Genetics* 62, 27-35.

Fadic, R., Russell, J.A., Vedanarayanan, V.V., Lehar, M., Kuncl, R.W. & Johns, D.R. (1997). Sensory ataxic neuropathy as the presenting feature of a novel mitochondrial disease. *Neurology* 49, 239-45.

Fang, W., Huang, C.C., Lee, C.C., Cheng, S.Y., Pang, C.Y. & Wei, Y.H. (1993). Ophthalmologic manifestations in MELAS syndrome. *Archives of Neurology* 50, 977-80.

Fischel-Ghodsian N. (1998). Mitochondrial mutations and hearing loss: paradigm for mitochondrial genetics. *American Journal of Human Genetics* 62, 15-9.

Franklin, R.J.M., Jeffery, N.D. & Ramsey, I.K. (1995). Neuroaxonal dystrophy in a litter of papillon pups. *Journal of Small Animal Practice* 36, 441-4.

Gago, M.F., Rosas, M.J., Guimarães, J., Ferreira, M., Vilarinho, L., Castro, L & Carpenter, S. (2006). SANDO: Two novel mutations in POLG1 gene. *Neuromuscular Disorders* 16, 507-9.

Goto, Y., Nonaka, I. & Horai, S. (1990). A mutation in the $tRNA^{Leu(UUR)}$ gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. *Nature* 348, 651-3.

Gray, M.W. (1993) Origin and evolution of organelle genomes. *Current Opinion in Genetics and Development* 3, 884-90.

Griffiths, I.R., Duncan, I.D. & Barker, J. (1980). A progressive axonopathy of Boxer dogs affecting the central and peripheral nervous systems. *Journal of Small Animal Practice* 21, 29-43.

Griffiths, I.R., McCulloch, M.C. & Abrahams, S. (1985). Progressive axonopathy: an inherited neuropathy of Boxer dogs. 2. The nature and distribution of the pathological changes. *Neuropathology and Applied Neurobiology* 11, 431-46.

Gruber, A.D., Wessmann, A., Vandevelde, M., Summers, B.A. & Tipold, A. (2002). Mitochondriopathy with regional encephalic mineralization in a Jack Russell Terrier. *Veterinary Pathology* 39, 732-6.

Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. *Journal of Gerontology* 11, 298-300.

Harman, D. (1992). Free radical theory of aging. *Mutation Research* 275, 257-66.

Hauswirth, W.W. & Laipis, P.J. (1982). Mitochondrial DNA polymorphism in a maternal lineage of Holstein cows. *Proceedings of the National Academy of Sciences of the United States of America* 79, 4686-90.

Hazlett, M.J., Smith-Maxie, L.L. & de Lahunta, A. (2005). A degenerative encephalomyelopathy in 7 Kuvasz puppies. *The Canadian Veterinary Journal* 46, 429-32.

HGEN: Commercially available genetic test for canine SAN. http://www.hgen.slu.se/ShowPage.cfm?OrgenhetSida_ID=10918, 2009

Holt, I.J., Harding, A.E. & Morgan-Hughes, J.A. (1988). Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. *Nature* 331, 717-9.

Holt, I.J., Harding, A.E., Petty, R.K.H. & Morgan-Hughes, J.A. (1990). A new mitochondrial disease associated with mitochondrial DNA heteroplasmy. *American Journal of Human Genetics* 46, 428-33.

Hudson, G., Keers, S., Yu Wai Man, P., Griffiths, P., Huoponen, K., Savontaus, M.L., Nikoskelainen, E., Zeviani M., Carrara, F., Horvath, R., Karcagi, V., Spruijt, L., de Coo, I.F.M., Smeets, H.J.M. & Chinnery, P.F. (2005a). Identification of an X-chromosomal locus and haplotype modulating the phenotype of a mitochondrial DNA disorder. *American Journal of Human Genetics* 77, 1086-91.

Hudson, G., Deschauer, M., Busse, K., Zierz, S & Chinnery, P.F. (2005b). Sensory ataxic neuropathy due to a novel *C10Orf2* mutation with probable germline mosaicism. *Neurology* 64, 371-3.

Hudson, G., Carelli, V., Spruijt, L., Gerards, M., Mowbray, C., Achilli, A., Pyle, A., Elson, J., Howell, N., La Morgia, C., Valentino, M.L., Huoponen, K., Savontaus, M.L., Nikoskelainen, E., Sadun, A.A., Salomao, S.R., Belfort Jr, R., Griffiths, P., Yu Wai Man, P., de Coo, R.F.M., Horvath, R., Zeviani, M., Smeets, H.J.T., Torroni, A. & Chinnery, P.F. (2007). Clinical expression of Leber hereditary optic neuropathy is affected by the mitochondrial DNA-haplogroup background. *The American Journal of Human Genetics* 81, 228-33.

Illa, I., Rojas, R., Gallardo, E., Serrano, C. & Graus, F. (2001). Chronic idiopathic sensory ataxic neuropathy: immunological aspects of a series of 17 patients. *Revue Neurologique (Paris)* 157, 517-22.

Inoue, K., Nakada, K., Ogura, A., Isobe, K., Goto, Y., Nonaka, I. & Hayashi, J.I. (2000). Generation of mice with mitochondrial dysfunction by introducing mouse mtDNA carrying a deletion into zygotes. *Nature* 26, 176-81.

Jaber, L., Shohat, M., Bu, X., Fischel-Ghodsian, N., Yang, H.Y., Wang, S.J, & Rotter, J.I. (1992). Sensorineural deafness inherited as a tissue specific mitochondrial disorder. *Journal of Medical Genetics* 29, 86-90.

Jansen, R.P.S. (2000). Germline passage of mitochondria: quantitative considerations and possible embryological sequelae. *Human Reproduction* 15, 112-28.

Jenuth, J.P., Peterson, A.C., Fu, K. & Shoubridge, E.A. (1996). Random genetic drift in the female germline explains the rapid segregation of mammalian mitochondrial DNA. *Nature* 14, 146-51.

Jolly, R.D., Burbidge, H.M., Alley, M.R., Pack, R.J. & Wilson, M.S. (2000). Progressive myelopathy and neuropathy in New Zealand Huntaway dogs. *New Zealand Veterinary Journal* 48, 188-91.

Kang, D. & Hamasaki, N. (2006). Mitochondrial disease: maintenance of mitochondrial genome and molecular diagnostics. *Advances in Clinical Chemistry* 42, 217-54.

Kim, K.S., Lee, S.E., Jeong, H.W. & Ha, J.H. (1998). The complete nucleotide sequence of the domestic dog *(Canis familiaris)* mitochondrial genome. *Molecular Phylogenetics and Evolution* 10, 210-20.

King, M.P. & Attardi, G. (1989). Human cells lacking mtDNA: Repopulation with exogenous mitochondria by complementation. *Science* 246, 500-3.

Kirby, D.M., McFarland, R., Ohtake, A., Dunning, C., Ryan, M.T., Wilson, C., Ketteridge, D., Turnbull, D.M., Thorburn, D.R. & Taylor, R.W. (2004). Mutations of the mitochondrial *ND1* gene as a cause of MELAS. *Journal of Medical Genetics* 41, 784-9.

Kirkman, M.A., Yu-Wai-Man, P., Korsten, A., Leonhardt, M., Dimitriadis, K., De Coo, I.F., Klopstock, T. & Chinnery, P.F. (2009). Gene-environment interactions in Leber hereditary optic neuropathy. *Brain* 132, 2317-26.

Kunz, W.S. (2003). Different metabolic properties of mitochondrial oxidative phosphorylation in different cell types – important implications for mitochondrial cytopathies. *Experimental Physiology* 88, 149-54.

Kärppä, M., Herva, R., Moslemi, A.R., Oldfors, A., Kakko, S. & Majamaa, K. (2005). Spectrum of myopathic findings in 50 patients with the 3243A>G mutation in mitochondrial DNA. *Brain* 128, 1861-9.

Larsson, N.G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., Barsh, G.S. & Clayton, D.A. (1998). Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nature* 18, 231-6.

Levine, J.M., Hillman, R.B., Erb, H.N. & de Lahunta, A. (2002). The influence of age on patellar reflex response in the dog. *Journal of Veterinary Internal Medicine* 16, 244-6.

Li, F.Y., Cuddon, P.A., Song, J., Wood, S.L., Patterson, J.S., Shelton, G.D. & Duncan, I.D. (2006). Canine spongiform leukoencephalomyelopathy is associated with a missense mutation in cytochrome *b*. *Neurobiology of Disease* 21, 35-42.

Lin, M.T. & Beal, M.F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature* 443, 787-95.

Liolitsa, D., Rahman, S., Benton, S., Carr, L.J. & Hanna, M.G. (2003). Is the mitochondrial complex I ND5 gene a hot-spot for MELAS causing mutations? *Annals of Neurology* 53, 128-32.

Ly, C.V. & Verstreken, P. (2006). Mitochondria at the synapse. *The Neuroscientist* 12, 291-9.

Mancuso, M., Ferraris, S., Nishigaki, Y., Azan, G., Mauro, A., Sammarco, P., Krishna, S., Tay, S.K.H., Bonilla, E., Romansky, S.G., Hirano, M. & DiMauro, S. (2005). Congenital or late-onset myopathy in patients with the T14709C mtDNA mutation. *Journal of the Neurological Sciences* 228, 93-7.

Matz, M.E., Shell, L. & Braund, K. (1990). Peripheral hypomyelinization in two Golden Retriever littermates. *Journal of the American Veterinary Medical Association* 197, 228-30.

McFarland, R., Elson, J.L., Taylor, R.W., Howell, N. & Turnbull, D.M. (2004a). Assigning pathogenicity to mitochondrial tRNA mutations: when "definitely maybe" is not good enough. *Trends in Genetics* 20, 591-6.

McFarland, R., Schaefer, A.M., Gardner, J.L., Lynn, S., Hayes, C.M., Barron, M.J., Walker, M., Chinnery, P.F., Taylor, R.W. & Turnbull, D.M. (2004b). Familial myopathy: New insights into the T14709C mitochondrial tRNA mutation. *Annals of Neurology* 55, 478-84.

Milone, M., Brunetti-Pierri, N., Tang, L.Y., Kumar, N., Mezei, M.M., Josephs, K., Powell, S., Simpson, E. & Wong, L.J.C. (2008). Sensory ataxic neuropathy with ophthalmoparesis caused by POLG mutations. *Neuromuscular Disorders* 18, 626-32.

MITOMAP: A human mitochondrial genome database. http://www.mitomap.org, 2009.

Moslemi, A.R., Tulinius, M., Holme, E. & Oldfors, A. (1998). Threshold expression of the tRNA^{Lys} A8344G mutation in single muscle fibers. *Neuromuscular Disorders* 8, 345-9.

Nardin, R.A & Johns, D.R. (2001). Mitochondrial dysfunction and neuromuscular disease. *Muscle & Nerve* 24, 170-91.

Ochs, S. & Ranish, N. (1970). Metabolic dependence of fast axoplasmic transport in nerve. *Science* 167, 878-9.

Okun, M.S. & Bhatti, M.T. (2004). SANDO: Another presentation of mitochondrial disease. *American Journal of Ophthalmology* 137, 951-3.

Olby, N.J., Chan, K.K., Targett, M.P. & Houlton, J.E.F. (1997). Suspected mitochondrial myopathy in a Jack Russell Terrier. *Journal of Small Animal Practice* 38, 213-6.

Paciello, O., Maiolino, P., Fatone, G. & Papparella, S. (2003). Mitochondrial myopathy in a German Shepherd dog. *Veterinary Pathology* 40, 507-11.

Polyak, K., Li, Y., Zhu, H., Lengauer, C., Willson, J.K.V., Markowitz, S.D., Trush, M.A., Kinzler, K.W. & Vogelstein, B. (1998). Somatic mutations of the mitochondrial genome in human colorectal tumours. *Nature Genetics* 20, 291-3.

Prezant, T.R., Agapian, J.V., Bohlman, M.C., Bu, X., Öztas, S., Qiu, W.Q., Arnos, K.S., Cortopassi, G.A., Jaber, L., Rotter, J.I., Shohat, M. & Fischel-Ghodsian, N. (1993). Mitochondrial ribosomal RNA mutation associated with both antibiotic-induced and non-syndromic deafness. *Nature* 4, 289-94.

Pulkes, T., Siddiqui, A., Morgan-Hughes, J.A. & Hanna, M.G. (2000). A novel mutation in the mitochondrial tRNA^{Tyr} gene associated with exercise intolerance. *Neurology* 55, 1210-2.

Raffelsberger, T., Rossmanith, W., Thaller-Antlanger, H. & Bittner, R.E. (2001). CPEO associated with a single nucleotide deletion in the mitochondrial tRNA^{Tyr} gene. *Neurology* 57, 2298-301.

RASDATA: A Swedish database for breed statistics. http://rasdata.nu, 2009

Sadun, A.A., Carelli, V., Salomao, S.R., Berezovsky, A., Quiros, P., Sadun, F., DeNegri, A.M., Andrade, R., Schein, S. & Belfort, R. (2002). A very large Brazilian pedigree with 11778 Leber's hereditary optic neuropathy. *Transactions of the American Ophthalmological Society* 100, 169-80.

Sahashi, K., Yoneda, M., Ohno, K., Tanaka, M., Ibi, T. & Sahashi, K. (2001). Functional characterisation of mitochondrial tRNA^{Tyr} mutation (5877G→A) associated with familial chronic progressive external ophthalmoplegia. *Journal of Medical Genetics* 38, 703-5.

Scaglia, F., Vogel, H., Hawkins, E.P., Vladutiu, G.D., Liu, L.L. & Wong, L.J.C. (2003). Novel homoplasmic mutation in the mitochondrial *tRNA^{Tyr}* gene associated with atypical mitochondrial cytopathy presenting with focal segmental glomerulosclerosis. *American Journal of Medical Genetics* 123A, 172-8.

Schaefer, A.M., Taylor, R.W., Turnbull, D.M. & Chinnery, P.F. (2004). The epidemiology of mitochondrial disorders – past, present and future. *Biochimica et Biophysica Acta* 1659, 115-20.

Sciacco, M., Bonilla, E., Schon, E.A., DiMauro, S. & Moraes, C.T. (1994). Distribution of wild-type and common deletion forms of mtDNA in normal and respiration-deficient muscle fibers from patients with mitochondrial myopathy. *Human Molecular Genetics* 3, 13-9.

Shoffner, J.M., Bialer, M.G., Pavlakis, S.G., Lott, M., Kaufman, A., Dixon, J., Teichberg, S. & Wallace, D.C. (1995). Mitochondrial encephalomyopathy associated with a single nucleotide pair deletion in the mitochondrial $tRNA^{Leu(UUR)}$ gene. *Neurology* 45, 286-92.

Sisó, S., Ferrer, I. & Pumarola, M. (2001). Juvenile neuroaxonal dystrophy in a Rottweiler: accumulation of synaptic proteins in dystrophic axons. *Acta Neuropathologica* 102, 501-4.

Sisó, S., Hanzlíĉek, D., Fluehmann, G., Kathmann, I., Tomek, A., Papa, V. & Vandevelde, M. (2006). Neurodegenerative diseases in domestic animals: A comparative review. *The Veterinary Journal* 171, 20-38.

Sligh, J.E., Levy, S.E., Waymire, K.G., Allard, P., Dillehay, D.L., Nusinowitz, S., Heckenlively, J.R., MacGregor, G.R. & Wallace, D.C. (2000). Maternal germ-line transmission of mutant mtDNAs from embryonic stem cell-derived chimeric mice. *Proceedings of the National Academy of Sciences of the United States of America* 97, 14461-6.

SKK, Swedish Kennel Club registry. http://www.skk.se, 2009.

Sudoyo, H., Marzuki, S., Mastaglia, F. & Carroll, W. (1992). Molecular genetics of Leber's hereditary optic neuropathy: study of a six-generation family from Western Australia. *Journal of the Neurological Sciences* 108, 7-17.

Summers, B.A., Cummings, J.F. & de Lahunta, A. (1995). Principles of neuropathology. Introduction to the central nervous system. In: Summers, B.A., Cummings, J.F. & de Lahunta, A., eds. *Veterinary Neuropathology,* St. Louis, Missouri: Mosby Year Book. Pp. 1-26.

Taivassalo, T., De Stefano, N., Argov, Z., Matthews, P.M., Chen, J., Genge, A., Karpati, G. & Arnold, D.L. (1998). Effects of aerobic training in patients with mitochondrial myopathies. *Neurology* 50, 1055-60.

Taivassalo, T., Gardner, J.L., Taylor, R.W., Schaefer, A.M., Newman, J., Barron, M.J., Haller, R.G. & Turnbull, D.M. (2006). Endurance training and detraining in mitochondrial myopathies due to single large-scale mtDNA deletions. *Brain* 129, 3391-401.

Tanji, K., Schon, E.A., DiMauro, S. & Bonilla, E. (2000). Kearns-Sayre syndrome: oncocytic transformation of choroid plexus epithelium. *Journal of the Neurological Sciences* 178, 29-36.

Tankisi, H., Pugdahl, K., Fuglsang-Frederiksen, A., Johnsen, B., de Carvalho, M., Fawcett, P.R.W., Labarre-Vila, A., Liguori, R., Nix, W.A. & Schofield, I.S. (2005). Pathophysiology inferred from electrodiagnostic nerve tests and classification of polyneuropathies. Suggested guidelines. *Clinical Neurophysiology* 116, 1571-80.

Tatuch, Y., Christodoulou, J., Feigenbaum, A., Clarke, J.T.R., Wherret, J., Smith, C., Rudd, N., Petrova-Benedict, R. & Robinson, B.H. (1992). Heteroplasmic mtDNA mutation (T→G) at 8993 can cause Leigh disease when the percentage of abnormal mtDNA is high. *American Journal of Human Genetics* 50, 852-8.

Tauro, A., Talbot, C.E., Pratt, J.N.J. & Boydell I.P. (2008). Suspected mitochondrial myopathy in a springer spaniel. *The Veterinary Record* 163, 396-7.

Taylor, R.W. & Turnbull, D.M. (2005). Mitochondrial DNA mutations in human disease. *Nature Reviews. Genetics* 6, 389-402.

Trifunovic, A., Wredenberg, A., Falkenberg, M., Spelbrink, J.N., Rovio, A.T., Bruder, C.E., Bohlooly-Y, M., Gidlöf, S., Oldfors, A., Wibom, R., Törnell, J., Jacobs, H.T. &

Larsson, N.G. (2004). Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature* 429, 417-23.

van den Ouweland, J.M.W., Lemkes, H.H.P.J., Trembath, R.C., Ross, R., Velho, G., Cohen, D., Froguel, P & Maassen, J.A. (1994). Maternally inherited diabetes and deafness is a distinct subtype of diabetes and associates with a single point mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene. *Diabetes* 43, 746-51.

Van Goethem, G., Martin, J.J., Dermaut, B., Löfgren, A., Wibail, A., Ververken, D., Tack, P., Dehaene, I., Van Zandijcke, M., Moonen, M., Ceuterick, C., De Jonghe, P. & Van Broeckhoven, C. (2003). Recessive *POLG* mutations presenting with sensory and ataxic neuropathy in compound heterozygote patients with progressive external ophthalmoplegia. *Neuromuscular disorders* 13, 133-42.

Van Goethem, G., Luoma, P., Rantamäki, M., Al Memar, A., Kaakkola, S., Hackman, P., Krahe, R., Löfgren, A., Martin, J.J., De Jonghe, P., Suomalainen, A., Udd, B. & Van Broeckhoven, C. (2004). *POLG* mutations in neurodegenerative disorders with ataxia but no muscle involvement. *Neurology* 63, 1251-7.

van Nes, J.J. (1986). Clinical application of neuromuscular electrophysiology in the dog: A review. *The Veterinary Quarterly* 8, 240-50.

Vermeersch, K., Van Ham, L., Braund, K.G., Bhatti, S., Tshamala, M., Chiers, K. & Schrauwen, E. (2005). Sensory neuropathy in two Border Collie puppies. *Journal of Small Animal Practice* 46, 295-9.

Vijayasarathy, C., Giger, U., Prociuk, U., Patterson, D.F., Breitschwerdt, E.B. & Avadhani, N.G. (1994). Canine mitochondrial myopathy associated with reduced mitochondrial mRNA and altered cytochrome *c* oxidase activities in fibroblasts and skeletal muscle. *Comparative Biochemistry and Physiology. Part A, Physiology* 109A, 887-94.

Wakshlag, J.J., Cooper, B.J., Wakshlag, R.R., Kallfelz, F.A., Barr, S.C., Nydam, D.V. & Dimauro, S. (2004). Biochemical evaluation of mitochondrial respiratory chain enzymes in canine skeletal muscle. *American Journal of Veterinary Research* 65, 480-4.

Wallace, D.C., Singh, G., Lott, M.T., Hodge, J.A., Schurr, T.G., Lezza, A.M.S., Elsas II, L.J. & Nikoskelainen, E.K. (1988). Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. *Science* 242, 1427-30.

Wheeler, S.J. (1987). Sensory neuropathy in a Border Collie puppy. *Journal of Small Animal Practice* 28, 281-9.

Wibom, R., Hagenfeldt, L. & von Döbeln, U. (2002). Measurement of ATP production and respiratory chain enzyme activities in mitochondria isolated from small muscle biopsy samples. *Analytical Biochemistry* 311, 139-51.

Wood, S.L. & Patterson, J.S. (2001). Shetland Sheepdog leukodystrophy. *Journal of Veterinary Internal Medicine* 15, 486-93.

Yokota, O., Tsuchiya, K., Terada, S., Oshima, K., Ishizu, H., Matsushita, M., Kuroda, S. & Akiyama, H. (2006). Frequency and clinicopathological characteristics of alcoholic cerebellar degeneration in Japan: a cross-sectional study of 1,509 postmortems. *Acta Neuropathologica* 112, 43-51.

Yu-Wai-Man, P., Griffiths, P.G., Hudson, G. & Chinnery, P.F. (2009). Inherited mitochondrial optic neuropathies. *Journal of Medical Genetics* 46, 145-58.

Zhang, J., Yoneda, M., Naruse, K., Borgeld, H.J.W., Gong, J.S., Obata, S., Tanaka, M. & Yagi, K. (1998). Peroxide production and apoptosis in cultured cells carrying mtDNA mutation causing encephalomyopathy. *Biochemistry and Molecular Biology International* 46, 71-9.

Zhivotovsky, B., Hanson, K.P. & Orrenius, S. (1998). Back to the future: The role of cytochrome c in cell death. *Cell Death and Differentiation* 5, 459-60.

Acknowledgements

These studies were performed at the Department of Clinical Sciences and the Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences, Uppsala, Sweden. The studies were supported financially by the Agria Insurance Foundation for Research, the Swedish Kennel Club and the Golden Retriever Club of Sweden.

During the years there have been many who have helped in one way or another in providing the platform for this thesis, and I would like to express my gratitude especially to the following persons:

Professor Åke Hedhammar, my main supervisor, for believing in me, for scientifically challenging discussions and for always having time to answer my questions, and at the same time granting me sufficient freedom during this work. That was exactly what I needed. I am grateful and honoured for being supervised by you.

Associate Professor Agneta Egenvall, associate supervisor, for your honesty, for the fruitful scientific co-operation we have had and for your close scrutinizing of everything I tried to express a little bit too fast and a little bit too incorrectly.

Associate Professor Øyvind Stigen, associate supervisor, for being so thorough and for the scientific talks we've had.

PhD student Izabella Baranowska, co-author, for your contagious enthusiasm and for everything you've taught me about the mitochondrial genome. You've really been a fantastic co-worker.

DVM Eva Örvind, co-author, for always being positive about examining these dogs electrophysiologically from the very first ataxic Golden Retriever.

Dr. Kaspar Matiasek, co-author for sharing your knowledge and expertise in neuropathology.

DVM Eva Johnsson, co-author and Golden Retriever breeder for your initiative that lead to this project.

Professor Kristina Narfström, for introducing me into the world of active research and the world of "diplomacy".

Professor Leif Andersson and Professor Göran Andersson, co-authors, for sharing your knowledge in molecular genetics with a novice.

PhD Rolf Wibom, Associate Professor Inger Nennesmo and Professor Nils-Göran Larsson, co-authors, for opening the door into mitochondrial medicine.

PhD student Sofia Malm and PhD Caroline Hahn, co-authors, for your contributions to the first steps in phenotyping this disease.

Professor Anders Oldfors and Associate Professor Ali-Reza Moslemi at Sahlgrenska University Hospital, Gothenburg for the single-fibre studies.

Professor Anne-Sofie Lagerstedt, Dr. Med. Vet. Ann-Margaret Grøndahl and Dr. Philos. Kristin Thorud, the respective leaders at the Department of Clinical Sciences, Division of Small Animals, Swedish University of Agricultural Sciences and the Department of Companion Animal Clinical Sciences, Norwegian School of Veterinary Science, for providing me with time to finish this project.

The librarians in Klinikcentrum, Swedish University of Agricultural Sciences and in the Norwegian School of Veterinary Science.

All the Golden Retriever breeders, owners, and veterinarians that have been involved and also the Swedish Golden Retriever Club for your kind co-operation through all steps in this work.

Colleagues and staff at the Department of Clinical Sciences, University Animal Hospital, Department of Animal Breeding and Genetics and National Veterinary Institute in Uppsala and at Norwegian School of Veterinary Science in Oslo who have been involved in different parts of this work.

Josefin, Jonas, Nora and Joachim for being the nice children you are, my father-in-law Truls for your never-ending enthusiasm and vigour, and my sister Anna and her family Michael, Elin and Mathilda for all the interesting in-depth discussions and laughter over the years.

Almost last but not least my best friend and beloved husband Truls for all the support, encouragement, help, care, attention and love you have given me.

Both last and least, a purr and mew to the ageing phenotype with fur that was sitting in my lap (or preferably on the laptop) when I wrote this thesis.