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Canine inherited retinal degenerations

a model for visual impairment in humans

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Cover: Zimba, a Labrador retriever working as a guide dog
(photo: Linda Mankefors/Falu Kuriren)

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Canine inherited retinal degenerations: a model for visual impairment in humans

Abstract

Inherited retinal degenerations (IRDs) form a clinically and genetically heterogeneous group of diseases, leading to visual impairment or blindness in both humans and dogs. The prevalence of IRDs is estimated at 1 in 2,000 in humans. In dogs, the exact prevalence is unknown, but close to 100 different breeds have been reported to be affected, many by more than one type of IRD. The identification of the underlying genetic variants is critical, as the results can be used to develop genetic tests, which allow breeders to make informed breeding decisions while preserving genetic variation.

In Labrador retrievers, a novel form of IRD was recently identified, with clinical signs indicating cone-rod photoreceptor degeneration. In this thesis, a whole-genome sequencing approach was used to identify a frameshift insertion leading to a premature stop codon in the canine *ABCA4* gene. In humans, mutations in the *ABCA4* gene are the major cause of Stargardt disease (STGD), an autosomal recessive retinal degeneration leading to central visual impairment. Transcript and protein level investigations showed that the canine *ABCA4* insertion is a loss-of-function mutation responsible for the novel canine IRD, and leads to a phenotype similar to STGD in humans.

Golden retrievers are affected by at least four different forms of IRD, one of which is associated with a deletion in the *TTC8* gene. Mutations in this gene in humans are involved in the Bardet-Biedl syndrome (BBS) with heterogeneous clinical signs. We were able to show that the canine deletion is a loss-of-function mutation resulting in a syndromic IRD similar to BBS.

Lastly, while the human retinal transcriptome has been extensively studied, less is known about the gene expression patterns in the canine retina. Using short- and long-read cDNA sequencing we characterized the canine retinal transcriptome, results that in the future can be used to identify and validate causative genetic variants for canine IRDs. The results of this thesis contribute to the understanding of two important IRDs affecting the health and welfare of both dogs and humans. In addition, the thesis highlights the importance of a well-characterized retinal transcriptome for successful identification of disease-causing alleles.

Keywords: dog, retina, PRA, retinopathy, ABCA4, Stargardt disease, TTC8, Bardet-Biedl syndrome, whole-genome sequencing, transcriptome

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Dedication

To my parents

To suppose that the eye with all its inimitable contrivances for adjusting the focus to different distances, for admitting different amounts of light, and for the correction of spherical and chromatic aberration, could have been formed by natural selection, seems, I freely confess, absurd in the highest possible degree.

Charles Darwin, 1859 in the Origin of Species

Contents

List of publications

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

I **Mäkeläinen S**., Gòdia M., Hellsand M., Viļuma A., Hahn D., Makdoumi K., Zeiss C.J., Mellersh C., Ricketts S.L., Narfström K., Hallböök F., Ekesten B., Andersson G., Bergström T.F. (2019). An *ABCA4* loss-of-function mutation causes a canine form of Stargardt disease. *PLOS Genetics*, 15(3): e1007873.

II **Mäkeläinen S**., Hellsand M., van der Heiden A. D., Andersson E., Thorsson E., Ström-Holst B., Häggström J., Ljungvall I., Mellersh C., Hallböök F., Andersson G., Ekesten B., Bergström T.F. (2020). Deletion in the Bardet-Biedl Syndrome Gene *TTC8* Results in a Syndromic Retinal Degeneration in Dogs. *Genes*, 11(9): e1090

III **Mäkeläinen S**., Wallerman O., van der Heiden A. D., Lindblad-Toh K., Ekesten B., Andersson G., Bergström T.F. (2020). Characterization of the canine retinal transcriptome using long- and short-read cDNA sequencing. (manuscript)

The contribution of Suvi Mäkeläinen to the papers included in this thesis was as follows:

I Took major part in planning the study, performed data analyses, performed most of the lab work, interpreted the results together with co-authors, had the main responsibility for writing the manuscript together with the corresponding author, and contributed to the correspondence with the journal.

II Designed the study together with the main supervisor, took part in the sampling and phenotypic characterization of the affected dogs, performed data analyses, interpreted the results together with co-authors, and had the main responsibility for drafting the manuscript, and, together with the corresponding author, for writing the final version of the manuscript and contributed to the correspondence with the journal.

III Designed the study together with the main supervisor, performed data analyses, interpreted the results together with co-authors, had the main responsibility for drafting the manuscript, and together with the corresponding author, for writing the final version of the manuscript.

Abbreviations

A2E	di-retinal-pyridinium-ethanolamine
A2PE	di-retinoid-pyridinium-phosphatidylethanolamine
AAV	adeno-associated virus
ADP	adenosine diphosphate
AMD	age-related macular degeneration
ATP	adenosine triphosphate
BAC	bacterial artificial chromosomes
BBS	Bardet-Biedl syndrome
bp	base pair
cDNA	complementary deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
COD	Cone degeneration
CRD	cone-rod degenerations
cSLO	confocal scanning laser ophthalmoscopy
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
ERD	early retinal degeneration
ERG	electroretinography
FCI	World Canine Organization
FERG	Flash electroretinogram
GC	guanine-cytosine
GCL	ganglion cell layer

GO	gene ontology
GPCR	G protein coupled receptors
GWAS	genome-wide association
ILM	internal limiting membrane
INDEL	insertion or deletion
INL	inner nuclear layer
IPL	inner plexiform layer
IRD	inherited retinal degeneration
IS	inner segment
LCA	Leber congenital amaurosis
LD	linkage disequilibrium
LHON	Leber hereditary optic neuropathy
MD	macular degeneration
mRNA	messenger ribonucleic acid
N-cis-R-PE	*N*-11-*cis*-retinylidene-phosphatidylethanolamine
N-trans-R-PE	*N*-retinylidene-phosphatidylethanolamine
NFL	nerve fiber layer
NGS	next generation sequencing
nm	nanometers
NMD	nonsense-mediated decay
nt	nucleotide
OCT	optic coherence tomography
ONL	outer nuclear layer
ONT	Oxford Nanopore Technologies
OPL	outer plexiform layer
OS	outer segment
PacBio	Pacific Biosciences
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PNA	peanut-agglutin
PRA	progressive retinal atrophy
RCD	rod-cone degeneration
RNA	ribonucleic acid
RP	retinitis pigmentosa

RPE	retinal pigment epithelium
RT-PCR	reverse transcription polymerase chain reaction
SNP	single nucleotide polymorphisms
SNV	single nucleotide variant
TPM	transcripts per million
UPS	ubiquitin–proteasome system
USH	Usher syndrome
UTR	untranslated region
WGS	whole-genome sequencing

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1 Introduction

Inherited retinal degenerations (IRDs) form a group of diseases characterized by deterioration of the retinal cells, resulting in visual impairment or blindness. The domestic dog has become an important comparative model for translational research of human genetic diseases. In particular for IRDs, where the dog has been instrumental for the development of gene-therapy based treatment strategies for human patients. A notable success story was the approval of a gene therapy protocol for treatment of Leber congenital amaurosis (LCA) in 2017. A defect in the *RPE65* gene was identified as the cause of LCA type 2, an early-onset retinal degeneration, in humans (Gu *et al.*, 1997; Marlhens *et al.*, 1997), and subsequently, using a candidate gene approach, in Briard dogs (Veske *et al.*, 1999). Lancelot, an affected Briard dog, became the first LCA-patient to be treated with gene therapy (Acland *et al.*, 2001), and the canine model was central for the preclinical development of the protocol.

In addition to *RPE65*, several other spontaneous canine IRDs of comparative interest for human ophthalmologists are now studied more in detail with the aim to provide gene therapy protocols (Winkler *et al.*, 2020). Currently, 32 genes involved in canine IRDs have been identified, of which 24 have been reported to be involved in similar diseases in humans so far. However, for many of the canine IRDs the underlying genetic cause remains unknown. The identification of novel causative genetic variants also enables the development of genetic tests which can be used to improve the health of the dogs.

In this thesis I used whole-genome sequencing, as well as transcript and protein level expression analysis to investigate two different IRDs in dogs, affecting the function of the photoreceptors in the retina and leading to visual impairment. In addition, I applied short-read and long-read cDNA sequencing technologies to characterize gene expression of the canine retina in an attempt to provide a better basis for research of canine inherited retinal degenerations.

2 Background

2.1 The retina

A landmark for retinal research was when the German physiologist Franz Christian Boll discovered the photopigment rhodopsin in the 1870's by bleaching red frog retinas with light exposure (Boll, 1877). The discovery came shortly after Max Schultze had proposed the duplex theory of vision (Schultze, 1866), suggesting that the vertebrate eye has two types of photoreceptor cells with different sensitivities to light (Ingram *et al.*, 2016). In the same time period, advances in cell staining methods developed by Camillo Golgi (Golgi, 1873), and the detailed descriptions of retinal cell layers by Santiago Ramón y Cajal (Ramón y Cajal, 1889) led to the understanding that the retina is part of the central nervous system. Both Golgi and Ramón y Cajal received the Nobel prize in Physiology or Medicine in 1906 for their important contributions. Almost a hundred years after the identification of rhodopsin, in 1967, George Wald was awarded the Nobel prize for his work elucidating the molecular components of the visual cycle (Wald, 1968), a prize shared with Ragnar Granit and Haldan Keffer Hartline "for their discoveries concerning the primary physiological and chemical visual processes in the eye".

2.1.1 The structure of the retina

The retina, situated between vitreous humour at its anterior side and choroid at its posterior side, is a thin, highly complex tissue layer lining the back of the eye (**Figure 1A)**. It consists of more than 60 distinct cell types (Masland, 2017). As part of the central nervous system, it receives information in wavelengths of visual light, and converts the light energy into electrical signals which can be interpreted by the brain (Tomita, 1970). The neuroretina consists of five major

neuronal cell types: retinal ganglion cells, amacrine cells, bipolar cells, horizontal cells and photoreceptor cells (Masland, 2012; Masland, 2011) as shown in **Figure 1B**. The photoreceptor cells are the most abundant cell type of the retina, in humans comprising approximately 120 million rod and 6 million cone cells (Molday & Moritz, 2015). Humans have approximately 5 million bipolar cells and 1 million ganglion cells (Sung & Chuang, 2010). The photoreceptor cells are highly specialized neuroepithelial cells converting light signals into neural impulses. The impulse then travels to the bipolar cells, and further to the visual cortex of the brain via the retinal ganglion cell axons, which form the optic nerve. Interneurons mediate lateral information flow from photoreceptor cells to bipolar cells (horizontal cells), and bipolar cells to ganglion cells (amacrine cells). Müller glial cells traverse through all retinal layers, and their function is to provide support and protection for the neurons.

The retinal cells are organized in layers (**Figure 1C**). The innermost boundary of the retina, the internal limiting membrane (ILM), is located between the vitreous humour and the retinal nerve fiber layer (NFL). The ganglion cell bodies form the ganglion cell layer (GCL), which is the proximal cell layer of the retina closest to the vitreous body. The ganglion, bipolar and amacrine cell synapses form the inner plexiform layer (IPL), followed by the inner nuclear layer (INL) consisting of cell nuclei of the bipolar, amacrine, horizontal, and Müller glial cells. The outer plexiform layer (OPL) is formed by the synapses between photoreceptor cells and bipolar cells, as well as horizontal cells. Photoreceptor cell nuclei are located at the outer nuclear layer (ONL) of the retina. The inner segments (IS) contain the biosynthetic machinery of the photoreceptor cell, including the rough endoplasmic reticulum (ER), free ribosomes, and the Golgi apparatus, and the distal end of the IS is densely packed with mitochondria (Molday & Moritz, 2015). The photoreceptor outer segments (OS) are embedded by the retinal pigment epithelium (RPE), a monocellular layer, which plays a critical role in maintenance of the photoreceptor cells, including the phagocytosis of the photoreceptor disks and renewing photopigments of the photoreceptor cells. The RPE, together with the choroidal vasculature, support the retina by bringing oxygen and glucose for the retinal cells and transporting waste products out of them. The choroidal vascular network is part of the systemic circulation, whereas blood vessels in the retina are restricted by the blood-retina barrier (Cunha-Vaz *et al.*, 1966; Palm, 1947).

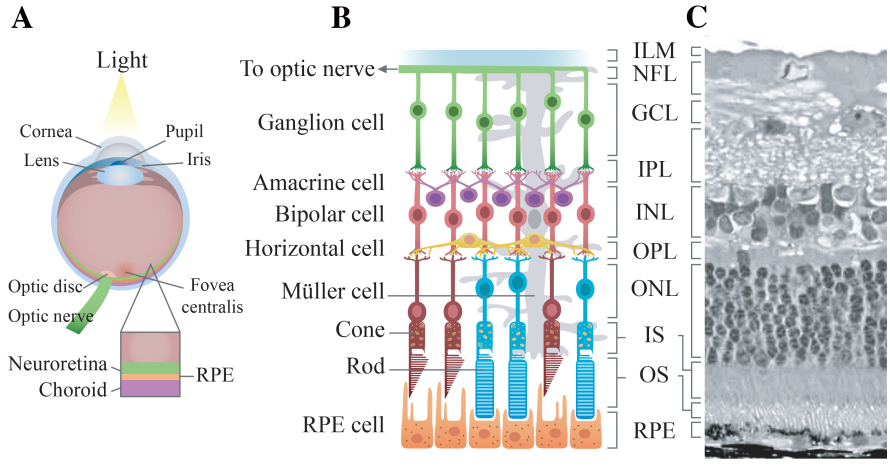

Figure 1. Structure of the retina. (**A**) Light enters the eye through the cornea, and is directed to the back of the eye, reaching the photoreceptor cells of the retina. (**B**) Schematic drawing of the arrangement of the five major neuronal cell types, non-neuronal Müller glial cells, and RPE cells. (**C**) Histology section of a normal canine retina where the different retinal layers are indicated. Illustrations by Anna Darlene van der Heiden
Histology image by courtesy of Dr. Simon Petersen-Jones, Michigan State University

2.1.2 Light is absorbed in the photoreceptor outer segments

The light absorption occurs in the disks of the photoreceptor outer segments, in the transmembrane domain of specific G protein coupled receptors (GPCRs) (Hara-Nishimura *et al.*, 1993), which span across the disk membranes in both rods and cones. The rod photoreceptor GPCR, a photopigment protein rhodopsin, is able to detect a single photon, and rods thus function in low light conditions (Baylor *et al.*, 1979). The peak sensitivity of the rod photoreceptors for light is at approximately 500 nm. Cones account for high acuity vision under daylight conditions and need more photons for activation compared to the rods. The GPCRs of cone photoreceptors are essential for our ability to discriminate objects based on their emission or reflection of different wavelengths of light. Thus, the cone photoreceptors also allow us to perceive colors.

Normal human color vision is trichromatic and based on the three different types of cone photoreceptors in the retina, each with different spectral sensitivity (Bowmaker & Dartnall, 1980). In humans, light absorption of long-wavelength cones (red cones, L) peaks at approximately 560 nm, in middle-wave cones (green cones, M) at 530 nm and short-wavelength cones (blue cones, S) at approximately 415-425 nm, enabling the normal human eye to see with a mixture of three spectral lights (Katayama *et al.*, 2019; Oprian *et al.*, 1991). Dogs, like most mammals, have dichromatic vision, with two types of cone

photoreceptors having spectral sensitivities peaking at approximately 429–435 nm (short-wavelength-absorbing cones or S-cones) and 555 nm (medium-to-long-wavelength-absorbing cones or M/L-cones) (Jacobs *et al.*, 1993; Neitz *et al.*, 1989). In dogs, the S-cones express opsin encoded by short wave sensitive opsin 1 (*OPN1SW*) and M/L-cone opsins are encoded by a gene termed long wave sensitive opsin 1 (*OPN1LW*). Dog lacks the third cone type, which in humans is encoded by medium wave sensitive opsin 1 (*OPN1MW*) (Nathans *et al.*, 1986).

2.1.3 Area centralis and the fovea

Not only the type of cone photoreceptor cells in the retina differ between species, but also spatial distribution and ratio between different photoreceptors vary. In the human eye, cone photoreceptor cells are outnumber by rods (1:20) in all other regions, except in the region for high acuity vision, the *fovea centralis* (fovea) in the *macula lutea* (macula) (Curcio *et al.*, 1990). The fovea harbors only cone photoreceptors, and the cell bodies of the cones proximal to fovea have been shifted to the side, creating a foveal pit (foveola), where light can enter the cone outer segments with minimal distortion. The fovea is also devoid of retinal blood vessels, and the foveal cones are connected to only one bipolar and one ganglion cell, unlike in the surrounding retina where each of the bipolar and ganglion cell receive signals from multiple photoreceptor cells (Provis *et al.*, 2013).

In dogs, a region similar to fovea, called the *area centralis* has higher cone density (cone-rod ratio 1:20) than the surrounding retina (1:40), and is devoid of the large retinal vessels (Mowat *et al.*, 2008). *Area centralis* is located in the temporal part of the visual streak, a region superior to the optic disc with high ganglion cell density (Peichl, 1992). In contrast to the human macula, the precise localization of *area centralis* in healthy dogs is not possible ophthalmoscopically, although it can be roughly estimated from the pattern of retinal vessels. In 2014, Beltran and colleagues showed that the canine *area centralis*, although lacking a foveal pit, does have a fovea-like region with localized thinning of the ONL (Beltran *et al.*, 2014). In comparison to the surrounding regions in the area centralis, the canine fovea-like region has a cone photoreceptor packing density which is at least 5-fold higher (Beltran *et al.*, 2014). Mice, although being important animal model for human IRDs, are nocturnal animals, and their retina is more rod dominated compared to the dog, with a cone-rod ratio of ~1:30 both in the peripheral as well as central retina (Carter-Dawson & LaVail, 1979). Mice also lack a cone dense *fovea* or *area centralis*, making the modelling of IRDs with initial cone degeneration challenging (Marmorstein & Marmorstein, 2007).

Many vertebrate species, excluding primates, have a light reflecting layer, the *tapetum lucidum*, at the back of the retina, enhancing the capture of light under dim light conditions by reflecting the scattered light back to the OS of the photoreceptors. In dogs, the tapetal cells are located in the choroidal cell layer (Lesiuk & Braekevelt, 1983), and the tapetum usually expands as a triangle shape above the optic nerve. In the non-tapetal region melanin granules in the RPE cells absorb the scattered light which is not absorbed by the photoreceptor cells. In contrast, RPE cells in the tapetal region are devoid of melanin, allowing light to reach the tapetal cells. The reflection from the tapetum creates challenges for fundus autofluorescence imaging, which in humans and non-tapetal region of the dogs can be used to investigate accumulation of lipofuscin, a pigment formed by oxidation of unsaturated fatty acids as a result of both normal aging and neurodegenerative diseases (Marani *et al.*, 2009).

2.1.4 Phototransduction and the visual cycle

In the absence of light, cyclic guanosine monophosphate (cGMP) is bound to the cGMP gated channels in the plasma membrane, keeping them open and ensuring the flow of ions (the dark current) into the photoreceptor cells (Fesenko *et al.*, 1985). Ions are simultaneously transported out of the cells by active transport of the ion pumps. Phototransduction, the process of light absorption and creation of a neural signal starts in the photopigments, when the chromophore, a vitamin A aldehyde (retinal), covalently bound to the photopigments, changes conformation from 11-*cis* retinal to all-*trans* retinal as a response to receiving a photon (Palczewski *et al.*, 2000; Yoshizawa & Wald, 1963). This photoexcitation triggers a signal transduction cascade which follows the same principle in both rod and cone photoreceptor cells, but is partly mediated by different proteins, encoded by members of related gene families specific for the cell type (Larhammar *et al.*, 2009). The conformational change of the photopigment initiates transducin-mediated signaling, where photoactivated rhodopsin (metarhodopsin II) catalyzes the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) of the transducin, which in turn activates cGMP phosphodiesterase (PDE6) (Hargrave *et al.*, 1993). PDE6 then hydrolyses cGMP to GMP (Azevedo *et al.*, 2014), and as a result, the cGMP gated ion channels close, which leads to the hyperpolarization of the cell. This change in the membrane potential is sensed by the synapses, which reduce their release of the neurotransmitter glutamate from the ribbon synapses to the bipolar cells. The decreased glutamate release in turn activates the bipolar cell.

After the photoexcitation, the photoactivated opsins are inactivated by phosphorylation and binding of arrestin (Nikonov *et al.*, 2008; Kühn & Wilden,

1987). This leads to the recovery of the cGMP, which binds to the ion channels finally reopen and restore the dark current. The photopigment is regenerated through the retinoid (visual) cycle (Wald, 1968; Wald, 1935), illustrated in **Figure 2**, where the all-*trans* retinal is first imported from the disk lumen into the cytosol by the ATP binding cassette subfamily A member 4 (ABCA4) protein (Quazi *et al.*, 2012; Molday *et al.*, 2000), and subsequently inactivated to all-*trans* retinol (vitamin A) in a reaction catalyzed by a NADPH-dependent all-*trans* retinol dehydrogenase (Parker & Crouch, 2010). All-*trans* retinol can then diffuse to the RPE cells with the help of the interphotoreceptor retinoid binding protein (IRBP) (Kiser *et al.*, 2012). In the RPE cells the photopigment is converted back into its 11-*cis* retinal, and transported back to the OS to reform an activatable rhodopsin. These steps include esterification of the all-*trans* retinol into retinyl esters by lecithin retinol acyltransferase (LRAT) (Saari & Bredberg, 1989), followed by the storage of retinyl esters in the retinosomes of the RPE (Imanishi *et al.*, 2004), or direct modification by RPE65 into 11-*cis* retinol, and oxidation into 11-*cis* retinal by RDHs (mostly RDH5) (Parker & Crouch, 2010), and the diffusion back to the OS with the help of IRBP.

In addition to the canonical RPE-mediated visual cycle, an alternative visual cycle has been proposed to supply 11-*cis* retinal to cone photoreceptors (reviewed in (Palczewski & Kiser, 2020; Wang & Kefalov, 2011)). In this pathway, a non-visual opsin termed the retinal G protein coupled receptor all-*trans* retinal (RGR) is needed to regenerate the chromophore independently of RPE65 (Zhang *et al.*, 2019).

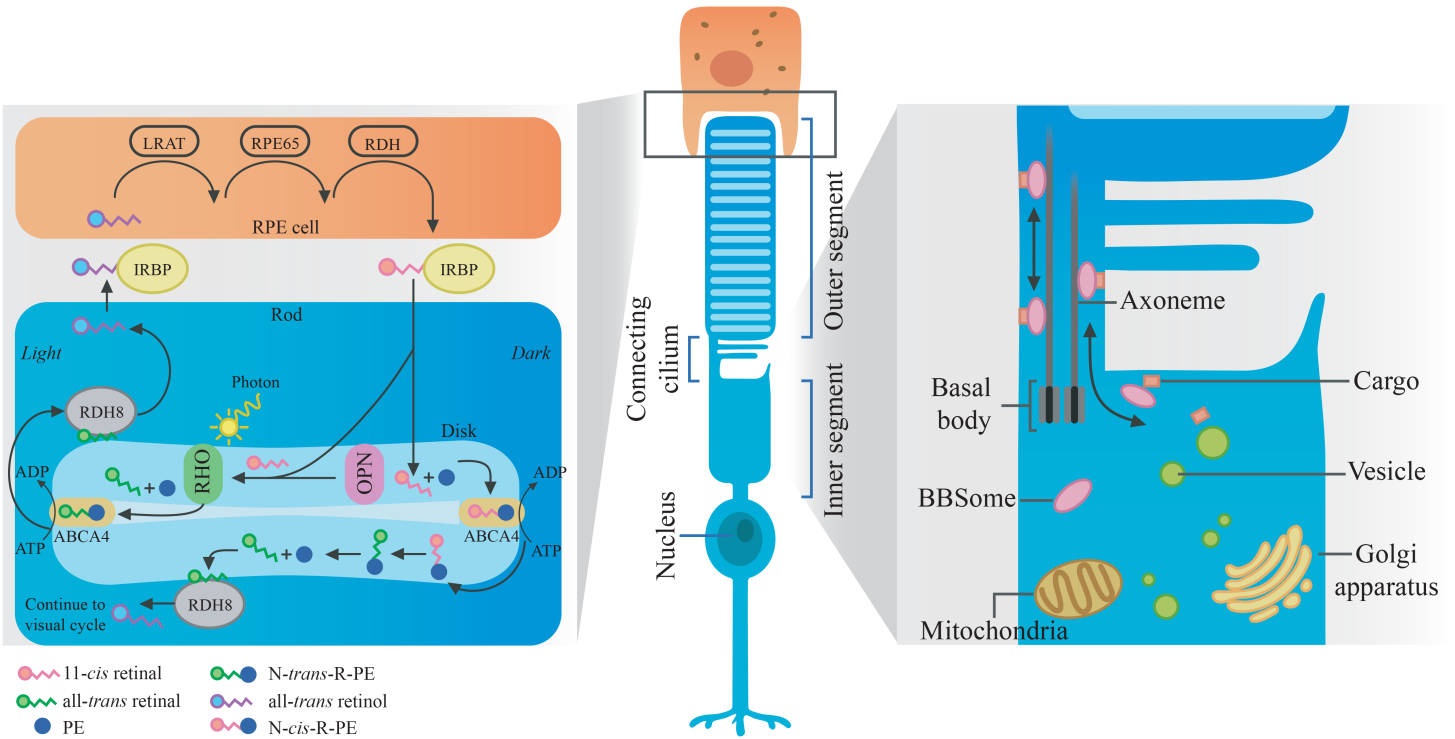

Figure 2. The visual cycle (left) and protein trafficking through the connecting cilium (right) in a rod photoreceptor. Illustration by Anna Darlene van der Heiden

2.1.5 Outer segments are specialized type of primary cilia

The first electron microscopic examinations of rod photoreceptor ultrastructure showed that the photoreceptor OS is composed of thousands of stacked disks (Sjöstrand, 1949), and suggested that there were similarities in the OS structure with that of the of cilium (De Robertis, 1956). We now know that the photoreceptors contain a specialized type of primary cilia (Wheway *et al.*, 2014), and in addition to the outer segment disks, they contain a microtubule-based axoneme and a connecting cilium. The axoneme begins at the basal body of the IS, stretches through the connecting cilium and continues up into the OS lining the disks (Roof *et al.*, 1991; Steinberg & Wood, 1975). The connecting cilium is equivalent to the transition zone of cilia, and acts as a bridge for proteins and lipids which are synthesized in the IS and transported into the OS (Röhlich, 1975). The basal body separates the outer segments and acts as a diffusion barrier, and thus the proteins of the OS need to be trafficked through the basal body with a protein complex termed the BBSome (Nachury *et al.*, 2007) acting as an adapter for the protein cargo, and intraflagellar transport (IFT) trains that move up and down the axoneme (van Dam *et al.*, 2013; Kozminski *et al.*, 1993), as shown in **Figure 2**.

The disks of rod photoreceptors are closed structures and have a distinct protein composition compared to the plasma membrane (Molday & Molday, 1987), whereas the disk membranes in the cone outer segments are continuous with the plasma membrane (Molday, 1998). In both rods and cones, the OS is renewed in a process where new disks are added from the base of the OS and aged disks are shed from the distal end of the cell (Young, 1967). The shed disks are phagocytized by the adjacent RPE cells (Young & Bok, 1969). The shedding of the photoreceptor disks occurs once a day, following a circadian rhythm (LaVail, 1980), and the process enables the outer segments to be completely renewed over a period of 10 days (Young & Bok, 1969; Young, 1967).

2.1.6 The metabolic ecosystem of the retina

The retina is a highly metabolically active tissue, and one of the most energy demanding tissues in the body. Similar to the brain tissue, the retina cannot store glucose in proportion to its demand. Therefore, it relies on transportation of metabolites via the systemic circulation (Kumagai, 1999). Glucose and oxygen mainly reach the retina via the choroidal blood vessel. Many mammals, including dogs, mice and primates, have additional blood vessels on the inner (vitreal) side of the retina (Yu & Cringle, 2001). Although the retinal vasculature

presumably affect the visual acuity by interfering with the pathway of light to the photoreceptors (Country, 2017), they increase the metabolic flow into the retina. The maintenance of membrane potential in darkness, the phototransduction, and the neurotransmission through the retina consume most energy (Ames *et al.*, 1992), but also the anabolic metabolism of OS renewal demands high amounts of energy (Chinchore *et al.*, 2017). Aerobic glycolysis with lactate formation accounts for a significant portion of the glucose consumption of the retina. For example, in the cat retina, aerobic glycolysis was found to account 78% of the glucose consumption (Wang *et al.*, 1997).

2.2 Inherited retinal degenerations

Retinitis pigmentosa (RP) was the first identified IRD in humans, named by Dutch ophthalmologist F.C. Donders (Donders, 1857), and at the time, RP was considered to be "one disease". However, is now well-established that RP by itself is a large group of diseases, caused by mutations in several genes and affecting approximately 1 in 3000-7000 people (Ferrari *et al.*, 2011). The first identified mutation, a non-synonymous substitution in the gene encoding for rhodopsin (*RHO*) was reported 30 years ago (Dryja *et al.*, 1990), and since then approximately 235 different *RHO* mutations (*The Human Gene Mutation Database, HGMD Pro 20.2*) and at least 89 different genes resulting in RP have been identified (*Online Mendelian Inheritance in Man, OMIM*).

2.2.1 Different types of IRDs

It is now recognized that in addition to RP, many other IRDs affect the retina, and form a genetically and phenotypically heterogeneous group of diseases. IRDs can be loosely categorized by the initially affected cell type, as well as onset and progression of the disease (Berger *et al.*, 2010). RP, the most common form of IRD, is an example of a progressive rod-cone degeneration (RCD), where the patients first experience visual problems in dim light resulting from loss of rod photoreceptors, and the cones are affected at a later stage of the disease. The opposite sequence of events is seen in the cone-rod degenerations (CRD), which are characterized by loss of visual acuity and defects in color vision due to primary cone involvement, followed by secondary loss of rod cells (Hamel, 2007). Cone degeneration (COD) and macular degeneration (MD), such as Stargardt disease, also affect cone photoreceptors, and deteriorate the central vision, but spare the rod dominated peripheral retina (Berger *et al.*, 2010). However, rod photoreceptors are often affected at a later stage of the disease. In addition to progressive IRDs, there are stationary IRDs where the disease state

does not change or changes very little after the initial onset of clinical signs (Berger *et al.*, 2010).

Moreover, some IRDs are syndromic, where the patients exhibit other clinical signs in addition to retinal degeneration. Examples of syndromic IRDs are Usher syndrome (USH) and Bardet-Biedl syndrome (BBS), the former including sensorineural deafness or hearing impairment (for review see: Mathur & Yang, 2015), and the latter a multitude of clinical signs including obesity, polydactyly, renal abnormalities and reproductive problems (Forsythe & Beales, 2013; Beales *et al.*, 1999). Most of the identified IRDs are monogenic and show an autosomal recessive mode of inheritance, but there are also autosomal dominant and X-linked IRDs. In addition, there are mitochondrially encoded IRD genes, resulting in conditions such as Leber hereditary optic neuropathy (LHON) (Wallace *et al.*, 1988). There are also complex IRDs, such as age-related macular degeneration (AMD) and diabetic retinopathy, where the disease phenotype results from a combination of genetic and environmental factors (Berger *et al.*, 2010). While the ultimate reason for retinal degeneration in most, if not all, IRDs is apoptosis of the photoreceptor cells (Travis, 1998), the steps leading to cell death vary between the different types of IRDs depending on the underlying genetic defect.

2.2.2 Genes involved in retinal degeneration

To date, genetic variants in at least 271 genes have been associated with human IRDs, listed in the Retinal Information Network database (RetNet) (**Figure 3**). The encoded proteins involve almost all aspects of cellular structure and function, and many have expression patterns, which are not restricted to retina (Wright *et al.*, 2010). The largest group, almost one quarter of known IRD genes, are associated with ciliary structure or function. Genes encoding key proteins for phototransduction and visual cycle together make the second largest group, followed by genes encoding for proteins associated with lipid metabolism, likely reflecting the composition of vast amount of lipids in the OS membranes, and genes encoding for ion channels (Wright *et al.*, 2010).

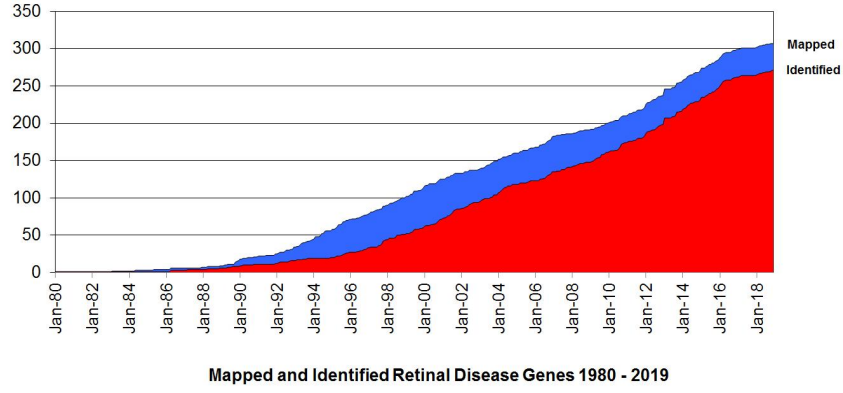

Figure 3. Accumulation of identified IRD genes in humans (RetNet, Retinal Information Network, by permission, January 2020, The Univ. of Texas Health Science Center Houston and Dr. Stephen P. Daiger)

2.2.3 Genes associated with canine IRDs

Currently, genetic variants in at least 32 genes have been identified to result in IRDs in dogs (**Table 1**). The canine equivalent of RP, termed progressive retinal atrophy (PRA), was first described 1911 in Gordon setters (Magnusson, 1911), and the responsible gene, photoreceptor cilium actin regulator (*PCARE*) (Downs *et al.*, 2013), now shown to be important for the expansion of the photoreceptor ciliary membrane when the OS is renewed (Corral-Serrano *et al.*, 2020). A genetic variant c.5G>A in the gene photoreceptor disc component (*PRCD*) is the most common cause for PRA in dogs, and interestingly, exactly the same mutation was found to cause RP in humans (Zangerl *et al.*, 2006).

Eight of the canine IRD genes have not been identified in similar diseases in humans to date). Two of them, coiled-coil domain containing 66 (*CCDC66*) (Conkar *et al.*, 2017) and microtubule associated protein 9 (*MAP9*) (Forman *et al.*, 2016) localize to the connecting cilium and the IS. *CCDC66* is associated with PRA in the Dutch sheepdog (schapendoes) (Dekomien *et al.*, 2010), and *MAP9* is believed to be a modifier of *RPGRIP1*-associated CRD. NECAP endocytosis associated 1 (*NECAP1*) gene is associated with PRA in giant schnauzers. The function of the protein encoded by the gene is not known, but the protein product localizes to clatherin-coated vesicles (Murshid *et al.*, 2006; Ritter *et al.*, 2003), and could therefore be involved in the intracellular trafficking of proteins into and out of the OS (Kwok *et al.*, 2008).

Table 1. *Identified canine IRD genes and associated canine and human IRDs.*

Gene	Canine IRD	Human IRD	Reference*
ABCA4	STGD	STGD, RP, CRD	(Mäkeläinen *et al.*, 2019)
ADAM9	CRD	COD, CRD	(Goldstein *et al.*, 2010b)
BBS4	PRA, BBS	BBS	(Chew *et al.*, 2017)
BEST1	CMR	MD, RP	(Petrukhin *et al.*, 1998)
CCDC66	PRA		(Dekomien *et al.*, 2010)
CNGA1	PRA	RP	(Wiik *et al.*, 2015)
CNGA3	ACHM	COD, CRD	(Tanaka *et al.*, 2015)
CNGB1	PRA	RP	(Winkler *et al.*, 2013)
CNGB3	ACHM	COD, CRD	(Sidjanin *et al.*, 2002)
FAM161A	PRA	RP	(Downs & Mellersh, 2014)
HIVEP3	PRA		(Kaukonen *et al.*, 2020)
IQCB1	CRD	LCA, Syndromic RD	(Goldstein *et al.*, 2013b)
LRIT3	CSNB	CSNB	(Das *et al.*, 2019)
MAP9	CRD*		(Forman *et al.*, 2016)
MERTK	PRA	RP	(Ahonen *et al.*, 2014)
NECAP1	PRA		(Hitti *et al.*, 2019)
NPHP4	CRD	Syndromic RD	(Wiik *et al.*, 2008)
PCARE	RCD	RP	(Downs *et al.*, 2013)
PDC	PD		(Zhang *et al.*, 1998)
PDE6A	RCD	RP	(Tuntivanich *et al.*, 2009)
PDE6B	CRD	CSNB, RP	(Suber *et al.*, 1993)
PPT1	PRA		(Murgiano *et al.*, 2019)
PRCD	PRA	RP	(Zangerl *et al.*, 2006)
RD3	RCD	LCA	(Kukekova *et al.*, 2009)
RHO	PRA	CSNB, RP, RP	(Kijas *et al.*, 2002)
RPE65	LCA	LCA, RP	(Veske *et al.*, 1999)
RPGR	XLPRA	COD, CRD, MD, XLPR	(Zhang *et al.*, 2002)
RPGRIP1	CRD	COD, CRD, LCA, Syndromic RD	(Mellersh *et al.*, 2006)
SAG	PRA	CSNB, RP	(Goldstein *et al.*, 2013a)
SLC4A3	PRA		(Downs *et al.*, 2011)
STK38L	ERD		(Goldstein *et al.*, 2010a)
TTC8	PRA, BBS	BBS, RP	(Downs *et al.*, 2014)

*Reference for the first identification of canine IRD associated with each gene.

Serine/threonine kinase 38 like (*STK38L*) gene is likely a regulator of amacrine cells causing LCA-like early retinal degeneration (ERD) in Norwegian elkhound (Léger *et al.*, 2018; Goldstein *et al.*, 2010a). An intronic splicing variant in an enhancer gene HIVEP zinc finger 3 (*HIVEP3*), is associated with one of the

forms of miniature schnauzer PRA (Kaukonen *et al.*, 2020). In addition to *HIVEP3*, the genes *PPT1* and *PDC* are reported to be associated with PRA in miniature schnauzer, but in both cases the association has been questioned (Kaukonen *et al.*, 2020; Murgiano *et al.*, 2019; Zhang *et al.*, 1998). Finally, solute carrier family 4 member 3 (*SLC4A4*) is an anion exchanger, possibly located in the plasma membrane of Müller and horizontal cells (Alvarez *et al.*, 2007), and, together with tetratricopeptide repeat domain 8 (*TTC8*) and *PRCD*, one of the three known genes associated with IRDs affecting golden retrievers.

2.3 Mapping of the causative genetic variants

The first efforts to find underlying genetic causes for IRDs were based on linkage analysis, followed by fine mapping in large pedigrees and finally Sanger sequencing of the linked locus (Claussnitzer *et al.*, 2020). The Human Genome Project, launched in 1990, was a 13-year-long "Apollo-project" of biology and medicine in an international effort to sequence the complete human genome. The costs of the project were 3 billion dollars, translating roughly to a dollar per base-pair. The Human Genome Project resulted in countless spinoffs in the field of computational biology (bioinformatics) and developments in sequencing technologies. By the time of the conclusion in 2003, the number of genes implicated in human disease had increased by fourfold (Claussnitzer *et al.*, 2020). Based on the initial sequencing, a human reference genome sequence was made publicly available to serve as an index for genetic features. This was followed by a flow of other mammalian reference genomes. The reference genome for the domestic dog was published 2005, completed with the same method as the original human genome project, Sanger sequencing of overlapping clones of bacterial artificial chromosomes (BAC), which included pieces of the genome from a female boxer called Tasha (Lindblad-Toh *et al.*, 2005).

2.3.1 Genome-wide association studies

In parallel to sequencing the canine genome, Lindblad-Toh and colleagues used dogs from 11 different breeds to identify single nucleotide polymorphisms (SNPs), and established the first high-density SNP map across the reference genome (Lindblad-Toh *et al.*, 2005). This paved the way for the use of genome-wide association studies (GWAS) to identify genetic loci harboring variants associated with disease phenotypes. In dogs in particular, this method has been successful because of the limited locus heterogeneity, a topic which will be revisited in paragraph 2.4, and it has been shown that only 10-20 affected and

unaffected individual dogs are needed for mapping autosomal recessive traits (Karlsson *et al.*, 2007).

2.3.2 High-throughput sequencing

Shortly after the completion of the Human Genome Project, a massively parallel sequencing technique, often referred to as next generation sequencing (NGS), revolutionized the field of DNA sequencing. In NGS, the target DNA is first fragmented into millions of short fragments which are then sequenced in parallel using real-time monitoring of the complementary strand biosynthesis. The first commercial NGS method on the market was the 454-pyrosequencing approach, by the time offering a 100-fold increase in output over the Sanger sequencing technology (Margulies *et al.*, 2005). Today, the Illumina sequencing platforms dominate high-throughput sequencing due to the low error rate (Pfeiffer *et al.*, 2018). These platforms are able to sequence a mammalian genome in a day, and produce millions of short fragments, which are then pieced together with bioinformatic analyses by mapping the sequence reads against the reference genome sequence. Since the introduction of NGS in 2005, the number of genes implicated in human IRDs has almost tripled (**Figure 3**). The advent of high-throughput sequencing has drastically reduced the costs related to genome sequencing. We are now approaching a "hundred-dollar genome", which has created two other types of challenges. These platforms generate unprecedented amounts of genomic data, which needs to be analyzed by bioinformatic tools in an effective and reproducible way. Secondly, the reproducibility means that these vast amounts of sequence data need storage solutions where the data can safely be deposited and efficiently retrieved and used for future analyses.

2.3.3 Whole-genome sequencing

As the costs of NGS have decreased, whole-genome sequencing (WGS) has become an attractive alternative to identify causative variants for Mendelian diseases with or without a prior GWAS. For example, WGS can be used to sequence one affected individual after a locus of interest has been identified by GWAS, but this approach requires a large variation database to exclude common variants in the population. An alternative approach, used in this thesis, is to sequence an affected individual and both parents (family trio sequencing) followed by careful bioinformatic analysis to filter for candidate variants. As a proof-of-principle study in dogs, this approach was used to identify a genetic variant associated with inherited footpad hyperkeratosis in Kromfohrländer dogs (Sayyab *et al.*, 2016). Instead of a trio, alternative family combinations can be

used, if available. In paper I of this thesis, a family quartet (including two affected siblings) was sequenced, and in other projects we have used several different family combinations depending on the available material.

The family (trio) sequencing approach relies on the principle, that by sequencing close relatives, not affected by the disease, we can filter away variation which the affected and unaffected individuals share by-descent, but which is not responsible for the clinical phenotype. To do this, we use conditional filtering depending on the expected mode of inheritance. For example, when looking for an autosomal recessive variant, only the homozygous variants of the affected individual for which the parents are heterozygous, are left for subsequent downstream analyses. To do this, we established a bioinformatic pipeline for dogs, including quality control and trimming of the raw sequence reads, mapping them against the reference genome, followed by variant calling. In dogs, this typically results in ~2 million small insertions or deletions (INDELS) and ~6 million single nucleotide variants (SNVs). Next, the variants are annotated to classify them into exonic, intronic or intergenic variants, as well as variants predicted to affect splice-sites and untranslated regions (UTRs). After this, exonic variants (normally comprising between 4,000-6,000 INDELS and 40,000-60,000 SNVs), and thereafter other types of variants, are analyzed by appropriate conditional filtering to extract candidate variants. Depending on the sequencing depth and the number of sequenced individuals, the candidate variants typically comprise around 20-100 INDELs and 300-1,000 SNVs.

Each variant is then compared to known variation in the canine reference genome (Ensembl), with the assumption that common variants in the population, are unlikely candidates for rare diseases. However, this step is not used to discard, but to prioritize variants, as the variation in the refence genome does not include phenotypic information. In addition, the candidate variants are prioritized based on their predicted effect on the protein product, and known association with similar phenotypes in other species, using for example the RetNet database (RetNet) and Ensembl BioMart tool (Kinsella *et al.*, 2011).

2.3.4 Long-read sequencing technologies

The input genomic DNA in NGS is first sheared into fragments of 350-550 bp, which are then sequenced from both ends (paired-end) with a typical read-length of 150 bp. This approach results in high-quality sequence, and is sufficient for identification of INDELs and SNVs. However, the identification of complex structural variation can be challenging using short reads, where long-read sequencing platforms, also referred to as the third-generation sequencing

technologies, can improve the detection of the variants. For retinal research, these technologies may provide useful as 9% of the underlying variation is predicted to be caused by structural variation, such as copy-number variation in the genome (Zampaglione *et al.*, 2020). Long-read sequencing using platforms from Pacific Biosciences (PacBio) (Eid *et al.*, 2009) and Oxford Nanopore Technologies (ONT) (Clarke *et al.*, 2009) can sequence unfragmented input DNA. PacBio has a lower error rate and produces reads spanning over kilobases (Ardui *et al.*, 2018). ONT platform has a higher error rate, but has been shown capable of sequencing read-lengths over a megabase (Payne *et al.*, 2019).
ONT sequencing, used in paper II and III in this thesis, is based on protein nanopores embedded on a synthetic membrane, thorough which an ionic current is passed, causing DNA fragment to move through the pore towards positive charge. Nucleotides passing the pore disrupt the flow of ions through the channel, and this change in current is then interpreted (basecalled) into nucleotide sequence (Deamer *et al.*, 2016; Jain *et al.*, 2016; Loman & Watson, 2015).

2.3.5 Transcriptome sequencing

The genome sequence works as a blueprint for the complex architecture of different tissues in the body. Each tissue, and the cell types within, possess a distinct gene expression pattern depending on which genes and transcripts are active at any given time. Thus, the transcriptome, the collection of mRNAs and all other RNAs expressed in the tissue, presents a dynamic reflection of the functions of the tissue, changing during the development, and as response to changes in the environment. As an example, the transcriptome profile of the retina changes constantly during the development, but also in adulthood as a response to circadian rhythms, lighting conditions, as well as the health status of the eye (McMahon *et al.*, 2014). These changes are achieved with interaction of transcription factors, enhancers and silencers acting within complex feedback-loops and regulating gene expression. Only 1% of the genome is translated into protein (Birney *et al.*, 2007), and the function of the non-coding part of the genome is incompletely understood, but biological function has been assigned to at least 80% of the human genome (ENCODE, 2012).

High-throughput sequencing methods for transcriptome sequencing are generally referred to as RNA sequencing (reviewed by Wang *et al.*, 2009). When sequencing the protein-coding genes of the genome, the mRNA is selected from the total RNA of a sample using selection based on polyadenylation, addition of a poly(A) tail to a mature mRNA transcript. In NGS-based RNA sequencing, the poly(A)-selected RNA is first reverse transcribed to cDNA, which is then used

for the preparation of sequencing libraries. ONT based transcriptome sequencing can be made using three different alternatives using either cDNA with or without prior PCR amplification, or native RNA as a starting material (Workman *et al.*, 2019; Garalde *et al.*, 2018).

2.4 Dog as a model for human inherited retinal degeneration

Comparative animal models are of high importance when studying the function of genes, and investigating the cellular and molecular mechanisms of disease. These animal models are particularly useful for the development of therapeutic strategies allowing testing the specificity, efficacy and potential side effects of therapeutic methods. Rodents and mice in particular, have been used extensively and successively to study the effect of different IRD mutations (Veleri *et al.*, 2015). Their small size, short generation interval and large litter size are advantages, that make them attractive for studies which require controlled laboratory environment and large number of animals. In comparison, the management of a colony of large animal models such as dogs, cats, pigs or non-human primates is relatively expensive, and requires large facilities, and is slower due to the longer generation interval. However, large animal models like the domestic dog have several significant advantages which complement the use of small animal models.

2.4.1 Naturally occurring large animal model for IRD

Dogs are affected by spontaneous, naturally occurring IRDs without the need for introducing the mutation artificially. Many aspects of these diseases can be studied without a colony, by recruiting pet dogs to the study. The dog patients live at home within a family, and are therefore exposed to similar environmental factor as human patients. Dogs have a longer lifespan than rodents, which allows for follow up studies monitoring the disease progression. The size of the canine eye globe becomes particularly important in the development of translational therapy methods, when surgical delivery approaches can be directly translated from dog to human (Winkler *et al.*, 2020). Moreover, modelling of IRDs where the initial effect of the disease is on high-acuity daylight vision is challenging in rodents, since they are nocturnal animals, and lack a cone dense region similar to fovea in humans and *area centralis* in dogs.

2.4.2 The landscape of the domestic dog genome

Pedigree dogs are particularly prone to IRDs, and not by coincidence. The dog is one of the oldest, if not the oldest domesticated animal species, and has been our companion for thousands of years. Although the exact time-point of the domestication is debated, fossil evidence of ancient dog-like remains from Altai Mountains of southern Siberia, have been dated back 33,000 years ago (Druzhkova *et al.*, 2013; Ovodov *et al.*, 2011). It has been suggested, that the modern dog might originate from several independent domestication events in various places. Originating from wolf, these domesticated lineages form the basis of the modern-day domestic dog (*Canis lupus familiaris*) (Vilà *et al.*, 1997). The early domestication events resulted in loss of genetic variation between individuals compared to the ancestral wolf population, referred to as a genetic bottleneck. To date, 353 dog breeds are recognized by the World Canine Organization (FCI), and together with the unofficial breeds, the number of breeds is close to 400. Most of the modern breeds have been created during the last 200 years by choosing a small number of dogs, and each breed now represents a genetically isolated population (Parker *et al.*, 2004). This breed formation represents a second bottleneck (Marsden *et al.*, 2016), and the genetic pool of the dogs has been further decreased by the intensive use of individual breeding sires, some of which may have hundreds of offspring, as well as by strong artificial selection and mating between close relatives to achieve homogeneous phenotype. Today, the domestic dog is a species characterized by extensive amount of phenotypic variation between the breeds, but very little variation within the breeds, which has led to a rich source of monogenic diseases facilitating genetic studies (Parker *et al.*, 2017).

The domestication and breed forming processes have left a unique mark in the canine genome where individuals of different breeds share short ancestral haplotype blocks and individuals of the same breed share long haplotypes where linkage disequilibrium (LD) stretching over several megabases of sequence, and the canine genome includes long regions of homozygosity (Lindblad-Toh *et al.*, 2005). Therefore, GWAS, have been successful in identifying genomic regions associated with disease phenotype. As a consequence of the extensive degree of LD and the long haplotypes in the megabase range and sometimes several megabases long within a breed of these regions makes it difficult to find the causative variants. The genetic landscape of the dog, the shared environment with humans, the size as well as the morphology of the canine eye, and the spontaneously occurring retinal diseases with equivalent genetic background to that in humans have contributed to the value of the dog as a large animal model. By studying the genetics of the canine retina, we can increase our understanding of the biological mechanisms underlying visual impairment. Ultimately, this

information has the potential to increase the health and welfare of both humans and dogs.

3 Aims of the thesis

The overall aim of this thesis was to increase our understanding of the genetic basis underlying inherited retinal degenerations in dogs.

The specific aims were to:

- Establish a whole-genome sequencing pipeline for mapping monogenic diseases in dogs.
- To identify the genetic cause of a novel inherited retinal degeneration in Labrador retrievers.
- Investigate if a single bp deletion in the *TTC8* gene, previously implicated in non-syndromic PRA in golden retrievers, results in a syndromic ciliopathy similar to Bardet-Biedl syndrome in humans.
- Define the expression pattern of *TTC8* transcripts in the canine retina.
- Characterize the canine retinal transcriptome in terms of expressed genes and their expression levels.
- Provide a retinal transcriptome resource to be used for validation of candidate variants for retinal disease.

4 Summary of studies (I-III)

4.1 Study I – An *ABCA4* loss-of-function mutation causes a canine form of Stargardt disease

In this study, we used whole-genome sequencing (WGS) to identify the genetic cause for a previously undescribed, slowly progressing retinopathy in Labrador retrievers. The disease was assumed to be very rare, as only two cases, a sib-pair, had been diagnosed. A novel retinopathy was suspected, because the parents of the sib-pair had been tested negative for the genetic variant in the gene photoreceptor disc component (*PRCD*), known to cause progressive retinal atrophy in the breed. The male sibling, diagnosed at the age of 5 years, was reported to have impaired day-light vision, suggesting a cone-rod degeneration, but was still able to function as a field-trial dog. His female sibling was diagnosed four years later, and similarly reported to have visual problems in day-light. It should, however, be noted that the owner of the female dog had noticed that the dog did not perform as expected during field marking drills when she was younger. At that time, both dogs were subject to routine eye screening, but no more rigorous clinical examination was conducted.

To identify the causative gene variant, we sequenced the family quartet, including the sib-pair as well as the unaffected parents, and used conditional filtering for autosomal recessive variants. This led to the identification of a one bp insertion in the ATP binding cassette subfamily A member 4 (*ABCA4*) gene. In humans *ABCA4* mutations have been shown to cause Stargardt disease (Nasonkin *et al.*, 1998; Allikmets *et al.*, 1997), a macular degeneration affecting central vision of the patients with an onset at late childhood or early adulthood.

To investigate if the canine phenotype resembled Stargardt disease, the affected siblings and a third litter-mate, an unaffected male who was genotyped heterozygous for the insertion, as well as an unaffected, age-matched Labrador

retriever were examined with electroretinography (ERG) to measure the electrical responses of the retinal neurons to light stimulus. The ERG results strengthened the view that the cone photoreceptor function was severely compromised, which explained the visual impairment under daylight conditions. The process of rod dark-adaptation was also substantially slower than normal, similar to what is seen in human Stargardt patients. While the heterozygous litter-mate had no visual impairment, his ERG results were borderline normal. In addition to testing retinal function with ERG, we were able to examine the retinal thickness and the fundus of the dogs using optic coherence tomography (OCT; **Figure 4**) and confocal scanning laser ophthalmoscopy (cSLO). The OCT examination indicated that the ONL of the affected dogs were severely thinner compared to the heterozygous litter-mate and the unaffected dog. The affected retina was also more autofluorescent than in age-matched controls, and we suspected that this could be due to abnormal accumulation of autofluorescent lipofuscin, a by-product of normal retinal metabolism, into the RPE.

Figure 4. An unaffected Labrador retriever in optical coherence tomography (OCT) examination.

The accumulation of lipofuscin is a hallmark of Stargardt disease (Delori *et al.*, 1995), caused by defects in the function of ABCA4 protein. After the photoisomerization of 11-*cis* retinal to all-*trans* retinal, the chromophore spontaneously reacts with phosphatidylethanolamine (PE), forming a reversible adduct termed *N*-retinylidene-phosphatidylethanolamine (*N*-Ret-PE, also called *N*-*trans*-R-PE). ABCA4 is a transmembrane protein located in the rim regions

(the hairpin loop) in the disk membranes of the rod and cone photoreceptor outer segments (Molday *et al.*, 2000; Illing *et al.*, 1997; Papermaster *et al.*, 1978), and it functions as a flippase, importing *N*-*trans*-R-PE from the disk lumen into the cytoplasm of the photoreceptor cell, where the adduct can dissociate, and all-*trans* retinal can be reduced to all-*trans* retinol, and subsequently diffuse to RPE (Quazi *et al.*, 2012), as illustrated in **Figure 2**. In addition, ABCA4 has been shown facilitate the clearance of excess 11-*cis* retinal, which also spontaneously forms an adduct with PE (*N*-11-*cis*-retinylidene-phosphatidylethanolamine; *N*-*cis*-R-PE) (Quazi & Molday, 2014). A defective ABCA4-mediated transport traps *N*-ret-PE inside the disk lumen, where it together with 11-*trans* retinal forms di-retinoid-pyridinium-phosphatidylethanolamine (A2PE). A2PE is then further hydrolyzed to phosphatidic acid (PA) and a toxic bisretinoid, di-retinal-pyridinium-ethanolamine (A2E), a major component of lipofuscin (Ben-Shabat *et al.*, 2002; Mata *et al.*, 2000). When the disks are shed at the tips of the OS, lipofuscin accumulates in the RPE cell layer. This results in toxification of the RPE cells, which no longer are able to sustain the adjacent photoreceptor cells that degenerate as a result.

The identified one bp insertion in the canine *ABCA4* gene (c.4176insC), located in exon 28, was predicted to shift the reading frame and result in a translation stop codon two amino acids downstream of the insertion. If the resulting mRNA was translated, it would lead to a truncated protein product lacking the second half of the protein, which includes most of the second extracellular domain and the second nucleotide-binding domain of the protein. We hypothesized that the mRNA with the premature stop codon would be targeted by nonsense-mediated decay (NMD) (Lykke-Andersen & Jensen, 2015). To show this, we designed primers amplifying parts of the canine *ABCA4* gene, and in the absence of retinal tissue, we extracted RNA from whole-blood of the two affected siblings, heterozygous litter-mate and unaffected dogs. However, we learned that the *ABCA4* gene is expressed in a highly cell type-specific manner, and the expression levels in blood of the unaffected dogs were not enough to amplify any transcripts. There were indications that *ABCA4* may be expressed in the hair follicles (Haslam *et al.*, 2015), and we therefore extracted RNA from the hair roots of whiskers from unaffected and affected dogs. Despite the recent report that *ABCA4* is highly expressed in human eyebrow hair follicles (Ścieżyńska *et al.*, 2020), our attempts to amplify normal canine *ABCA4* mRNA from the hair roots were not successful. Shortly after these attempts, the now 12-year-old heterozygous litter-mate and some months later an unaffected 10-year-old Labrador, and the affected male dogs were euthanized due to reasons not related to the study. We were able to extract RNA from the retinas of all three dogs, and could show that while *ABCA4* mRNA was

expressed in the retina of the unaffected dog, the heterozygous sibling had lower *ABCA4* expression, and the expression levels of the affected male were approximately one third of the wild-type expression. This let us conclude that a large portion of the transcripts with premature stop codon were likely degraded by NMD.

We also extracted protein from the retina, and, using western blotting, showed that a full-length ABCA4 protein product was completely missing from the protein extract of the affected retina. Interestingly, the ABCA4 protein levels in the heterozygous retina were again lower than the wild-type expression level, but because we only had access to one heterozygous individual, we cannot say if this represents normal variation between the dogs or a result of the insertion in one allele. However, there are indications that some heterozygous *ABCA4* mutations result in a molecular phenotype, but do not affect vision noticeably in humans (Kjellström, 2015), and it would be interesting to study the heterozygous dogs further in the future.

Finally, because the western blotting of the large transmembrane protein ABCA4 proved to be non-trivial, we also collected an eye from the affected male for fluorescence histochemistry. Using the same anti-ABCA4 antibody as in the western blot, we were able to show that, in contrast to the retinal sections of a wild-type and the heterozygous litter-mate, immunoreactivity to ABCA4 was not detected in the affected retina. In addition, using peanut-agglutin (PNA) which selectively binds to cone photoreceptors, we could show that the affected retina was largely devoid of cone photoreceptors. In addition, the affected retina was autofluorescent, confirming the autofluorescence seen on the OCT examinations *in vivo*. Finally, histopathological examinations of the affected retina confirmed the OCT observation of the retinal thinning, showing that the ONL layer was severely affected.

Before the publication of the results, the public annotation of the canine genome reference sequence (CanFam3.1 Ensembl build 78) had been updated from the time of the identification of the insertion to Ensembl build 88. While testing the updated WGS analysis pipeline with the updated annotation, we identified a non-synonymous substitution in the gene usherin (*USH2A*), which had not been captured by the previous pipeline. The subsequent analysis showed that this variant was predicted to have a deleterious effect on the resulting protein product. In humans, mutations in *USH2A* cause Usher syndrome causing hearing problems and visual impairment (Eudy *et al.*, 1998), and are also associated with non-syndromic RP (McGee *et al.*, 2010). While the phenotype of the human patients with *USH2A* mutations was not consistent with the clinical signs of the affected dogs, we needed to validate that this variant was not contributing to the visual impairment of the affected dogs. During the course of the study, we had

identified eight additional affected dogs, free from the *PRCD* variant, and these dogs were genotyped for *USH2A*. However, the variant was not found homozygous in any of the eight unaffected dogs, but instead found homozygous in four unaffected dogs, and was therefore discarded from the further analysis. The *ABCA4* variant was in turn found homozygous in all of these additional affected dogs, and in none of the unaffected dogs (13 in total). This allowed us to conclude that the one bp insertion in the canine *ABCA4* gene is causative for the clinical signs of the affected dogs, and results in a canine form of Stargardt disease.

4.2 Study II – Deletion in the Bardet-Biedl syndrome gene *TTC8* results in a syndromic retinal degeneration in dogs

The tetratricopeptide repeat domain 8 (*TTC8)* gene encodes for one of the eight proteins forming an octameric protein complex termed the BBSome (Nachury *et al.*, 2007), and has in humans been implicated in Bardet-Biedl syndrome (BBS). BBSome functions in the trafficking of proteins in the primary cilia, a signaling organelle which is found in almost all cell types (Seeley & Nachury, 2010), where they are involved in sensory reception and cell proliferation, as well as developmental signaling pathways (Marshall & Nonaka, 2006). The dysfunction of primary cilia results in syndromic ciliopathies such as the BBS, with heterogenic clinical signs that vary between patients. In addition to *TTC8* and other BBSome genes, mutations in 16 additional genes have also been associated with BBS. In dogs, a genetic variant in one of these genes, Bardet-Biedl syndrome 4 (*BBS4*), has previously been identified in the Hungarian puli, and is associated with a phenotype including retinal degeneration, obesity and morphologically abnormal sperms (Chew *et al.*, 2017).

In 2014, Downs et al. identified a one bp deletion in the canine *TTC8* gene, associated with a form of progressive retinal atrophy (PRA) in golden retriever dogs (Downs *et al.*, 2014). At the time of the identification, however, a rigorous clinical characterization of the phenotype of these patients was not possible, although there were indications that affected dogs may also showed other clinical signs besides PRA. To obtain further information about the possible additional clinical signs, a questionnaire was designed (Downs *et al.*, 2014) and sent to owners of affected dogs, but only two owners responded.

Since 2014, genetic testing has identified eight additional genetically affected dogs from Sweden and Finland. We interviewed the owners of these dogs, as well as the responders from the earlier study, using the previously developed questionnaires. For two of the dogs, a sister and brother, we were able to follow

the progression of PRA during two years, as well as examine the cardiac status and collect semen from the male dog. Both dogs were finally euthanized on the owners' request due to problems caused by the rapidly progressing visual impairment. Necropsies were conducted and tissue samples were collected for macroscopic and microscopic analysis.

In humans, the symptoms of BBS are highly variable, even between individuals of the same family having the same causative variant. A BBS diagnosis is based on the presence of at least four primary characteristics or on three primary and two secondary characteristics. The primary characteristics include retinal degeneration, obesity, polydactyly, renal abnormalities, learning disabilities or cognitive impairment, hypogonadism in males, and genital abnormalities in females. The secondary features include speech delay, developmental delay, behavioral abnormalities, other ocular abnormalities, brachydactyly/syndactyly, ataxia/poor coordination/imbalance, short stature, mild hypertonia, diabetes mellitus, orodental abnormalities, cardiovascular anomalies, situs inversus, hepatic involvement, craniofacial dysmorphism, Hirschsprung disease, and anosmia (Forsythe & Beales, 2013; Beales *et al.*, 1999). Of the primary symptoms, retinal degeneration is seen in 93% of all BBS patients, and we therefore carefully investigated the retinal disease in the affected dogs.

All ten affected dogs were diagnosed with PRA, and the average age at diagnosis was 4 years and 8 months (range: 2 years 9 months to 6 years 6 months). At the first ophthalmic examination of the affected sib-pair, both dogs showed signs of visual impairment in dim light conditions, whereas their daylight vision was considered normal. The dim-light visual impairment was more pronounced in the male dog than of the female. OCT examination of the female dog showed considerable (outer) retinal thinning, and rod responses were already non-detectable on FERG. The male dog had bilateral cataracts which hindered OCT examination, and due to signs of more advanced retinal degeneration on ophthalmoscopy, he did not undergo the ERG examination. Two years after the initial exam, both dogs had a severe visual impairment under both daylight and dim light conditions.

The semen analysis of the affected male strongly indicated infertility. The female dog had not been in heat before the age of two years when she was neutered. It is therefore unclear if she had reproductive problems, although most female dogs have first heat around one year of age. One female dog was reported to only have been in heat twice at the age of six years by the owner, and three of the male dogs were said not to be interested in females.

In our study, the body condition score for the male dog indicated obesity, and the female was classified as heavy. Obesity occurs in 72–92% of human patients

(Weihbrecht *et al.*, 2017). BBS mouse models suggest that the obesity could be related to lack of melanin-concentrating hormone receptor 1 (Mchr1), which in healthy mice is located in the primary cilia of the neurons, and regulates food intake and energy homeostasis (Berbari *et al.*, 2008). Five out of the other eight dogs in the study were either considered as heavy or obese by their owners. While the dogs were said to gain weight easily and most of them were constantly hungry, they also benefitted from dietary restrictions, as seen in human patients (Wingfield *et al.*, 2018). It is therefore possible, as suggested by the mouse model, that neuronal signaling of satiety could play a role in the obesity of BBS patients and the dogs.

Renal failure is the most common cause of mortality among BBS patients (Imhoff *et al.*, 2011). While the dogs in this study were euthanized earlier than the breed average, only one of them was euthanized due to kidney failure. This dog was unfortunately not available for necropsy. The kidneys of the affected sib pair were examined after necropsy. Macroscopically, the kidneys of the affected male revealed suspected chronic infarcts bilaterally, although these could not be confirmed histologically in the left kidney. The female showed bilateral signs of mild, chronic glomerulonephritis.

Interestingly, while almost 95% of BBS patients have polydactyly or other digit anomalies (Mockel *et al.*, 2011), none of the affected dogs in this study had any digit malformations. This is in line with the mouse models of BBS, which do not show polydactyly (Tadenev *et al.*, 2011; Kulaga *et al.*, 2004). Polydactyly is believed to result from defective sonic hedgehog signaling during development (Bimonte *et al.*, 2011), and it would be interesting to investigate if this observation is coincidental, or a result of biological differences in the sonic hedgehog signaling pathway during development.

Cardiovascular anomalies are considered a secondary characteristic of BBS. To investigate the cardiac status of the affected siblings, both dogs were subjected to echocardiography. However, results were unremarkable. In the *post-mortem* gross examination of the affected male, a mild myxomatous valvular degeneration was observed in the heart. The finding is, however, common in dogs (Fox, 2012). It has, however, recently been shown that ciliary diseases may lead to myxomatous mitral valve disease in both humans and mice (Toomer *et al.*, 2019). It is unclear if the observation is related to the *TTC8* deletion, and this requires further studies.

Next, we investigated the effect of the mutation at the molecular level. We first sequenced retinal cDNA from the affected female and two unaffected female dogs using Oxford Nanopore long-read sequencing (ONT). Quantification of the expression level of genes, which are known to be expressed in specific retinal cell types, indicated that the expression of rod photoreceptor

specific genes (*PDE6A*, *PDE6B*, *CNGB1*, *GNAT1* and *CNGA1*; (Kaewkhaw *et al.*, 2015; Downes & Gautam, 1999) was considerably lower in the affected compared to the unaffected dogs. Quantitative RT-PCR results indicated that the expression of *TTC8* was only 10-20% of the expression observed in the unaffected dogs, and rhodopsin was expressed below the detection level. In contrast, expression of glial fibrillary acidic protein (*GFAP*) was approximately 50 times higher in the affected female. *GFAP* expression is known to be elevated in retinal degeneration and stress (Lewis & Fisher, 2003; Sarthy *et al.*, 1991). The lack of rhodopsin expression is not surprising, because the retinal degeneration was advanced.

TTC8 is known to have a retina-specific transcript, which is the most abundant transcript in the photoreceptor cells. An alternatively spliced transcript lacks the retina-specific exon 2a. Both these known transcripts, as well as a third transcript including a short 22 nt long exon 1b were identified in the unaffected canine retina using ONT sequencing. In contrast, the *TTC8* reads in the affected dogs appeared to be only partly spliced, and none of the reads reached full-length over all exons, indicating that the transcripts including the deletion, which subsequently leads to a premature stop codon, are targeted by NMD (Lykke-Andersen & Jensen, 2015).

Based on our results, we concluded that the deletion can result in a canine form of BBS. As in humans, BBS in dogs appear to be a heterogenous disorder with variable clinical signs. Primary cilia are referred to as the antenna of the cell, and given its broad functions in sensory signaling (Marshall & Nonaka, 2006), it is not surprising that cilia defects result in complex phenotypes and a multitude of clinical signs. However, the affected dogs showed a variable number of clinical and other phenotypical signs, varying between one (one dog) and four (four dogs) primary signs, and from one to six secondary signs of BBS. Given, that all ten dogs were homozygous for the same genetic variant in the *TTC8* gene, and were of the same breed, the variation in the clinical signs is remarkable, and indicates that the genetic background of individual dogs may affect the manifestation.

Taken together, our results suggest that the *TTC8* deletion results in a syndromic IRD similar to Bardet-Biedl syndrome. A canine model may help to elucidate the underlying molecular mechanisms of specific BBS characteristics and ultimately, be of importance for therapeutic management of BBS patients.

4.3 Study III – Characterization of the canine retinal transcriptome using long- and short-read cDNA sequencing

The retina consists of more than 60 different cell types (Masland, 2017). The retinal transcriptome refers to all the transcripts from genes which are active in the retina. To understand the molecular basis of IRDs, it is critical to have an accurate characterization of the genome as well as the transcriptome. However, such a resource is lacking for the domestic dog. Moreover, when using the bioinformatic pipeline developed in study I, and used also in other IRD projects, several inaccuracies in the current genome annotation for the dog became evident. The most striking example was the lack of the *RHO* gene, despite the fact that a mutation in the gene has been implicated in an autosomal dominant form of PRA already 20 years ago (Kijas *et al.*, 2002). In paper III, we therefore aimed to describe the genes and pathways, which are active in the normal adult canine retina by means of both long- and short-read cDNA sequencing (**Figure 5**).

Figure 5. ONT sequence reads capture the entire 49 exons of *ABCA4* gene in a single read. The one bp insertion identified in paper I is located in exon 28 (arrow).

For this purpose, we used a combination of Oxford Nanopore Technologies (ONT) long-read sequencing and Illumina short-read platform Novaseq 6000 to sequence the retinal mRNA of a healthy 12-year-old female beagle. Illumina and ONT quantification results correlated with a Spearman correlation coefficient of 0.82. In total, mRNA from 61% of the annotated protein-coding genes were shown to be expressed in both datasets over a detection threshold of 0.5

transcripts per million (TPM). Illumina sequencing detected on average of 2.2 transcripts per gene (same as annotation average), whereas only 1.9 protein-coding transcripts were identified using ONT. In contrast, ONT detected more other biotypes than protein-coding genes. As suggested by the correlation, the quantification based on the two technologies showed similar trends of mRNA expression, although the TPM values in ONT were in general somewhat lower compared with Illumina, resulting in a smaller portion of protein-coding genes being expressed above the threshold. If the annotation does not reflect the full range of transcripts for a gene, the longer sequence reads are less likely to find a matching alignment against the reference, resulting in lower TPM values. Non-coding genes have on average 1.5 alternative transcripts per gene, whereas the protein-coding genes have 2.2 (Ensembl build 101), which could help to explain why the non-coding biotypes were detected with higher TPM levels compared to Illumina. Future studies will, however, be needed to test this hypothesis.

Among the genes which were expressed in the top 1000 in both datasets, mitochondrial genes had the highest expression, which is not surprising given that the retina is one of the most energy demanding tissues in the body (Country, 2017; Wong-Riley, 2010), and is in line with studies of human retinal transcriptome (Pinelli *et al.*, 2016; Farkas *et al.*, 2013). DAVID functional annotation and GO-term enrichment analyses of the highly expressed genes identified an enrichment of genes involved in the ubiquitin–proteasome system (UPS), the main intracellular pathway for protein degradation (Lobanova *et al.*, 2018; Campello *et al.*, 2013). UPS was previously shown to be enriched in mouse rod photoreceptors (Blackshaw *et al.*, 2001), and its proper function has been shown important for retinal homeostasis (Toulis & Marfany, 2020). Unsurprisingly, GO-terms related to phototransduction were enriched among the most highly expressed genes.

The quantification results between the two technologies resulted in similar expression patterns of well-annotated protein-coding genes. More variation was observed in novel genes, such as the eighth (ENSCAFG00000044879) and ninth (ENSCAFG00000012371) highest expressed genes in ONT. We did not find support for these two genes based on the genomic alignments of the data, indicating that the reads likely aligned better to a region missing from the reference transcriptome. BLAST-analysis identified the ninth most expressed gene as 60S ribosomal protein L38, encoded by the gene *RPL38*, as the most similar human protein (Identity: 84.7%). In mice, a genetic variant in *Rpl38* has been shown to result in developmental defects including eye abnormalities by causing selective reduction in the translation of a subset of *Hox* mRNAs (Kondrashov *et al.*, 2011). In their study, Kondrashov and colleagues showed that *Rpl38* was highly expressed in the neuroretina of a mouse embryo. It is

possible, that *RPL38* could be highly expressed in the adult canine retina, but further studies are needed to investigate the aberrant expression levels between ONT and Illumina datasets. A second gene with deviating expression levels, ENSCAFG00000044879, was the 8th most expressed ONT gene (12823.2 TPM). The expression level of this gene in the Illumina data was 211.9 TPM (538th most expressed gene in Illumina). The UniProt annotation of the gene is ATP synthase membrane subunit 6.8PL (*ATP5MPL*), and in the Human Protein Atlas this gene is expressed in the retina according to mouse RNAseq. More studies will be needed to investigate the reasons for this discrepancy.

Finally, the genes known to harbor variants associated with canine and human IRDs, and genes associated with human or mouse retina-related phenotype were listed (n=382). Most of the genes were expressed on medium (TPM 10-1,000) expression level in the canine retina. The top 23 genes, expressed with more than 1000 TPM, included four canine IRD genes (*RHO, PDC, SAG and PDE6A*), as well as a *RBP4*, which has been recently been implicated in congenital eye malformation in Irish soft-coated wheaten terriers (Kaukonen *et al.*, 2018).

Taken together, our results provide an in-depth characterization of the canine retinal transcriptome and represents a first step towards its detailed annotation. Although RNA sequencing of retinas obtained from additional individuals will be needed for a more comprehensive annotation, this study indicates that a combination of ONT long-read sequencing and Illumina short-read sequencing can be used to quantify mRNA expression levels of well annotated protein-coding genes. The characterization of genes and their expression levels can be used in future to prioritize candidate variants for IRDs and will be of importance for both canine and human IRD research.

5 General discussion and future perspectives

The investigations presented in this thesis have to a large extent been possible due to the rapid technological advances in high-throughput DNA sequencing technologies. In 2015, the whole-genome sequencing of a family quartet, presented in paper I, was a pilot project run on an Illumina NextSeq500 sequencing platform, and resulted in an average coverage of 18X per individual. Today, it would be possible to sequence 20 dogs with similar output and the same costs. Moreover, the long-read sequencing platforms have improved in terms of accuracy and throughput and have for some applications become an attractive alternative to short-read sequencing. These platforms can, for example, be used to map structural variation, increase contiguity of the reference genome sequence, or to sequence over high GC rich, repetitive or paralogous regions in the genome (Pollard *et al.*, 2018). For transcriptome sequencing, single reads spanning the full length of a transcript present a possibility to access the complexity of expressed transcripts without the bias of assembling short NGS reads (Byrne *et al.*, 2017).

The power of long-read transcriptome sequencing was illustrated in papers II and III in this thesis. We first sequenced the retinal transcriptome of an affected dog homozygous for the *TTC8* mutation, as well as two dogs homozygous for the wild-type allele. Encouraged by the achieved sequencing depth, number of full-length reads spanning over entire gene loci, and the high correlation in quantification results between the dogs, aside from the effects of NMD on *TTC8* transcripts and photoreceptor loss in the affected retina, we then moved on to combine ONT and Illumina transcriptome sequencing in paper III. While the two approaches generally agreed in terms of expression levels, quite some challenges still remain to digest and differentiate the biological meaning from the technological differences between the ONT and Illumina datasets. Currently, the bioinformatic tools for processing ONT reads are under rapid development,

and the bug fixes, version updates and lack of best practices hampers the reproducibility of the analyses (Amarasinghe *et al.*, 2020). The direct comparison of the ONT and the Illumina transcriptome sequencing was, however, not the main purpose for using the combination of the two technologies. Rather, the purpose was to complement the long-read sequencing, because Illumina RNA sequencing has become the gold standard of transcriptome quantification.

From a biological perspective, future studies should focus on updating the annotation to correctly include all the genes important for retinal function and structure. To complement the pan-retinal transcriptome with single-cell resolution would increase our understanding of the transcriptional differences between canine retinal cell types. An attractive future direction would also be to use spatial transcriptomics (Vickovic *et al.*, 2019; Ståhl *et al.*, 2016) to elucidate the gene expression profiles of different topographical regions in the retina. The single-cell as well as spatial information would help us to understand how the gene expression changes between the distinct topographical regions of the retina, and possibly illuminate some of the biological processes leading to different types of IRDs with discrete regional changes.

One such example of regionally restricted effect of photoreceptor degeneration is Stargardt disease which in humans typically starts as a macular dystrophy affecting central cone vision, and only later may progress to a phenotype also involving rod photoreceptors and peripheral vision, although the phenotype differs between individuals and different mutations in the gene (Fujinami *et al.*, 2013). Dogs homozygous for the *ABCA4* insertion, may show abnormal appearance of the fundus already before the age of one year. The changes start in the cone-rich foveal area in the center of area centralis and then spread to more peripheral parts of the retina (Ekesten *et al.*, 2020).

The two spontaneous canine IRD models presented in this thesis may eventually be used for developing protocols for gene therapy. Currently, there are no large animal models for STGD and BBS. For STGD, two *ABCA4* gene therapy mouse models have recently been published (McClements *et al.*, 2020; Dyka *et al.*, 2019). Due to the large size of *ABCA4* gene, both of these strategies are based on a dual Adeno-associated virus (AAV) vector, where the cDNA of the gene is packaged into two separated vectors. For *TTC8*, no model for gene therapy is available. Ciliopathies such as Bardet-Biedl syndrome are challenging to treat due to the early onset of diseases owing for the involvement of primary cilia in development. (Datta *et al.*, 2020). In addition to gene therapy, pharmacological treatments can be beneficial to BBS patients and help to manage, although not cure, the disease (Forsythe *et al.*, 2018). Regardless of treatment strategy, dog models can be used to complement the research made

with mouse models. The size of the canine eye and other organs makes it possible translate the methodology, such as gene-delivery techniques, to human patients, and the longevity of the dog compared to mouse make longitudinal studies over several years possible.

The definition for a rare disease in the European Union is 1 affected in 2,000 people (Institute of Medicine Committee on Accelerating Rare Diseases Research, 2010). Using this definition, IRDs can collectively be classified as a rare disease with 1:2,000 affected people (Berger *et al.*, 2010). The prevalence of individual IRD is even lower. Stargardt disease, for example, affects 1 in 8,000 to 10,000 people (Blacharski, 1988). Bardet-Bield syndrome is very rare in Europe and the United States (1:100,000), although the prevalence shows regional differences (Forsythe *et al.*, 2018). In dogs, the exact number of individuals affected by IRDs in general is not known, but more than 100 different dog breeds are affected (Downs *et al.*, 2013). The preliminary estimate for the frequency of the defective allele of *ABCA4* in the Swedish Labrador retriever population is approximately 0.6. With random mating this would result in one affected dog born per 150 to 300 individuals. In golden retrievers, almost 20% of the more than 2,000 genotyped dogs carry at least one *TTC8* or *SLC4A3* allele associated with IRD. In addition, *PRCD* PRA also affects both Labrador retrievers and golden retrievers, although the prevalence is considered very low in golden retrievers. Therefore, even though any one IRD is rare in the dog population in general, they are collectively common and can have a high prevalence within a breed. The development of diagnostic DNA tests to identify carriers of disease mutation helps the breeders to make informed breeding decisions to avoid affected offspring, but at the same time to keep as many dogs in the breeding population as possible, and maintain the genetic diversity in the breed. However, it is of utmost importance, that these tests are validated and accurate, and do not lead to false negatives and confusion among breeders.

The basis of any undertaking to find causative genetic variants for IRDs is a rigorous characterization of the phenotype. The manifestation of the clinical signs, age of onset, rate of progression and family history are critical parameters that need to be considered when designing the most effective strategy for genetic studies. Dog as a patient present some challenges for determining the age of onset. Study I in this thesis presents an excellent example for this. The disease was first considered to be very rare and only two field-trial Labrador siblings had been diagnosed. Despite the visual impairment later in life, these dogs were successfully used for hunting and in field-trials during their early years. The subsequent genotyping of additional dogs showed, that the disease is not restricted to field-trial Labradors retrievers, but also affects dual-purpose and show lineages of the breed. The visual impairment is easier to notice, when the

dog is expected to perform tasks which require good vision. In contrast, a slowly progressing visual impairment might go unnoticed for the owner if the dog is not exposed to unfamiliar environments and this, combined with the perception that older dogs often loose visual acuity, may lead to that the disease progression goes unnoticed by the owners.

Despite the successful identification of the *ABCA4* variant in study I, the whole-genome sequencing framework has not been successful in identification the causative variant for additional canine IRD projects. In humans, genetics behind 60 to 85% can be detected using NGS (Daiger *et al.*, 2019). At present, the percentage is likely lower for dogs, given the resolution of the canine genome reference sequence (CanFam3.1), and the identified shortcomings in the annotation. The detection of structural variation, such as copy number variation, and complex variants like inversions, duplications and large insertions/deletions, can benefit from the recent developments in long-read sequencing technologies. For example, copy number variation has been estimated to explain 9% of the genetic variants associated with IRD (Zampaglione *et al.*, 2020). Recently, two canine IRD variants have been identified where the causative variant encodes for modifiers and transcription enhancers (*HIVEP3* (Kaukonen *et al.*, 2020), *MAP9* (Forman *et al.*, 2016)). The latter is also an example of a complex variant and its detection required a correction to the reference genome sequence. Taken together, it is possible that the current whole-genome sequencing framework has missed similar structural variation, small regulatory RNAs (e.g. miRNAs), lncRNAs or regulatory mutations. Our future work will concentrate on tuning the pipeline for the identification of different kinds of regulatory mutations and non-coding variants. In addition, the pipeline should be expanded to include whole-genome sequencing data from long-read technologies to enhance the detection of structural variants.

6 Concluding remarks

The domestic dog has become an established large animal model for comparative genomic research, and in particular for IRDs. In this thesis, a whole-genome sequencing framework was established for the identification of variants implicated in Mendelian diseases in dogs.

As a proof-of-principle study, we used whole-genome sequencing (WGS) to identify the genetic variant responsible for a novel form of inherited retinal disorder (IRD) in Labrador retrievers. A one bp frame-shift insertion in the *ABCA4* gene was detected and subsequently validated as a loss-of-function mutation. We also carefully investigated the clinical phenotype of the affected dogs, and concluded that the canine IRD has similarities with Stargardt disease in humans. Based on the results of this study, we have established genetic testing for this mutation, assisting the breeders to make informed breeding decisions when choosing the parents for the next generation.

Next, we investigated the clinical manifestation of dogs homozygous for a one bp frame-shift deletion in the *TTC8* gene, previously associated with PRA in golden retrievers. The effect of the mutation was investigated on the transcriptome level, and our results suggest that the transcripts including the defective allele are degraded by nonsense-mediated decay. A thorough clinical characterization and necropsy were performed, the results of which indicated that the phenotype of the affected dogs has similarities to Bardet-Biedl syndrome in humans.

Study III presents the first characterization of the canine retinal transcriptome using short- and long-read sequencing. This study will serve as a catalog of genes and enriched pathways active in the adult canine retina, and the results have the potential to aid the validation and prioritization of candidate variants from whole-genome sequencing studies.

The results presented in this thesis can be used to establish two canine models for comparative studies of biological mechanisms underlying normal and

degenerating retina. Ultimately, these results can be used to improve the health and wellbeing of dogs and humans.

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Popular science summary

Vision is an important sense for both dogs and humans, and for us to perceive images, we depend on an intrinsic biological system that converts incoming light to electrical neural impulses that are interpreted by the brain. One important part of the visual system is the retina which is a light sensitive tissue at the back of the eye. However, there are inherited retinal diseases that lead to visual impairment and even blindness in both humans and dogs. These diseases are collectively called inherited retinal degenerations (IRDs), and are generally rare diseases in humans, affecting about 1 in 2000 people, but in dogs they may be ten times more common. To date, more than 270 different genes have been implicated in different forms of human IRDs and approximately 30 have been identified in dogs. For most of these diseases, there is no cure, but in recent years there has been promising development using gene-therapy.

This thesis focuses on two different IRDs in dogs. This was done by reading the genome sequence, consisting of 2.4 billion letters (A, C, G and T) of DNA, that is present in every cell in the body. By comparing the sequence from affected and unaffected individuals, the work led to the identification of a mutation in the canine *ABCA4* gene that in humans causes Stargardt disease. In humans, this is the most common IRD among children and young adults affecting about 1 in 8,000-10,000 people world-wide. The disease primarily affects the central vision in daylight, and the patients have problems performing tasks where high acuity vision is needed, such as reading. Similar to humans, the affected dogs also have problems in daylight, and especially at later stages of this progressive disease, vision was clearly impaired.

Most of the IRDs are only affecting the patient's vision but there are also syndromic forms of IRDs where impaired vision is only one of several clinical signs and symptoms. In 2014, a mutation in the *TTC8* gene causing IRD in golden retriever dogs was identified. In humans, this gene is involved in a syndromic form of IRD termed Bardet–Biedl syndrome (BBS) but it was unclear if the mutation in the *TTC8* gene cause a syndromic or non-syndromic form of

IRD in golden retrievers. The investigations presented in this thesis showed that many of the dogs indeed had other characteristics that were similar to human BBS-patients. It was also shown that dogs with the mutation in the canine *TTC8* gene will not be able to produce a TTC8 protein.

Lastly, the thesis characterizes which genes are active in the retinal tissue. This is important for future research on IRDs in dogs, because the knowledge about which genes are turned on in the retina helps to identify the proteins which are important for retinal function. The results of this thesis can contribute to the understanding of Stargardt disease and Bardet-Biedl syndrome, and make it possible to develop a large animal model for these diseases. Ultimately, these results can be used to improve the health and wellbeing of dogs and humans.

Populärvetenskaplig sammanfattning

Syn är ett viktigt sinne för både hundar och människor. För att kunna se är vi beroende av ett biologiskt system som omvandlar inkommande ljus till elektriska nervimpulser som tolkas av hjärnan. En viktig del av det visuella systemet är näthinnan som är en ljuskänslig vävnad på baksidan av ögat. Det finns dock ärftliga näthinnesjukdomar som leder till synnedsättning och i vissa fall till blindhet hos både människor och hundar. Ärftlig näthinnedegeneration är i allmänhet sällsynta sjukdomar hos människor, och drabbar cirka 1 av 2,000 personer. Bland hundar kan det dock vara tio gånger vanligare. Hittills har mer än 270 gener kopplats till i olika former av humana näthinnedegenerationer och cirka 30 hos hundar. För de flesta av dessa sjukdomar finns det inget botemedel men under de senaste åren har det varit en lovande utveckling inom genterapi för att bota patienter.

Denna avhandling fokuserar på två olika ärftliga näthinnesjukdomar hos hundar. Detta gjordes genom att läsa hundarnas hela arvsmassa (genomsekvensen), bestående av 2,4 miljarder bokstäver (A, C, G och T), som finns i nästan alla celler i kroppen. Genom att jämföra sekvensen från drabbade och opåverkade individer ledde arbetet till identifiering av en mutation i hundens *ABCA4*-gen som hos människor orsakar Stargardts sjukdom. Hos människor är detta den vanligaste näthinnesjukdom bland barn och unga vuxna och drabbar cirka 1 av 8,000-10,000 människor världen över. Sjukdomen påverkar främst den centrala synen i dagsljus, och patienterna har problem med att utföra uppgifter där syn med hög skärpa behövs, såsom läsning. Liksom människor har de drabbade hundarna också problem i dagsljus, och särskilt i senare skeden av denna progressiva sjukdom var synen tydligt nedsatt.

De flesta ärftliga näthinnesjukdomar påverkar endast patientens syn men det finns också syndromiska former där nedsatt syn bara är ett av flera kliniska tecken och symtom. År 2014 identifierades en mutation i genen *TTC8* som orsakade näthinnesjukdom hos hundrasen golden retriever. Hos människor är denna gen involverad i en syndromisk sjukdom som kallas Bardet-Biedls

syndrom (BBS) men det var oklart om mutationen i *TTC8*-genen orsakade en syndromisk eller icke-syndromisk form av ärftlig näthinnesjukdom bland golden retrievers. Undersökningarna som presenteras i denna avhandling visar att många av hundarna har kliniska tecken som uppvisar stora likheter med de som BBS patienterna har. Resultaten visade också att hundar med mutationen i *TTC8*-genen inte kommer att kunna producera ett TTC8-protein.

Slutligen karakteriserar avhandlingen vilka gener som är aktiva i näthinnan. Detta är viktigt för framtida forskning om ärftliga näthinnesjukdomar hos hund, eftersom kunskapen om vilka gener som slås på och av i näthinnan hjälper forskare att identifiera de proteiner som är viktiga för näthinnans funktion. Resultaten av denna avhandling kan bidra till förståelsen av Stargardts sjukdom och Bardet-Biedls syndrom, och göra det möjligt att utveckla en djurmodell för dessa sjukdomar. I slutändan kan dessa resultat användas för att förbättra hälsa och välbefinnande hos både hundar och människor.

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Båne, a Labrador retriever who participated in the studies.
Photo: Stig Persson

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Inherited retinal degenerations (IRDs) form a clinically and genetically heterogeneous group of diseases, leading to visual impairment or blindness in both humans and dogs. This thesis establishes a whole-genome sequencing framework for the identification of genetic variants underlying IRDs in dogs, which was then applied to find the genetic cause for a novel IRD in Labrador retrievers. In addition, the thesis investigates a syndromic form of IRD in golden retrievers, and characterizes the canine retinal transcriptome for the benefit of future IRD research.

Suvi Mäkeläinen received her postgraduate education and the Department of Animal Breeding and Genetics, SLU. In 2015, she obtained her MSc degree at Wageningen University, the Netherlands.

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