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Department of Animal Science and Food Processing

Evaluation of genetic variability parameters in small populations of local breeds of canids

Ph.D. THESIS

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Author: Ing. Silvie Neradilová

Chief supervisor: Mgr. Barbora Černá Bolfíková, Ph.D.

Declaration

I hereby declare that I have written this Ph.D. thesis entitled “Evaluation of genetic variability parameters in small populations of local breeds of canids” independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged using complete references and according to Citation rules of the FTA.

In Prague 6.9.2022

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Ing. Silvie Neradilová

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Abstract

This dissertation thesis summarizes the history of dog breeding and the problems of modern breeds. Especially small population-sized breeds require special management to maintain the level of genetic variability as high as possible. The loss of genetic variability closely correlates with an inbreeding rate. Inbreeding causes an increase in homozygosity of the population and thus, a higher risk of genetic diseases occurrence.

We have examined genetic variability parameters in a small population-sized dog breed (Cesky Fousek) and compared them to other similar breeds. The specific type of breeding in CF seems to be working well since the genetic variability in the breed is comparable to other dog breeds, often with a much larger population. However, in the whole CF population there have been 25 carriers detected of two genetic diseases – Degenerative Myelopathy (n = 20) and Hyperuricosuria, Hyperuricemia and Urolithiasis (n = 5). It is recommended that CF breeding individuals are tested for these genetic diseases and carefully managing the breeding population to ensure that two carriers are not bred together.

We have investigated causative factors of a complex genetic disease – alopecia - in the CF population. The disease is likely polygenic, with incomplete penetrance. We have been able to identify 144 GWAS and 236 strongly differentially expressed candidate genes and four major metabolic pathways connected to alopecia in CF - collagen formation, muscle structure/contraction, lipid metabolism, and the immune system. More samples are needed to pinpoint specific mutations.

To determine longevity in CF and the most common cause of death (COD) we performed a survey study on the CF breed. The median longevity in the whole CF population is 11.24 years and the most common causes of death were cancer or movement impairment. The mean coefficient of inbreeding calculated from pedigrees (Fx) was 6.11 %, and from genotyping data was calculated as mean COI = 14.3 %. A significant dependence of COD on the age of individuals was found, as well as for a difference between the inbreeding rate in Czech dogs and dogs from abroad.

Key words: canine small population, SNP, alopecia, genetic variability, microsatellites, genetic disease, health, longevity

Contents

List of tables

Table 13. Table showing basic descriptive parameters of the CF population from survey data in connection to the value of inbreeding (Fx) coefficient. N – number of observations; % - percentile of observations; Mean – mean Fx; Median – median Fx; Min – minimal Fx; Max – maximal Fx; SD – standard deviation; SE – standard error of the mean; VC – coefficient of variation; Q1 – 25% quantile; Q3 – 75% quantile; First section – cause of death; Second section – sex; Third section – assignment to Czech or abroad population

List of figures

from affected animals (blue) are clearly separated from the clusters representing normal skin but show a higher inter-group variability

Figure 10. Interactions of GWAS candidate genes (green) and STRING-associated strongly differentially expressed genes colored by their level of expression. We used only medium confidence associations and higher (increasing thickness of lines connecting genes indicates greater confidence). Colorful bubbles represent the metabolic pathways common for each cluster of genes.

Figure 11. Five-generation pedigree of two affected litters. Full black squares and circles represent aRFA-affected individuals (square-male, circle-female); red color represents genotyped individuals. In red writing, there are marked several matings of the same parents (repeated mating – RM).

Figure 12. A simple illustration of the hair follicle and its surroundings. Shown are stages of the hair cycle (anagen – active growth, catagen – transition stage, telogen – resting stage), sebaceous glands, dermal papilla, and arrector pili muscles attached to the dermal papilla. The picture was taken from [https://smartgraft.com/hair-growth-cycle/].

List of the abbreviations used in the thesis

Ar – allelic richness

aRFA – atypical recurrent flank alopecia

BWPGCA – Bohemian Wirehaired Pointing Griffon Club of America

CEA – Collie Eye Anomaly

CF – Cesky Fousek

CFNA – Cesky Fousek North America

COD – cause of death

COI – coefficient of inbreeding calculated from SNP data

CT – Copper Toxicosis

DD – Deutch Drahtahaar

DEGs – differentially expressed genes

FCA – factorial correspondence analysis

FDR – false discovery rate

F_{IS} – inbreeding coefficient calculated from STR

F_{ST} – fixation index

Fx – Wright coefficient of inbreeding calculated from pedigrees

GLM – generalized linear model

GSP – German Shorthaired Pointer

GWAS – genome-wide association study

GWP – German Wirehaired Pointer

HC – hair cycle

H_E – expected heterozygosity

HF – hair follicle

H_O – observed heterozygosity

H-W test - Hardy-Weinberg test

ISAG – International Society for Animal Genetics

K – cluster

KCHCF – Klub chovatelů českých fousků (Czech Cesky Fousek Breed Club)

LD – linkage disequilibrium

MAF – minor allele frequency

MCMC – Markov Chain Monte Carlo

N_e – effective population size

OCD – *osteochondritis dissecans*

PCA – principal component analysis

QGWAS – quantitative GWAS

RIN – RNA integrity number

RM – repeated mating

RSPO2 – R-spondin-2 gene

SC – stem cell

SNP – single nucleotide polymorphism

STR – short tandem repeat (microsatellites)

WPG – Wirehaired Pointing Griffon

This Dissertation thesis is based on the following publications:

Neradilová S, Connell L, Hulva P, Černá Bolfíková B. Tracing genetic resurrection of pointing dog breeds: Cesky Fousek as both survivor and rescuer. Palsson A, editor. PLOS ONE. 2019;14: e0221418. doi:10.1371/journal.pone.0221418

Neradilová S, Schauer AM, Hayward JJ, Brunner MAT, Bohutínská M, Jagannathan V, et al. Genomic and Transcriptomic Characterization of Atypical Recurrent Flank Alopecia in the Cesky Fousek. Genes. 2022;13: 650. doi:10.3390/genes13040650

1. Introduction and Literature Review

1.1. How it all began

Dogs (*Canis lupus familiaris*) have been beside humans since the Pleistocene, for approximately 15 000 - 25 000 years [1–4]. They were domesticated from one common ancestor, a grey wolf (*Canis lupus)*, earlier than any other animal [1,3,5] and it is the only large carnivore that has ever been domesticated [4,6]. It is believed that there were several domestication centers, however, exactly how many and when the domestication happened remains unknown [1,3]. Some proposed domestication centers (Belgium, Altai Mountain) are believed to be a "dead end" and the dogs from these sites probably went extinct [5]. Other centers, however, such as Mid- and Eastern Asia, Western Eurasia, and Siberia seem to be more relevant [1,4,7,8] and the dispersal of dogs from these sites was then strongly dependent on human migration all around the world [1,3,5,7].

It is believed, that the specific wolf population which the dogs emerged from became extinct. The wolves at the time of domestication had much higher genetic variability than extant population [1,4,9,10]. During the domestication process, dogs went through several bottlenecks, each such event reduced their genetic variability [4]. Shortly after the diversification from wolves, the bond between dogs and humans was still rather weak, allowing the early dogs to hybridize with wolves. This enriched the genetic variability of dogs and brought new variants to the population [3]. However, even though the bottleneck effect was reduced by this, the loss of genetic variability at this point was strong [5]. Later, the reproductive isolation from wolves was more profound and the genetic variability was reduced even more due to both, artificial selection and natural effects such as genetic drift [7]. Another strong bottleneck in dogs occurred when the modern dog breeds were being created, a few hundred years ago [5].

Dogs show incredible phenotypic variation unlike any other animal. They vary in size and shape, as well as in their behavioral characteristics [7]. There are certain differences in behavior between wolfs and dogs. While the behavior evolution of wolves was driven mainly naturally, in dogs the behavior was shaped mainly by human-driven artificial selection [3,11].

Ultimately, during the last few hundred years, dogs´ behavior has been shaped into specialization to a specific purpose and gave rise to breeds with diverse behaviors such as gundogs, herding dogs, guard dogs, retrievers, terriers, companions, etc. [3,8].

1.2. How the dog breeding advanced since the "first dog"

The phenotype of the first dogs was certainly wolf-like. Which morphological changes appeared first after their divergence from wolves remains unknown [4]. Humans have been choosing dogs for specific purposes and their morphology has changed accordingly [8]. Despite the evidence of morphological changes in dogs throughout the domestication process, there is no evidence, that people were intentionally breeding dogs until approximately 2000 years ago [12] when the first groups of specialized dogs started to appear. During the last 200 years these groups of dogs were divided into specific breeds, however, before this time the admixture of morphologically distant dogs may have been extensive [13]. This may explain why some breeds today share the same deleterious alleles even though they sometimes appear genetically very distant [8]. Nearly 360 dog breeds are now recognized by different canine organizations, such as the Fédération Cynologique Internationale (FCI), the American Kennel Club (AKC), the Canadian Kennel Club (CKC), the United Kennel Club (UKC), and others. Each breed is phenotypically adjusted to its original purpose and represents a set of defining traits which are summarized in a document called the "breed standard". The standard of a breed describes the ideal individual of that particular breed, its phenotype, and behavior. In some cases though, the standards are not well written and can contain contradictory information paradoxically leading to health issues [14,15]. Such standards should be changed.

Of the total of approximately one billion dogs in the World, only 20 % of them are purebred with a clear purpose. The rest are free-living dogs (village dogs), without human control [3,12].

The beginning of modern dog breeding is considered to be the 19th century, what in the UK is called the Victorian era, when the setting of the society has changed allowing people to have more free time [16]. Although dogs had been kept as pets or working dogs already for several centuries, only at this time did the breeders began to improve their

dogs/breeds in terms of phenotype [16]. The primary dog event at that time was a dog show, where the breeders could present their dogs in competition based on appearance. The owner of the best dog received a certain prestige and subsequently could benefit from higher stud fees and sales of puppies. Dog breeding had become a tradition but also a way of profit [16]. Even in the more recent past dog breeding was influenced mainly by fashion than function [17].

The first kennel club stud books were established during the 19th century where records of dogs and their pedigrees were kept [16,18], including dog show or performance titles. These pedigrees were used by breeders to identify traits that they valued in blood lines [16,19]. The word "blood" had a different meaning at that time, it was close to nowadays term "genes". During this time it was widely believed that conception happen from a female's blood and male's semen, also derived from blood, in the womb [16]. This incorrect belief changed with the onset of genetics at the beginning of the 20th century, but it gave rise to an understanding of heredity and the fact, that the parents of the offspring are important for the improvement of the next generation [16]. In addition, as many other things, dog breeding was a man's privilege. With the growth of the women's rights movement, there was a stronger position for women in dog breeding as well. Women improved the hygiene and welfare in the dog breeding as well as they arose discussion about banning some practices in certain breeds that they considered animal abuse – e.g. tail docking or ear cropping [16].

The breeding for certain phenotypes in some breeds has led to a high incidence of health issues connected to those exaggerated phenotypes. In pugs, for example, the breeding for wide and globular eyes has led to exophthalmos and exposure keratopathy. English Bulldogs bred for wide skulls led to dystocia (problematic parturition) because the skull of the fetus is so wide it cannot fit through the mother's pelvis during parturition. A high incidence of prolapsed intervertebral discs due to breeding for a long body occurs in Dachshunds [14]. Nowadays, some Kennel Clubs are taking precautions to overturn these breeding practices.

The overall focus during the age of modern dog breed development was conformation, however, there have always been some breeders who bred for working abilities [12]. These breeders kept records of the abilities in dogs and their offspring and tried to improve them in each generation. To fix the desirable traits they sometimes bred

closely related individuals (e.g. parent to offspring or siblings to one another). Of course, the knowledge of genetics was not as advanced as today and it is only more recently that breeders understood the importance of genetic variability and inbreeding rate of their dogs.

Until recently the pedigree data was the only source of information for estimation of genetic variability of dog breeds. The first studies of genetic variability using pedigrees started about 30 years ago [18]. Most pedigrees can be traced back to the 1960s or 1970s [20,21], but in some breeds can be the pedigree data traced back to the 19th century [22]. However, there might be errors in the pedigrees, missing records, and/or undetected matings. The completeness of the records as well as the number of known generations correlates with the accuracy of the genetic parameters calculations, the less data we have, the less precise the results. The error rate in dog pedigrees, though, is under 10 %, less than in cattle pedigrees [18]. The molecular data that came into use in the last 25 years has allowed us to estimate the genetic variability of populations even if the pedigree is unknown. The calculations of probability are made based on a kinship matrix calculated directly from the genotypes of individuals. But this method also has its limits such as the sampling effect. If the dataset has a low number of samples, or the samples do not represent the population correctly, the results might be biased. The accuracy of genetic parameters estimation highly depends also on the markers chosen. If the wrong markers are chosen for the particular study (microsatellites (STRs), SNPs, mitochondrial markers, etc.), their informative value may be too low to address the question asked. The best option is a combination of both, molecular and genealogical data.

1.2.1. Modern dog breeds – the price for pure-breeding

The reduction in population size caused by the "purification" of breeds subsequently may result in the reduction in genetic variability within breeds unless countermeasures were applied. Each modern dog breed has been established using a limited number of founding individuals and only some animals in the following generations were allowed to procreate. The entire population of each breed contains the genetic variability of these ancestors only and it stays the same or, more often, diminishes with each generation. In some breeds, the number of founders is known, especially if the breed is young. The younger the breed the higher the chance that records are complete

and the founders are known. For example in the Kooiker dog breed (FCI recognized 1990), with a current population of approximately 5000 animals, was the number of founding individuals 15 (nine dams, six sires) [23]. In Icelandic Sheepdog (UKC recognized 1996) the number of founding individuals was 36. However, the current population of approximately 3000 individuals genetically corresponds to only 2.4 unique individuals [24].

In most breeds, the founders are unknown due to lost or poorly kept records. But current genetics allows us to estimate the number of founding individuals from genomic and/or pedigree data. For example, in Braque Saint-Germain and Barbet, the identified number of founders was 49 and 13, respectively [25]. In Polish Hunting Dog with a population of approximately 1400 individuals, the number of founders was estimated to 26, although genetically represented by only 4.17 individuals [26]. In Polish Tatra Shepherd dogs 44 founders were identified and only four ancestors could explain 50 % of the overall genetic variability [27]. In Lancashire Heeler with a population size of approximately 3000 individuals today, only five individuals represent 50% of the overall genetic variability, even though founded by 15 individuals [20]. In some breeds the situation is critical. For example in Nova Scotia Duck Tolling Retriever founded by 10 individuals has a large population size of approximately 25 000 individuals, however, 50% of genetic diversity is represented only by 2 individuals [20]. It is evident that the number of individuals in the population is not necessarily an indicator of the level of genetic variability. Even with a large population, its genetic variability might be very low.

Ideally, each individual in the population should pass their alleles to the next generation to maintain the genetic variability as high as possible. But in reality, that does not happen because each breed has its own breeding conditions and only some animals are allowed to enter the breeding system. For example, in Nederland, only 3 – 5 % of all registered dogs are used for breeding [28]. These animals form the effective population (N_e). N_e in the modern dog breeds was found to rage from 53 to 230 individuals [29].

The pure-breeding practice often causes a loss of genetic variability, thus, increasing homozygosity of the population and the probability of fixation of deleterious alleles, such as genetic diseases, hidden close to the genetic regions under strong artificial selection (so-called genetic hitchhiking) [7,30]. But if the population is large enough, the deleterious alleles do not have to be necessarily a problem because the chance of mating

of two carriers of the deleterious allele is not high. The problem starts when the relatedness between individuals rises due to a small population size or due to a long-term closed population. In these cases, the chance that two recessive alleles meet rises [19]. This is the principle of genetic disease occurrence in dog breeds. Each breed has its own health problems, sometimes several and in a high incidence [19,31–33]. Some are based on the Mendelian segregation rules, creating only three possible genotypes – healthy, carrier, affected, and are relatively easy to fight with (e.g. Hyperuricosuria (HUU) or Degenerative Myelopathy (DM)) [34,35]. Others, however, are more complex in nature, and thus, it is more difficult to find the causative factors and eliminate the disease/problem from the population (e.g. joint disorders such as hip and elbow dysplasia or *osteochondritis dissecans* (OCD); behavioral disorders such as fearfulness or aggressivity; or skin disorders such as different types of alopecia) [23,32,36–38].

The level of relatedness of individuals in the breed/population can be expressed by the coefficient of inbreeding. If the coefficient rises, the fitness of offspring declines, and the risk of occurrence of accompanied problems increases. Aside from the recessive genetic diseases, there can start to appear problems in reproduction, such as reduction in litter sizes, shorter life span, and immune system disorders (e.g. allergies, cancers, bacterial and viral infections). This is also known as inbreeding depression [39].

1.3. Small population-sized dog breeds and their problems

Many dog breeders make the same mistakes whether it is a small- or a large population-sized breed. The difference is, however, that in the small population the effect of these mistakes are stronger [40,41]. The most common mistakes breeders make are over use of popular sires, breeding of too closely-related individuals, repeated mating of the same parents, and breeding for only one or few traits (appearance). On a larger scale, also strictly closed studbooks, over time, lead to the reduction of the genetic variability of the breed [42–44]. Even if the breeders avoid the above-mentioned mistakes, there is always a natural way of variant loss or fixation, a random chance causes some rare genetic variants to disappear from the population or others (often harmful) to get fixed in the population (genetic drift) [42,45]. All these effects are lowering the genetic variability of the population and increasing its homozygosity and inbreeding level unless countermeasures are applied.

Inbreeding has been for a long time an unknown term. In Europe, the breeders started to learn more about this topic, and apply precautions in their breeding at the beginning of the 21st century when relevant literature became widely available (e.g. [42]). Around the same time, many scientific studies have been made to estimate inbreeding levels and other parameters of genetic variability in many breeds. At that time, usually a set of STR primers was used for this purpose. In dogs a standard ISAG panel containing 18 STR loci (e.g. a study of Tatra Shepherd Dog [46] or our study of Cesky Fousek (CF) [47]) was widely used. But also more STRs could be studied [48]. Using the combination of STR and pedigree data it was found that in all studied breeds the overall genetic variability tends to decline even though, there might be visible a recent increase in the number of individuals and a bit lower homozygosity level in some of them, compared to the situation of 50 to 60 years ago [23,49]. An average increase in the inbreeding level of modern dog breeds was estimated to be 0.66 % per generation. [50]. It was also found that the higher level of inbreeding of a litter, therefore also of the parents, is connected to smaller litter sizes as well [23,51]. It also increases rates of cancers and shortens lifespan [30,52–55]. If such signs of inbreeding depression start to appear in the population, it is needed to apply countermeasures to increase the genetic variability.

To establish an average lifespan of the CF breed, we conducted an owner survey. The results are reported in the "Results" section of this Dissertation. In general, the smaller breeds live longer than the large ones [30], females in some breeds live longer than males [30,56] and in some breeds, even some color variants live longer than others [57]. Mainly the body size, but also the inbreeding level, might have a negative impact on the life expectancy in dogs [30]. CF is a middle-sized breed with small population size and a specific type of breeding (controlled line-breeding).

Despite the knowledge of inbreeding and other genetic parameters that has been spreading across the community of breeders for quite some time, there are still many breeders, that either ignore or do not understand the risks. The consequences of the "effect of popular sires" can be found in many breeds, some sires can have more than 2500 offspring [50]. Usually, it is an exceptional individual that was successful in either dog shows or working tests, or rarely both. The breeders believe, that the exceptional father makes exceptional offspring as well. However, that is often not the case, and even a champion sire might give under-average puppies. One example of a popular male over

use is in the American population of Basenji. The breed has been introduced to the US around 1941 and one particular male of those founding individuals creates 30.3 % of the genetic diversity of the current Basenji population in the US [29]. In Bracco Italiano only 9 ancestors explain 50 % of the overall genetic variability of the breed [58] and in Kooiker dogs 12 frequently used sires were identified. Since these males had high inbreeding levels themselves and were related to each other, the decrease in genetic variability, in this case, was strong [23]. The over use of popular sires is probably also the reason for the high inbreeding level in Sharpei (11 %) [13].

Breeding of closely-related individuals is sometimes a useful tool but must be used carefully. In many breeds the breeding of closely related individuals to fix some desirable traits in the next generation has been used, especially at the beginning of breed development. But we need to bear in mind that just as it fixes the desirable traits, it can "trigger" harmful ones as well [19]. By breeding close relatives, we increase the homozygosity of the offspring and the chance of meeting of two harmful recessive alleles rises exponentially. If this breeding practice is used too often, it significantly increases the risk of inbreeding depression.

Sometimes, repeated mating is used to obtain offspring with the same qualities. However, due to random sampling of alleles, the second litter might be very different compared to the first one. Using the same sire does not decrease the genetic variance per se, however, it may lead to overrepresentation of alleles from few individuals which later leads to decreased heterozygosity. An example of repeated mating is shown in a CF pedigree in the Discussion section (Figure 11).

The genetic variability can be quickly lost if the breeders breed for just one or a few traits, usually appearance for dog shows. In some breeds a show line and a working line of individuals exist. It has been shown, that dogs bred for conformation are much less genetically variable than dogs bred for work [59]. Breeding for conformation is relatively easy compared to behavior. The appearance is encoded by a small number of genes so the response in breeding is faster. On the contrary, breeding for working abilities is much more difficult. Behavior is encoded by a large number of genes, the environment and training have a great influence, and the response in breeding is slow.

Genetic drift causes the fixation or extinction of alleles in the population. If there are a large number of deleterious alleles in a population, there is a high chance that some of them get fixed in the population by chance, especially in populations with low N_e [18].

At the end of the 19th and beginning of the 20th century when most of the modern dog breeds were established is detectable a strong genetic bottleneck. This bottleneck led to higher levels of inbreeding in many breeds because the studbooks were closed for outcrosses from other breeds [49]. Many breeders are against outcrosses because they feel their breed would not be "pure" anymore. However, to keep the breed closed to outcrosses for too long causes the loss of genetic variability because of both, the breeding practices used and genetic drift.

The frequently used parameters for estimation of genetic variability are coefficient of inbreeding (Fx, COI, F_{IS}), heterozygosity (expected vs. observed), number of alleles per locus, number of private alleles, and allelic richness. Even though the trend is slowly changing in terms of markers used for these kinds of studies, these parameters are strong enough to estimate the genetic variability of a canine population. Until several years ago STR markers were primarily used, however, declining price and higher availability now allow researchers to use single nucleotide polymorphism (SNP) genotypes or the whole-genome sequences to estimate genetic variability.

There are two main ways of calculating of the coefficient of inbreeding, a) from pedigrees or b) from genomic data. The inbreeding coefficient calculated from pedigrees is also known as Wright's coefficient of inbreeding (Fx) [60]. The coefficient of inbreeding calculated from genomic data is usually marked F_{IS} or COI. F_{IS} is calculated from STRs and it can range from values of -1 to 1. COI is calculated directly from SNP genotypes or sequencing data and it is expressed as a percentage. All coefficients give us the same information, they show the probability of inheritance of an allele from the common ancestor. But their values are not comparable. Of the two, the COI is more precise [51] because it is calculated directly from a large number of SNP markers [61] but the precision of the result depends on the reference population against which the individual is tested. F_{IS} is highly dependable on the markers used, the STRs must be carefully chosen for each species. Fx is highly dependent on the pedigree depth and the number of generations used for calculation [51] and it also neglects the effects of recombination [62] unlike COI. Often the Fx using three to five generations does not

change much but with a higher number of generations the value rises exponentially and it gives us a more relevant picture of the overall inbreeding value of the individual/population. Breeders usually use the Fx for up to five generations to keep track of inbreeding values, but this value has its limits and it does not have to truthfully reflect the inbreeding level of the individual/population. Unfortunately, to calculate Fx for more than five generations can be problematic because few breeders have access to a complete database of individuals and extended pedigrees.

Due to the above-mentioned breeding practices, and thus high inbreeding rate, many dog breeds suffer from genetic diseases [15,19]. Examples of breeds where the high value of inbreeding somehow influenced the occurrence of a genetic disease are the Bouvier Belge des Flandres where in animals with higher inbreeding level (Fx = 6.4 – 12.5 %) diseases such as osteochondrosis, food allergy, autoimmune disease, neoplasm, and hypoplastic trachea occurred [63]. In the Polish Tatra Shepherd dog where the mean Fx was calculated to be 7.4 % (reaching up to 25% in males and 19.5% in females), hip dysplasia (HD) is prevalent [27]. The same health problem was detected in Icelandic Sheepdog, where the inbreeding level goes up to 21 %. It has been shown that highly inbred individuals are more likely to suffer from HD [64].

In some breeds the genetic testing helped to reduce the occurrence of a genetic disease. As an example can be used Collie Eye Anomaly (CEA) in herding dogs. The frequency of affected animals that used to reach up to 97% of tested individuals [65] reduced significantly in recent years [66]. The only exception is the Rough Collie where the incidence is still rather high, however, the number of samples in the study might have biased the results [66]. But even in CEA-affected individuals was recently detected that the affection in single individuals is not as severe as it used to be in the past [https://www.colliehealth.org/cea-mutation/].

Fighting with genetic disorders is a never-ending effort as proved for example by Bedlington terriers suffering from an autosomal recessive disease - copper toxicosis (CT). CT was identified to be caused by a mutation in COMMD1 gene. In 2000 the breed club decided to exclude all heterozygotes and recessive homozygotes from the breeding system to radically reduce the CT occurrence in the population. Although four rare alleles have been lost, the parameters of genetic variability were otherwise not significantly different between a control group (healthy animals) and cases (recessive homozygotes).

At that time the researchers recommended to include carriers into the breeding system as well since the overall heterozygosity was low [67]. The hard selection helped to decrease the incidence from 46 to 11% in Dutch population [68]. Later was found that the COMMD1 mutation is probably not the only allele/gene involved in CT in Bedlington terriers since there were identified dominant homozygous animals in COMMD1 showing clinical signs of the disease [68,69]. Currently is the CT under research again to identified the additional variants involved in the disease occurrence in Bedlington terriers [68,69].

1.3.1. What might help?

What can the breeders do to keep the genetic variability of their small population-sized breed as high as possible, or even increase it?

Individuals that are geographically and genetically distant from a target population can be used. It can be an animal from the same breed but for example from a different country. However, this option might not be possible if the breed does not have another such population available, especially in the case of a local breed.

Another way of introducing new alleles in a breed is an outcross. It was shown that outcrosses have played an important role in the development of many breeds [8] but also an outcross in an established breed is usually highly beneficial for fitness [30]. However, the choice of the specific individuals used for the outcross is very important and it is usually done by a breed club. There exists a danger of the introduction of deleterious alleles into the recipient population through the outcross. Each breed and even each individual carries several deleterious alleles [70]. For example, the German Shepherd was identified with 58 non-conformational genetic disorders, Golden Retriever with 50, the Irish setter with 33, etc. [19]. However, if the individual is properly tested by available genetic tests, the danger of introgression of such alleles is reduced. Besides, just as there is a risk of introgression of harmful alleles, there is also a chance of introgression of beneficial ones as well. In terms of breed, when a similar breed is chosen, the gain of different alleles might not be as high, but it will not disrupt the appearance of the following generations so significantly. If a phenotypically different breed is chosen, very different alleles might be introduced into the target population, but it may cause changes in appearance that can be highly significant. In this case, a high risk exists that none of the F1 individuals passes the breeding conditions in terms of standard appearance. There

is also a question of the number of outcrosses performed. If the outcross is used only once in a population, the new genetic variants might be bred out in a few generations. To reduce the increase of inbreeding level in each generation, repeated outcrosses are more effective [70]. An example of successfully used outcross can be found in the American population of Wirehaired Pointing Griffon (WPG) [71]. The WPG is a breed that was bred for a long time as "pure". The origin of the breed starts with Dutchman Eduard Karel Korthals who developed this breed mainly in France and Germany around 1880 using dogs from wirehaired background. After Korthals died in 1896, part of the breeders refused to use outcrosses in the breed and closed the studbook. Since the beginning of the 19th century, the breed spread around Europe and it was introduced to the USA as well. The WPG individuals have been used for hunting in the USA under the umbrella organization Wirehaired Pointing Griffon Club of America (WPGCA). Over time, however, a part of the Club in North America concluded that there was a high rate of genetic disease occurrence (especially OCD), increasing relatedness of individuals, temperament issues (fearfulness), and vanishing working abilities. In 1984 it was decided to use a similarly looking breed, CF, as an outcross. There were imported three CF individuals (one male, and two females) and the results were visible immediately. The working abilities, temperament, and coat quality improved significantly, and the health as well [47,71]. Over time the outcrosses continued and were more frequent and the population of American WPG slowly transformed into the CF breed [47]. The umbrella club has changed its name to nowadays Cesky Fousek North America (CFNA).

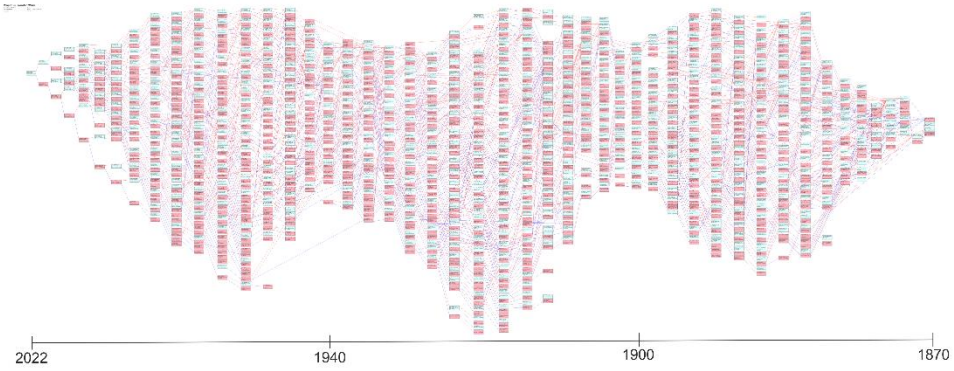
Another example of outcross use can be found in our national dog breed – the CF. In 2000, two males of different breeds (GSP and DD) have been used in the breeding system of CF [47]. CF is an old, versatile, small population-sized breed with a complex history. The breeding is realized using line-breeding [72] and use several breed wardens to carefully maintain the breed's genetic variability. The main reasons for the outcross use was improvement of coat quality, pointing, and a boost of genetic variability to help reduce the occurrence of alopecia in the breed. The outcrosses were used in two out of eight lines and the results were quickly visible. The visualization of one of the outcrosses is shown in Figure 1. Under the development and maintenance of CFNA, an international database of pedigrees for the whole CF population has been established. Currently the database contains over 35 000 individuals and continues to grow. It does not contain only CF individuals, going further to the past, there start to appear Deutsch Drahthaar (DD),

Deutsch Stichelhaar, WPG, even Spinone. Figure 1A shows a pedigree of a CF individual with a recent GSP outcross and one CF without the recent outcross (Figure 1B). Figure 1C shows a pedigree of a WPG individual. It is also one of the most complete pedigrees in the database. Although the pedigrees of these individuals might not be complete, especially in the oldest generations, it is visible how much potential variation comes to the pedigree if an outcross is used (Figure 1A). Alternatively, in the pedigree of the WPG individual there is visible a relatively small number of ancestors, thus, indicating a lower amount of genetic variability in the population/individual (Figure 1C). In the pedigrees, especially 1B, are also visible effects of both World Wars. The number of individuals declined during these periods. The genetic relationships between CF, WPG, GSP, and other related breeds of pointing dogs have been explored in one of our studies [47].

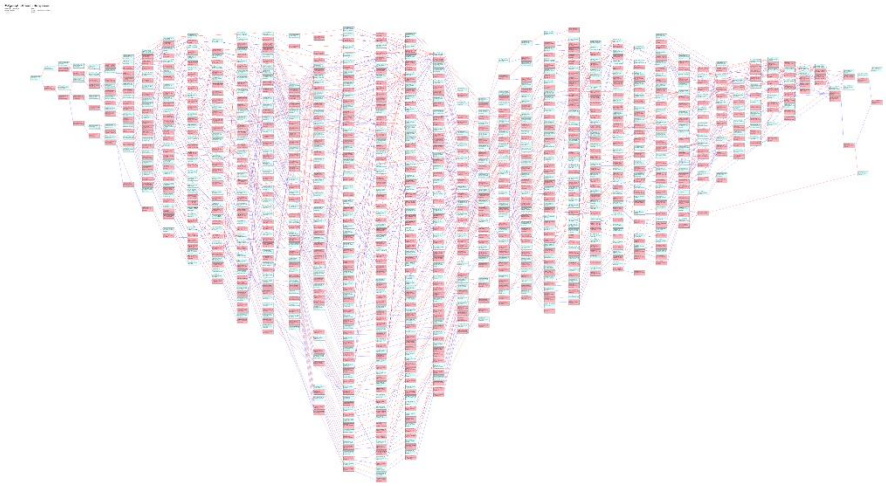
Figure 1. Pedigrees of three individuals from the Cesky Fousek database. A) pedigree of a CF female Bonny; there was recently (in 2000) used an outcross to GSP, marked by a red box in her third generation. B) pedigree of a CF female Quanta; there was not recently used the outcross to GSP. C) pedigree of a WPG female Mahaska; this is one of the most complete pedigrees in the database.

A)

B)



C)



When the outcrosses in CF were used, there was no available testing for genetic diseases. In GSP was later found 26 different genetic disorders [19], for example, 8 % of the population are carriers for Factor VII Deficiency [73], so there was a risk of introduction of at least some of these harmful alleles to the CF population. If that is the case remains to be seen, however, according to our results of CF health screening, the GSP individual did not bring any (known) new harmful alleles to the CF population. One of the problematic aspects of an outcross can be seen in the CF population as well. The outcross with a breed of a different coat type also means a higher differentiation in the descendants' phenotype. For example, after the GSP outcross into the CF population, the coat length varied in offspring. In some individuals, it was too short, and these individuals were not chosen for the breeding system. The same holds for shorthaired breeds where a longhaired outcross is used. Fortunately, in this case, the loci responsible for the long coat are known [74], so we can test the puppies and choose the animals for the breeding system accordingly. In the wire-haired breeds, the situation is different, the coat characteristics are encoded by more genes, some still unknown, such as in CF. However, despite these risks of outcrosses, the gain of genetic diversity is worth the subsequent difficulties.

Another option to keep the genetic variability of the small population-sized breed as high as possible is to create separate lines in the population. As mentioned before, there exist breeds that contain show lines and work lines. But there is another option of how to create lines in the breed that help to contain more overall genetic variability of the population, as mentioned above for the CF breed. In this case each line is based on either a blood line (usually paternal), kennel, or a specific trait [75–77]. All individuals have the same breeding conditions, but they are restricted to mating with individuals of the same line only. This, of course, requires a discipline of breeders, and thus, the breed clubs usually establish a net of breed advisors, that control the breeding and will not allow breaking the rules of line-breeding. In the CF breed there are currently eight active lines that are not supposed to mix with each other [77] although, in reality, there does not exist a dog that would be 100 % pure in one line. The breed wardens sometimes allow interline breeding and the offspring then goes back to the line, it might be considered an outcross on a smaller scale.

The breed clubs have different breeding approaches. Some clubs leave the composition of mating pairs to the breeders entirely. Some have one or more breed

wardens that can advise the breeders if they want to. And some clubs have established controlled breeding - the female owner gets to choose from only a few breeding males (usually three). Another option of control by a breed clubs is to limit of breedings for each male. Both, controlled breeding and a limit of breedings (up to 4 per male per year), are used by the Czech CF breed club to prevent the effects of overusing only one or few males repeatedly. Controlled breeding is managed by informed people who have the necessary knowledge to avoid bad breeding practices.

Other options for maintaining genetic diversity involve avoiding the above-mentioned mistakes. Each breeding individual should procreate, there should not be used matings of too closely related individuals and repeated matings of the same parents. Breeders should not overuse only one or a few sires. If the breeders breed only for conformation, they should consider using an animal from the work line every once in a while, even though it might mean the coat or other elements of appearance might get worse in the following generation. The breed clubs should closely watch the genetic variability of the breed and in case of need allow an outcross to avoid the consequences of inbreeding depression.

1.4. Dogs as a model organism for genetic studies

Given the long co-evolution with humans, dogs are a great model organism for understanding human diseases [7]. Living in a similar environment, often sharing similar food sources, and highly developed health care have led to changes in dog genome and longer longevity of many dog breeds [8]. Throughout the domestication process, bottlenecks, and breed creation the dog genome lost much of its original variability, haplotype homozygosity increased and long regions of linkage disequilibrium (LD) appeared [4,78]. Studies have shown that there is a small number of haplotypes within regions of ~10–15 kb shared by the majority of breeds. However, to discover haplotypes specifically for a particular breed, we need to study large haplotypes [8]. Dogs are also physiologically and clinically more similar to humans than mice [79,80]. Even if the canine disease does not have a direct equivalent in humans, the understanding of complex genetic diseases might indirectly help in human medicine as well. The advantage of canine genomic studies is that the number of samples required is much smaller even for complex genetic diseases [81].

We have performed a study of a polygenic canine genetic disorder that occurs in high prevalence in the CF breed. It is a type of alopecia, hair loss, with an unknown cause. A similar disorder, called Alopecia Areata, occurs in humans as well [82,83]. In the CF breed, the alopecia resembles Recurrent Flank Alopecia, however, it is atypical due to missing hyperpigmentation of the skin, thus, we call it atypical Recurrent Flank Alopecia (aRFA) [36].

With the development of the methods in genetics and genomics, it is now possible to study canine equivalents of human mental and developmental disorders as well [79,80,84,85]. However, these studies still require large sample numbers, thus, the research is very demanding in terms of financial support.

Similarly, the behavior of dogs is complex and polygenic, influenced by many small-effect genes and the environment. Studies of behavior also require several hundreds to thousands of surveys and samples to ensure strong enough data for relevant results [12]. Until recently was the GWAS method usually used to compare populations of breeds, however, sometimes it is needed to use individuals, regardless of the breed, with the same trait to get a stronger significance of associated variants [12]. Some kinds of behavior can be found, in varying prevalence, in all breeds suggesting these behaviors have been developed deep in the past in dogs´ ancestors during the domestication process [12]. There have already been, for example, identified DNA regions associated with fear and aggression in dogs [37].

In dogs, we can also study longevity and aging which might have an impact on the human population as well. First genomic regions have been identified as having an association with longevity in dogs [86]. Mixed-breeds were found to live longer than pure-bred animals [30,86].

Aims of the Thesis

This thesis aimed to evaluate parameters of genetic variability on a model of a small canine population, identify causative factors of one of the complex genetic diseases that occur in the population, and find the most significant cause of death and longevity.

Objectives Study 1: Genetic variability of a small canine population (Appendix 1)

i) to assess genetic diversity and describe genetic parameters of a small canine population and compare the parameters to other, similar breeds;

ii) to evaluate the level of genetic divergence and differentiation between studied breeds;

Objectives Study 2: Causative factors of a complex genetic disease (Appendix 2)

iii) to analyze the population genotypic structure associated with the complex genetic disease;

iv) to establish a histological phenotype for diagnosing this disease;

v) to identify specific dysregulated genes and metabolic pathways involved in the pathomechanism of the disease;

Objectives: Additional health analyses (paper in prep.)

vi) to screen for an extensive panel of known genetic diseases, coefficient of inbreeding, and maternal and paternal haplotypes present in the CF population;

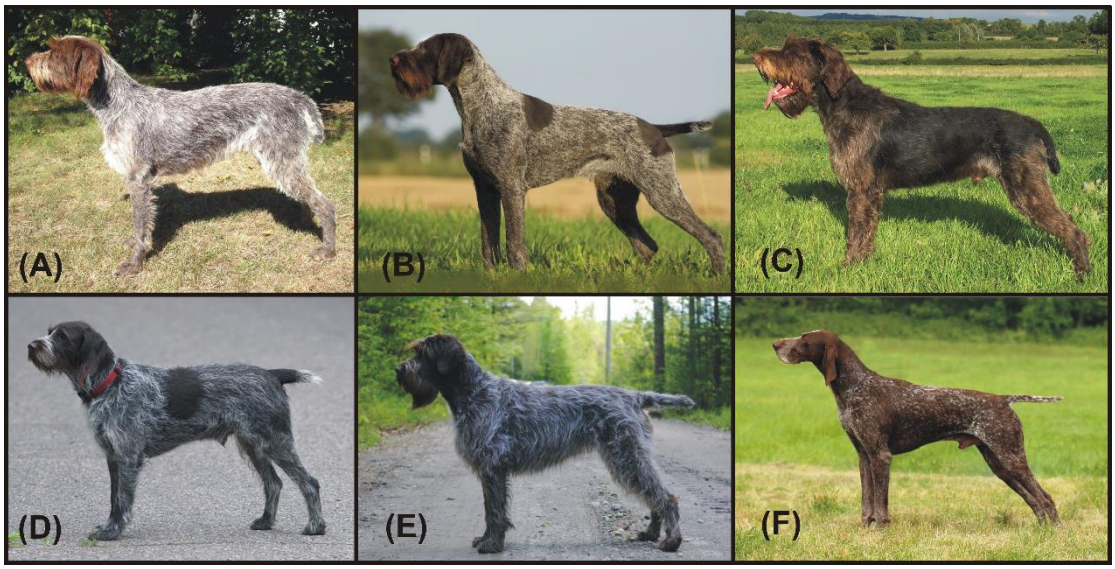
vii) identify the most common cause of death of CF individuals and the average longevity of the breed;

2. Methods

2.1. Methodology – Study 1: Genetic variability of a small canine population

To assess the genetic variability of a small population-sized dog breed we chose a Czech dog breed (CF) and we compared it with other breeds of pointing dogs (Figure 2) with a similar- or larger-sized populations and similar appearance [47] (Appendix 1).

Figure 2. The general appearance of six studied breeds. (A) Cesky Fousek; (B) Deutsch Drahthaar; (C) German Wirehaired Pointer; (D) individual of Bohemian Wirehaired Pointing Griffon Club of America; (E) Wirehaired Pointing Griffon; (F) German Shorthaired Pointer. Tail docking is allowed for hunting breeds in the countries of origin for these animals which were: the Czech Republic (A; F), Germany (B; C), and the USA (D; E).



2.1.1. Sampling

Samples were collected from 405 individuals representing six pointing breeds: Cesky Fousek (CF; n = 193), Deutsch Drahthaar (DD; n = 87), German Wirehaired Pointer (GWP; n = 26), individuals of Bohemian Wirehaired Pointing Griffon Club of America (BWPGCA[1]; n = 38), Wirehaired Pointing Griffon (WPG; n = 20) and German

[1] Note that the name of the American breed club has changed since the time the study was performed. The original name Bohemian Wirehaired Pointing Griffon Club of America (BWPGCA) has changed to Cesky Fousek North America (CFNA) and it is used in the subsequent papers.

Shorthaired Pointer (GSP; n = 41), during years 2012–2016. Samples were taken as buccal swabs (FLOQSwabs1) with the agreement of the dog owners during dog shows, hunts, and hunting competitions. The origin of the samples is given in the S1 Table. Samples from the Czech Republic and the Netherlands were taken by SN, BČB, MJ, and PH (n = 255); samples from other countries were obtained directly from owners (n = 150). These owners were instructed how to take the samples correctly to avoid contamination. Several samples were obtained from the Cornell Veterinary Biobank (n = 27), which provided 23 samples of BWPGCA individuals and four samples of WPG individuals. Some individuals sent by BWPGCA were imported individuals from the Czech Republic; these samples were classed with the pure CFs from the Czech Republic to avoid biased results. DNA from buccal swabs was extracted using Genomic DNA Mini Kit (Geneaid Biotech Ltd., New Taipei, Taiwan) for tissue and saliva according to the manufacturer's protocol.

2.1.2. PCR and fragmentation analysis

We have selected nuclear microsatellites as the genetic marker type for this study due to their high polymorphism, neutrality with respect to the selection, and good statistical power to detect recent population structure. A commercially available microsatellite genotyping kit (Canine Panel 1.1; ThermoFisher Scientific) was used to amplify 18 microsatellite markers (AHTk211, CXX279, REN169O18, INU055, REN54P11, INRA21, AHT137, REN169D01, AHTh260, AHTk253, INU005, INU030, FH2848, AHT121, FH2054, REN162C04, AHTh171, and REN247M2). Fragmentation analysis was processed on ABI Prism 3100 Avant Genetic Analyser (Applied Biosystems) using polymer POP-4tm separation matrix with DS-33 matrix standard size and Gene Scan TM 500 LIZ (Applied Biosystems) size markers.

2.1.3. Data and statistical analysis

The length of each allele was scored and binned in GENEIOUS R10 [87]. FSTAT was used to estimate allelic richness (Ar) based on minimal population size from the smallest group in the study (18 individuals). Ar describes genetic variation while eliminating the effect of the sample size. Estimates of expected heterozygosity (H_E), observed heterozygosity (H_O), inbreeding coefficient (F_{IS}), and a number of private alleles for each population were calculated in software GENEALEX 6.501. Pairwise fixation

index values (F_{ST}) and Hardy-Weinberg (H-W) test for heterozygote deficiency were calculated in GENEPOP software [88,89]. Exact p-values for the H-W test were calculated using a Markov chain algorithm [90] with 1000 dememorization steps for 500 batches and 1000 iterations per batch. Software POPULATIONS [91] was used to compute a matrix of minimum genetic distances according to Nei [92] for all individuals. This matrix was used to construct a phylogenetic neighbor-joining tree of relationships among populations and individuals. The tree was graphically visualized in FIGTREE [93]. Visualization of genetic relationships between individuals was processed in GENETIX software [94] using factorial correspondence analysis (FCA). To assign particular genotypes to respective clusters (K) and to assess substructure within the dataset, the Bayesian clustering approach implemented in software STRUCTURE [95] was used. The number of tested K ranged from 1 to 10. For each value of K, five runs were performed with a burn-in period of 300 000 and 1 000 000 MCMC (Markov chain Monte Carlo) repetitions. The best support for the number of clusters (K) was combined in STRUCTURE SELECTOR [96] using the Evanno method of ΔK [97] and MedMed K, MedMean K, MaxMed K, and MaxMean K statistics [98] which are more accurate for unequal population sample sizes.

2.2. Methodology – Study 2: Causative factors of a complex genetic disease

CF is known to suffer from alopecia, a hair loss that affects mainly the body's sides. The type of alopecia in CF is atypical Recurrent Flank Alopecia (aRFA), and its cause is unknown. We attempted to discover variants associated with this disease [36] (Appendix 2).

2.2.1. Blood Sample Collection

Altogether, 216 samples (non-affected n = 116, affected n = 100) were collected (189 from the Czech Republic and 27 from Cesky Fousek North America (CFNA)); 72 males and 144 females (Table S3). Relatives were not excluded from the dataset. The blood draws were done in cooperation with the Czech Cesky Fousek Breed Club (KCHCF) and CFNA during 2016–2019. Blood samples were shipped to the Cornell Veterinary Biobank, where DNA extraction was performed by standard salt precipitation

and the DNA was then stored at −20 °C. The level of severity was determined by a responsible member of the breed club (KCHCF). In the past, the club has developed a protocol for the identification of all aRFA levels and this protocol was also followed in our study. The affliction of individuals was marked during sample collection, and their affected status and severity were updated throughout the study's duration.

2.2.2. *Biopsy Sample Collection*

Six-millimeter punch biopsies were taken under local anesthesia for histological evaluation and RNA extraction (seven control dogs and seven affected dogs). From the seven control dogs, two 6 mm punch biopsies were taken from sites close to each other. From the seven dogs affected with aRFA, two neighboring biopsies were taken from a completely alopecic site and two from a distant, fully haired area (shoulder). One biopsy from each site was fixed and stored in buffered Formafix 10%® (Formafix AG, Hittnau, Switzerland). The second biopsy from each site was collected in RNAlater (Invitrogen, CA, USA) and stored at −20 ◦C until RNA extraction was performed. Based on the histological evaluation of the samples and the diagnosis of hypothyroidism in one of the dogs, we had to exclude some samples from further analysis.

2.2.3. Genotyping

Genotyping was performed on a semi-custom 220k CanineHD array (Illumina, CA, USA), currently available as the Embark genetic test [www.embarkvet.com, accessed on 19 February 2022]. In total, 216 samples were genotyped: 47 samples in 2016 with 214,582 markers and 169 samples in 2018–2019 with 239,490 markers. The number of markers differs due to the upgrading of the genotyping array. The positions of the markers are listed in CanFam3.1.

PLINK [99,100] datafiles were generated and the data were checked for errors in sex and genotype missingness. All samples had a genotyping rate higher than 95% so no samples were excluded at this point. Only markers with minor allele frequency (MAF) higher than 0.05 were included in the analyses. Data from the two different arrays were merged and discordant SNPs between duplicate samples, in accordance with a previous study [101], were removed from the datasets. Moreover, to avoid the resulting bias caused by the non-balanced sex of the individuals entering the analysis (Table S3), we also

filtered out the chromosome Y retrocopies [102] and sex-associated markers (a total of 96 SNPs). After filtering, the number of SNPs used for GWAS was 140,024.

Principal component analysis (PCA) was performed before the GWAS analyses to (i) check for population structure between the Czech Republic and USA samples, (ii) look for any batch effects due to the two genotyping arrays used, and (iii) identify any individual outliers. PCA was run on unlinked SNPs only, using PLINK command --indep 50 5 2, in the program EIGENSTRAT in the EIGENSOFT v5.0.1. package [103,104]. PCA plots were visualized in R i386 3.6.1 [105].

The population stratification of the data was corrected in GEMMA by including a relatedness matrix, calculated from genotypes, as a random effect. We calculated the genomic inflation factor (λ), based on p-values, in the R package snpStats [106]. Lambda inflation factor compares the median test statistic and expected null distribution and it detects the normality of the data distribution with a value of 1.0 representing no inflation. Manhattan and quantile-quantile plots of p-values were constructed in R. The significance thresholds for the GWAS analyses were set on Bonferroni correction on unlinked SNPs (using --indep 100 10 10 in PLINK). LD plots were created from LD analyses run in PLINK and using the Matplotlib library in the Jupyter notebook [107,108].

2.2.4. Case/Control GWAS

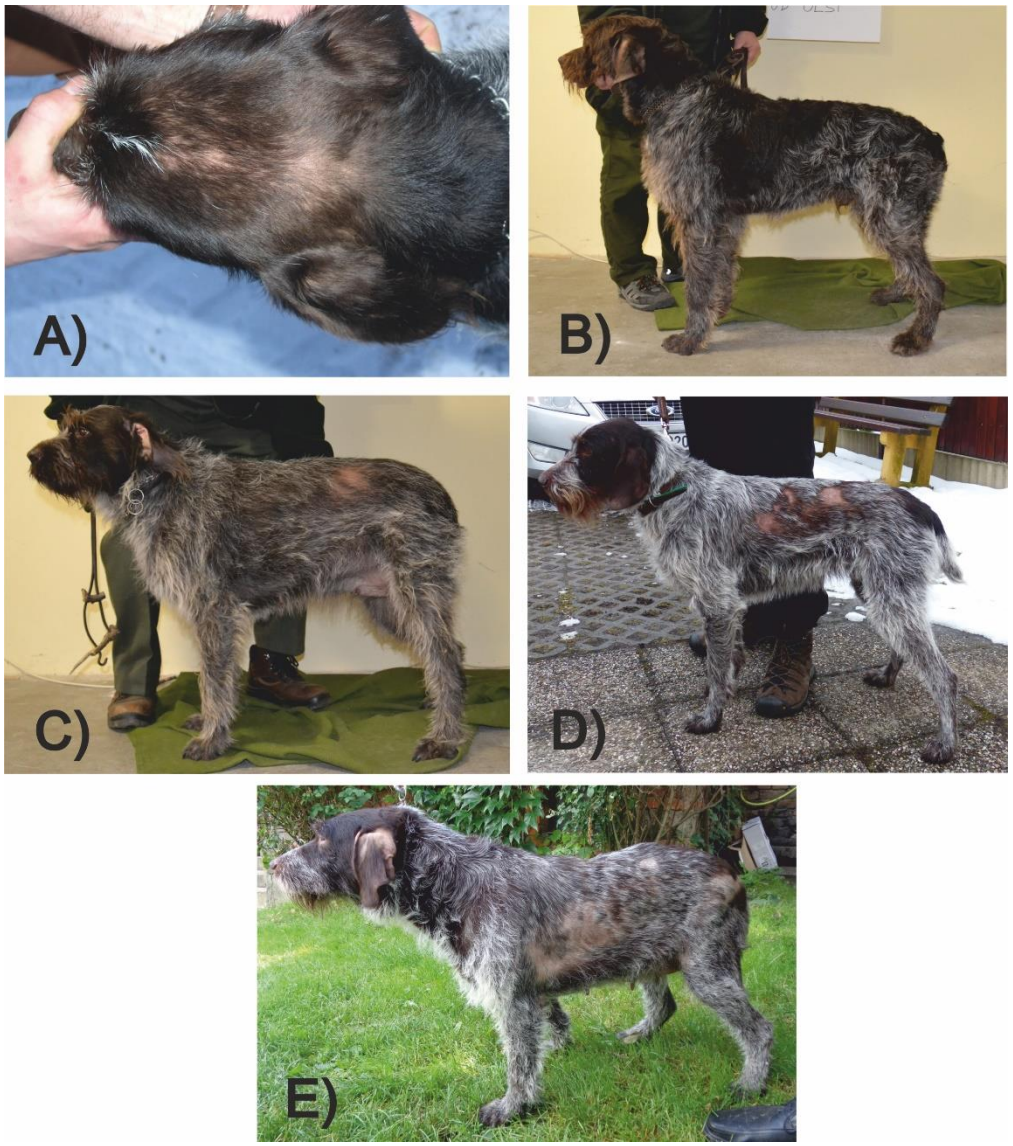
A genome-wide association study (GWAS) was conducted using a linear-mixed model in GEMMA v0.98.1 [109]. In total, 213 individuals out of 216 were used for the case/control GWAS study - three affected individuals were excluded from this analysis due to an unusual manifestation of alopecia (alopecia on the head), resulting in a dataset of 96 affected and 117 control individuals.

2.2.5. Quantitative GWAS (QGWAS) and Additional GWAS Analyses

To discover variants with a direct association with aRFA level, we performed a QGWAS analysis with 216 individuals. The three animals with the unusual manifestation of aRFA on their heads (Figure 3A) were included in this analysis. All individuals were divided into one of six phenotypic categories. The number of individuals in each category and the code of each category are: healthy (n = 111; code “0”), head affection (n = 3; code “0.1”), level 1 aRFA (n = 6; code “0.25”), level 2 aRFA (n = 28, code “0.5”), level 3

aRFA (n = 49; code “0.75”), level 4 aRFA (n =19; code “1”). Each sample was assigned to the corresponding category according to the aRFA level (Figure 3A-E): Head affection—the individual loses hair on the top of the head, ears, and sometimes the top of the nose. Level 1—the individual loses hair on the ears only (can enter the breeding program); Level 2—the individual loses hair on the body sides up to the size of approximately 10 × 10 cm; Level 3—hair loss on the body sides up to approximately 10 × 25 cm; Level 4—hair loss on the body sides up to approximately 10 × 40 cm; Level 5—hair loss on the body sides larger than 10 × 40 cm (this level was not represented in our dataset). Moreover, we conducted four additional GWAS analyses to identify specific variants that were associated with level 2 aRFA (28 individuals), level 4 aRFA (19 individuals), age of onset before 2 years of age (26 individuals), and age of onset at 6–8 years of age (20 individuals). The last two groups were also affected by level 2 aRFA or worse. We did not consider level 1 aRFA as “affected”. The control group for all the above-mentioned groups were composed of 35 individuals aged 10+ years in which the chances of developing aRFA were very low. The settings of the allelic and genotyping frequencies were the same as in the main case/control GWAS analysis.

Figure 3. Cesky Fousek individuals affected by aRFA. A) unusual manifestation on the head; B) level 1 aRFA—loss of hair on ears only; C) level 2 aRFA—loss of hair on the body sides up to approximately 10 × 10 cm; D) level 3 aRFA—loss of hair on the body sides up to approximately 10 × 25 cm; E) level 4 aRFA—loss of hair of the body sides up to approximately 10 × 40 cm. Pictures A and E were taken prior to the hair-loss peak in these individuals; alopecia worsened in the weeks after the pictures were taken.



2.2.6. Haplotype Identification

Before haplotype identification, we divided the genotyping data by chromosomes using PLINK v.1.9 [110,111] and subsequently phased each chromosome of interest, based on the case/control GWAS results, in SHAPEIT.v2.r837 [112]. The settings were left at their default levels with 7 MCMC burn-in iterations, 8 pruning iterations, and 20 main iterations. The number of conditioning states (K) was left at 100, the --window size setting was 2 Mb, and the genetic map was not provided, leaving the --rho value at its

default (0.0004). Each phased chromosome was then transferred to PED/MAP format and run in PLINK v1.07 [99,100] to estimate the haplotypes in both the case and control groups of individuals. The setting was set to --hap-window from 1 to 10 SNPs to obtain all one-, two-, and up to ten-SNP windows across the dataset (respecting the chromosome boundaries).

2.2.7. Histopathological Analysis

Formalin-fixed biopsies were processed for routine histological analysis by embedding in paraffin, microtoming (3 μm), and staining with hematoxylin and eosin (H&E). The samples were blinded and histopathological analysis was conducted to characterize specific histological features and patterns associated with this alopecic disease. Histological evaluation was also utilized to include or exclude samples not suitable for RNA extraction based on histological findings that might influence gene expression (e.g., secondary lesions such as inflammation). Based on this, we excluded the biopsies from one control dog, two lesional sites of alopecic dogs, and three normal skin sites from affected dogs from the analysis, resulting in a total of six samples from control animals (B2, B5, B6, B12, B13, and B14), five samples of alopecic skin (B3, B7, B9, B10, B11), and four samples of normal skin from affected dogs (B3, B9, B10, B11). Factors for exclusion included an endocrine imbalance in one of the alopecic dogs and pronounced inflammation in the biopsy of one control dog, three biopsies of normal skin of alopecic dogs, and one biopsy of alopecic skin of an affected dog. The list of individuals is stated in Table S4.

2.2.8. RNA Extraction and RNA-Seq Experiments

RNA extraction and cDNA sequencing experiments were conducted according to the protocol outlined previously in [38,113]. All 11 samples were of high quality with a RIN > 9. After sequencing, the Illumina BCL output files with base calls and qualities were converted to FASTQ file formats and demultiplexed.
All reads that passed quality control were mapped to the canine reference genome (CanFam3.1) by STAR aligner version 2.5.3.a, as described in [38,113]. The alignment of RNA-seq reads from each sample was summarized by the number of splice arrangements per sample. The read abundance was calculated using the count software HTseq [114] and an NCBI annotated GTF (release 103) file.

2.2.9. Differential Expression Analysis

The R DESeq2 package [115] was used for differential expression analysis as described in [38]. For each gene, normalized read counts were fit to a generalized linear model (GLM) with the design formula where the condition was the factor of interest in two states: control and affected. Transcripts were considered to be differentially expressed with a Benjamini and Hochberg false discovery rate (FDR) of <0.01. The differentially expressed genes (DEGs) were mapped to biological networks using open-source, open access, and manually curated pathway database called Reactome [https://reactome.org/ version 71; accessed on 12 January 2021]. Separate lists of upregulated and downregulated genes were uploaded separately into the database and were analyzed and matched with known biological processes and pathways.

2.2.10. Protein-Protein Interaction Analysis

We searched for potential functional associations among our GWAS and differentially expressed candidate genes using the STRING database [116], following the approach described in the study of Bohutínská et al. [117]. We were able to retrieve predicted protein-protein interactions for 132 out of 144 GWAS candidates and 144 out of 236 strongly DEGs (exceeding the Log2FC value of +/−2). We used the 'multiple proteins' search in *Canis lupus*, with text mining, experiments, databases, co-expression, neighborhood, gene fusion, and co-occurrence as information sources. We used minimum confidence of 0.4 and retained only 1st shell associations (proteins that are directly associated with the candidate protein: i.e., immediately neighboring network circles).

2.3. Methodology – Additional analyzes of health in CF (paper in prep.)

The results of these analyses have not been published yet. We have focused more broadly on the health status of the CF, its longevity, and the most common cause of death (COD). It is believed, that aside from the aRFA, CF is a healthy breed that does not suffer from genetic diseases. It is also the first study that focuses on the lifespan of this breed.

2.3.1. Genotyping data

We have been collecting genotypes of CFs since 2016. As of April 2022, we have been able to collect genotypes from 276 individuals and additional results for DM testing from 4 individuals. These results allowed us to screen for 154-210 genetic diseases in the tested population. The Embark array is being updated each year, thus, the earlier genotyped individuals have been tested for fewer genetic traits and diseases. In 2016 were the individuals (n = 47) tested using 214 582 markers for 154 genetic diseases and 27 phenotypic traits. In 2018-2019 were the individuals (n = 169) tested using 239 490 markers. Samples from 2020 and 2021 (n = 60) obtained 253,330 markers; tested were for 210 genetic diseases and 35 phenotypic traits.

We have examined the mean COI from the genomic data for the whole CF population, as well as the representation of maternal and paternal haplotypes in the population, and the occurrence of genetic diseases.

2.3.2. Surveys of longevity and cause of death

We have created a simple survey using Google Forms and distributed it among breeders and owners through social media, club magazine, and club websites during 2020-2022. In total, we collected 318 surveys from the Czech Republic (n=136) and from abroad (n=182). Abroad was represented by 10 countries – the United States (US; n=163), the Netherlands (NL; n=7), New Zealand (NZ; n=5), Finland (FIN; n=1), France (FR; n=1), Germany (GE; n=1), Slovakia (SK; n=1), Belgium (BEL; n=1), Poland (PL; n=1), and South Africa (SA; n=1). Most records from abroad have been collected from the Cesky Fousek Database (n = 135), however, these records were sometimes incomplete and, in some cases, did not show the COD, the type of housing, and/or environment. Three individuals from abroad have been stolen or lost and they are presumed dead. These animals have been excluded from the longevity calculation. CF is a hunting breed and due to this fact, there is a higher possibility of injury and/or early death. The COD for these animals has been marked as an “accident”. The environment where each dog lives was divided into two categories – rural, and suburban/urban. Housing was divided into three categories – inside the house, outside, and combination. COD was divided into five main categories – cancer; other health problem; accident (injury, poisoning); natural death; euthanasia due to old-age complications. The category of “other health problem”

was further divided into 15 subcategories – kidney/liver failure; movement issues; heart attack or other heart problem; stroke; swallowing a foreign object; infection; spine damage/spondylosis; seizure (diabetic or other); digestive problems; collapse; breathing issues; post-operation complications; cysts; mental issues; and an unspecified illness.

2.3.3. Statistics

The statistical analyses of the surveys have been done in Statistica 12 [118]. We have examined basic descriptive parameters of the CF population – mean, standard error of the mean, standard deviation, min, max, median, Q1, Q3, and coefficient of variation.

We also examined the possible dependence of age on housing, environment, COD, sex, and origin. Similarly, for Fx, we evaluated a possible dependence on COD, sex, and origin. We examined the correlation between age and Fx using the Spearman rank correlation coefficient and the Pearson correlation coefficient.

Using main effect ANOVA, we evaluated the difference between age and three effects – housing, environment, and COD. The assumption for ANOVA has been tested (normality of data and variance homogeneity).

The Fx calculated for 10 generations was taken from the CF database [ceskyfousekpedigrees.org/]. Origin was divided into two categories, Czech and abroad, according to the place where the dog lives/lived. For example, individuals exported from the Czech Republic to a different country were assigned to that country.

3. Results

3.1. Results – Study 1: Genetic variability of a small canine population (Appendix 1)

Each individual had maximally 20% of missing data (S1 Table). All genotypes can be found in Table S1. The highest number of alleles per locus (Na) was found in DD (Na = 6.222; Table 1). The highest Ar value was found in GSP (Ar = 5.304) and the lowest in BWPGCA (Ar = 4.723), with CF showing an intermediate value of Ar = 5.245 (Table 1). The H_O ranged between H_O = 0.669 (in CF; Table 1) and H_O = 0.639 (in BWPGCA).

Table 1. Descriptive genetic parameters for all studied breeds. CF = Cesky Fousek; DD = Deutsch Drahthaar; GWP = German Wirehaired Pointer; BWPGCA = individuals of Bohemian Wirehaired Pointing Griffon Club of America; WPG = Wirehaired Pointing Griffon; GSP = German Shorthaired Pointer; SE = standard error; n = number of individuals; Na = average number of alleles per locus; Ar = allelic richness; H_E = expected heterozygosity; H_O = observed heterozygosity; HWE = Hardy-Weinberg test for heterozygote deficiency; Np = number of private alleles; F_{IS} = coefficient of inbreeding; The significant values for heterozygote deficiency test are marked with asterisks: * P<0,05; ** P<0,01 ;** P<0,001; ns P>0.05.

Breed	n	Na	Ar	H_E	H_O	HWE	Np	F_{IS}
CF	193	6.111	5.245	0.673	0.669	*	3	0.005
SE		0.342		0.026	0.027			0.011
DD	87	6.222	5.117	0.676	0.660	***	5	0.022
SE		0.308		0.020	0.020			0.012
GWP	26	5.278	5.038	0.657	0.652	ns	5	0.014
SE		0.321		0.026	0.038			0.038
BWPGCA	38	4.889	4.723	0.639	0.639	ns	4	0.002
SE		0.241		0.028	0.033			0.028
WPG	20	5.222	5.142	0.683	0.644	*	6	0.061
SE		0.222		0.029	0.039			0.035
GSP	41	5.889	5.304	0.650	0.653	ns	4	-0.004
SE		0.322		0.040	0.043			0.022

The highest value of F_{IS} was found in the WPG breed (F_{IS} = 0.061; Table 1). The lowest value of F_{IS} was found in the GSP breed (F_{IS} = -0.004; Table 1). In CF, F_{IS} = 0.005. Values of the Hardy-Weinberg heterozygote deficiency test show that there is a significant lack of heterozygotes in DD. Values of F_{ST} calculated for each pair of populations are stated in Table 2. The values indicate that the breed of CF is less differentiated from BWPGCA (F_{ST} = 0.030) than from DD and GWP (F_{ST} = 0.086/0.077). The highest differentiation was found between the breed of GSP and BWPGCA (F_{ST} = 0.144).

Table 2. Pairwise differentiation index (F_{ST}) for all pairs of studied populations. CF = Cesky Fousek; DD = Deutsch Drahthaar; GWP = German Wirehaired Pointer; BWPGCA = individuals of Bohemian Wirehaired Pointing Griffon Club of America; WPG = Wirehaired Pointing Griffon; GSP = German Shorthaired Pointer.

Breed	CF	BWPGCA	WPG	DD	GWP
BWPGCA	0.030				
WPG	0.118	0.135			
DD	0.086	0.119	0.116		
GWP	0.077	0.110	0.124	0.036	
GSP	0.114	0.144	0.117	0.091	0.115

The genealogical tree shown in Figure 4 proposed three differentiated groups; one containing CF, and BWPGCA, where most BWPGCA are the inner lineage of CF. A second group contained WPG and GSP, where some GWPs are the inner lineage of GSP, but mainly from their own cluster. The last group consisted of DD and GWP (Figure 4).

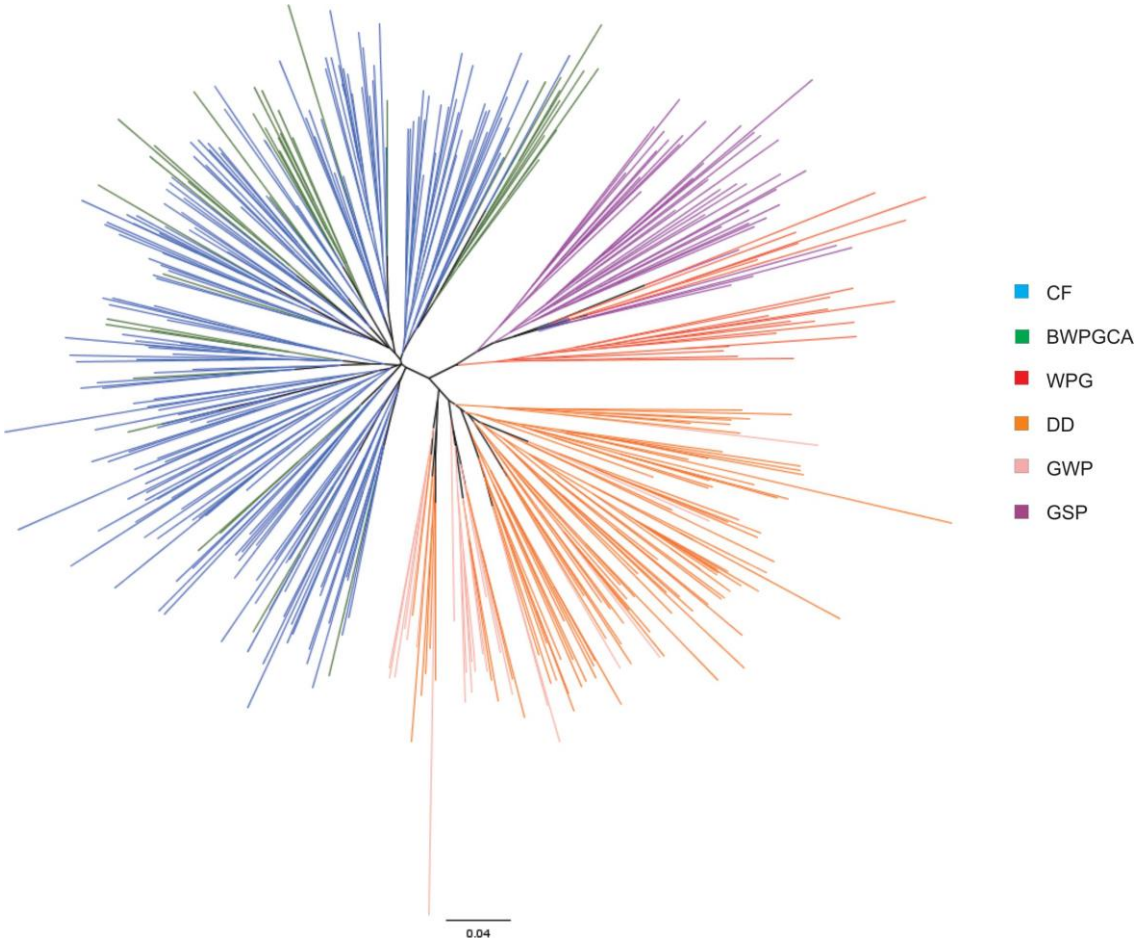


Figure 4. Genealogical tree of individuals based on a matrix of minimum genetic distances according to Nei (1972). Blue–Cesky Fousek, dark green–individuals of Bohemian Wirehaired Pointing Griffon Club of America, red–Wirehaired Pointing Griffon, orange–Deutsch Drahthaar, pink–German Wirehaired Pointer, purple–German Shorthaired Pointer.

All three groups are also differentiated by FCA, with a particular overlap of clusters (Figure 5). Although it seems that WPG and GSP breeds cluster together, from a different perspective we can see that they are well-differentiated (Figure S3).

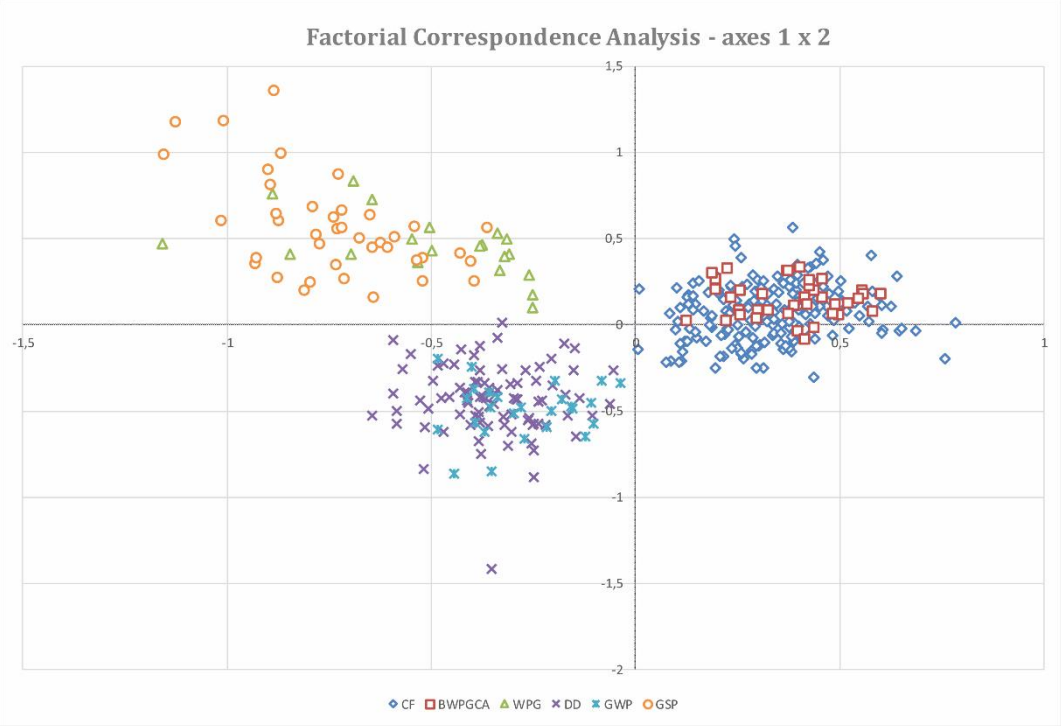


Figure 5. Genetic distances between individuals and populations, based on Factorial Correspondence analysis of 18 microsatellite loci performed in GENETIX software. CF—Cesky Fousek, DD—Deutsch Drahthaar, GWP–German Wirehaired Pointer, BWPGCA—individuals of Bohemian Wirehaired Pointing Griffon Club of America, WPG—Wirehaired Pointing Griffon, GSP—German Shorthaired Pointer.

A higher resolution was achieved using Bayesian clustering analysis in Structure. Using the method of Puechmaille [33], the highest support was obtained for K = 6 (Figure S2) where the mean membership coefficient for each cluster differentiated all breeds. Considering each individual separately, some degree of shared ancestry between CF and BWPGCA and between DD and GWP is visible (Figure 6). On the other hand, the method of Evanno [32] supported K = 2 as the best number of clusters, where the first group consisted of CF and BWPGCA and the second group consisted of the remainder of the studied breeds (Figure S2). The first group represented approximately half of the dataset which might bias the analysis.

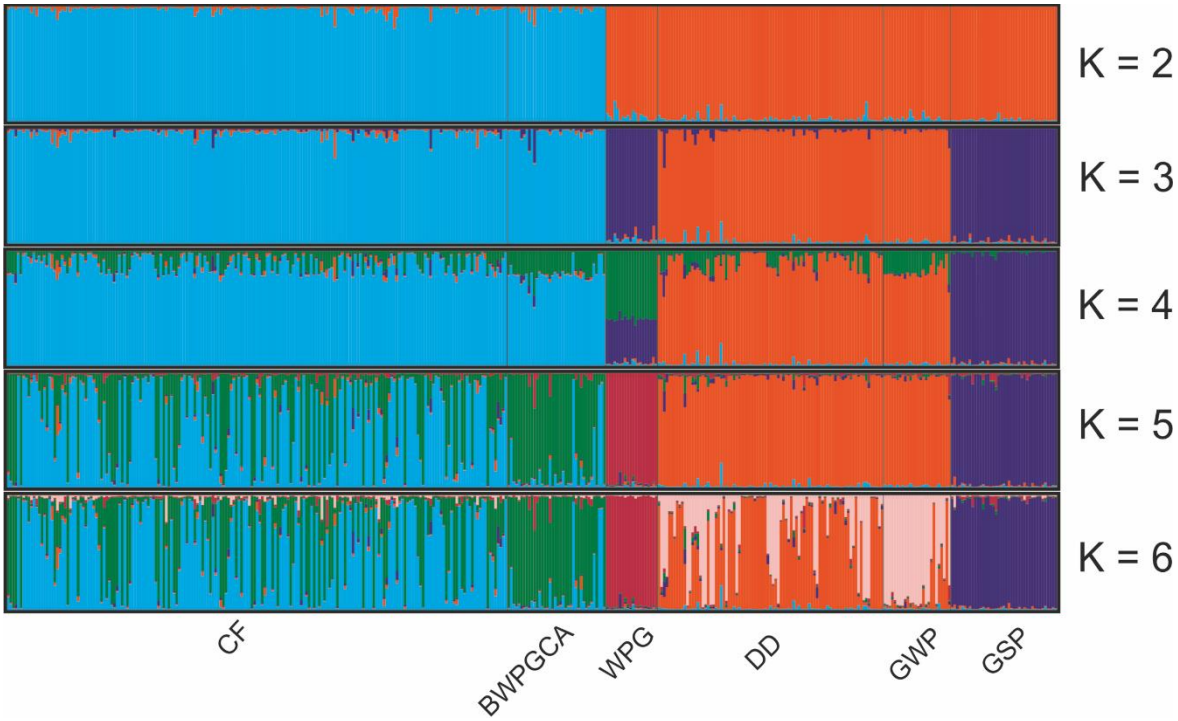


Figure 6. Bayesian clustering analysis of six studied breeds based on 18 microsatellite loci. CF—Cesky Fousek, DD—Deutsch Drahthaar, GWP–German Wirehaired Pointer, BWPGCA—individuals of Bohemian Wirehaired Pointing Griffon Club of America, WPG—Wirehaired Pointing Griffon, GSP—German Shorthaired Pointer.

3.2. Results – Study 2: Causative factors of a complex genetic disease (Appendix 2)

3.2.1. Population Genetic Structure of aRFA Affected and Control Individuals

We first inquired whether aRFA individuals appear evenly distributed among populations. Even though a certain level of genetic differentiation between the Czech and the North American populations exists [47], the case and control samples were evenly distributed across the whole dataset (Figure S4a); thus, we decided to use all samples for the GWAS analyses. We also checked for a possible batch effect since the samples were genotyped in different years and the genotyping array had been updated. No batch effect was identified, as shown in Figure S4b.

3.2.2. Case/control GWAS

To identify genetic variants associated with aRFA, we performed GWAS, using the presence/absence of aRFA as a predictor of phenotype. Our main within-breed

case/control GWAS analysis revealed a significant association with aRFA on chromosome 19 ($P = 1.08 \times 10^{-6}$) (Table 3; Figure 7). Of the top ten SNPs, six are located on chromosome 8 and five of these are within the region 43,341,000–43,490,000 bp. Genotypes and their frequencies for the top SNPs on chromosomes 19 and 8 are shown in Table 2. The lambda value ($\lambda = 1.01$) shows that the stratification correction worked well. The significance threshold was based on the Bonferroni correction (alpha = 0.1; cut-off = 1.16×10^{-6}). Only the chr19 association can be considered significant, while the other identified variants are considered suggestive.

The distribution of genotypes for the chromosome 19 association shows that 59% of controls are of genotype AA while only 27% of cases are of the same genotype, and nearly 19% of cases are GG compared to only 6% of controls. For the chromosome 8 association, the highest proportion of cases (70%) has the genotype GG compared to only 44% of controls (Table 4).

Table 3. Case/control GWAS results show chromosome (Chr), SNP name, position (bp), allele frequency, and raw *P*-value for the top twenty SNPs. One significant SNP was identified on chromosome 19. The interrupted line represents the Bonferroni cut-off.

Chr	SNP Name	Position (bp)	Allele Freq	*P*-value
19	BICF2G630255452	47,856,573	0.333	1.08×10^{-6}
8	BICF2P465820	43,487,284	0.262	3.10×10^{-5}
8	TIGRP2P114211_rs8542415	434,942,31	0.262	3.10×10^{-5}
8	BICF2S23110497	25,810,719	0.205	3.30×10^{-5}
36	BICF2P1194573	28,584,717	0.271	6.72×10^{-5}
30	BICF2G630401492	26,273,661	0.326	8.07×10^{-5}
8	BICF2P361090	43,341,287	0.233	8.90×10^{-5}
8	BICF2P543725	43,371,261	0.233	8.90×10^{-5}
8	BICF2S23137831	43,418,611	0.233	8.89×10^{-5}
13	BICF2P281837	63,012,417	0.057	9.61×10^{-5}
6	BICF2P742566	35,078,147	0.309	9.86×10^{-5}
8	BICF2P177234	43,520,222	0.235	1.10×10^{-4}
41	BICF2S23546044	18,45,101	0.310	1.11×10^{-4}
8	TIGRP2P114933_rs9187625	46,799,348	0.493	1.24×10^{-4}
31	BICF2P1368177	7,605,782	0.104	1.27×10^{-4}
31	BICF2S2443709	7,615,165	0.104	1.27×10^{-4}
13	BICF2G630745860	61,855,230	0.149	1.28×10^{-4}
14	BICF2G630521203	10,825,554	0.061	1.36×10^{-4}

30	TIGRP2P370921_rs8763952	26,977,673	0.233	1.36×10^{-4}
8	BICF2P1102123	43,411,814	0.255	1.54×10^{-4}

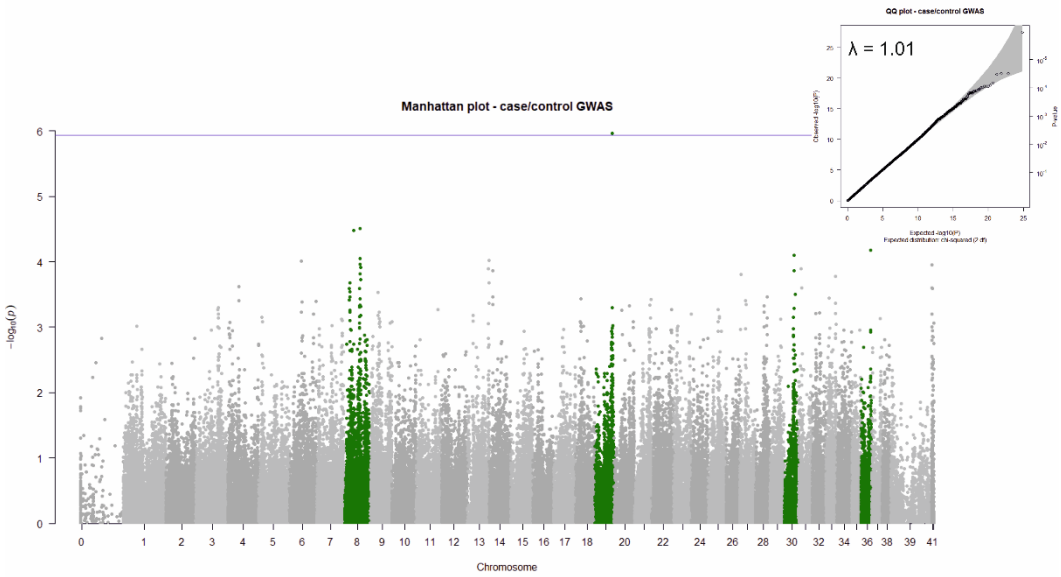


Figure 7. Manhattan and QQ plot for case/control GWAS. The chromosomes of the nine most significant SNPs are shown in green. The significance threshold (shown as a purple line) was set based on Bonferroni correction (cut-off = 1.16×10^{-6}). The lambda value is shown in the QQ plot.

Table 4. Genotypes of the two top SNPs from the case/control GWAS for chromosomes 19 and 8. The highest proportions in each phenotype group are underlined.

Chr (SNP)	Genotype	No. Controls	% Controls	No. Cases	% Cases
chr19 (BICF2G630255452)	AA	69	<u>59.0</u>	26	26.8
	GA	41	35.0	53	<u>54.6</u>
	GG	7	6.0	17	18.6
chr8 (BICF2P465820)	AA	16	13.7	1	1.0
	AG	50	42.7	27	27.8
	GG	51	<u>43.6</u>	68	<u>70.1</u>

3.2.3. Quantitative GWAS and Additional GWAS Analyses

Quantitative GWAS (QGWAS) and additional GWAS analyses were performed to find possible variants associated with the aRFA level of severity represented in our dataset, as well as the age of aRFA onset. A QGWAS analysis of six phenotypic categories showed that seventeen of the twenty top SNPs were on chromosome 8 (Table 5; Figure S5). Seven of the top ten SNPs overlapped with those identified in the

case/control GWAS. Although the significance level of the case/control GWAS was not met, we considered these suggestive associations relevant as well. The significance threshold was based on the Bonferroni cut-off (alpha = 0.05) for all analyses mentioned in this section.

Table 5. Quantitative GWAS results show associations with six phenotypic categories. Chromosome (Chr), SNP name, position (bp), allele frequency, and raw *P*-value for the top twenty SNPs. No variant reached the significance cut-off, and thus these variants are considered suggestive only.

Chr	SNP Name	Position (bp)	Allele Freq	*P*-value
8	BICF2P361090	43,341,287	0.235	4.81×10^{-6}
8	BICF2P543725	43,371,261	0.235	4.81×10^{-6}
8	BICF2S23137831	43,418,611	0.235	4.81×10^{-6}
8	BICF2P465820	43,487,284	0.263	5.56×10^{-6}
8	TIGRP2P114211_rs8542415	43,494,231	0.263	5.56×10^{-6}
8	BICF2S23110497	25,810,719	0.207	5.81×10^{-6}
8	BICF2P177234	43,520,222	0.236	7.36×10^{-6}
19	BICF2G630255452	47,856,573	0.335	9.05×10^{-6}
8	TIGRP2P114933_rs9187625	46,799,348	0.495	1.36×10^{-5}
8	BICF2S23235533	15,314,523	0.251	1.53×10^{-5}
8	BICF2S22921051	15,005,970	0.260	1.56×10^{-5}
8	BICF2P1102123	43,411,814	0.256	1.85×10^{-5}
8	BICF2P1109401	43,462,069	0.256	1.85×10^{-5}
8	BICF2P146090	43,425,554	0.256	1.85×10^{-5}
8	BICF2P396875	43,463,543	0.256	1.85×10^{-5}
8	BICF2P755461	43,441,286	0.256	1.85×10^{-5}
8	BICF2P762487	43,454,904	0.256	1.85×10^{-5}
8	BICF2S22932019	46,809,268	0.493	2.18×10^{-5}
31	BICF2P1368177	7,605,782	0.102	2.58×10^{-5}
31	BICF2S2443709	7,615,165	0.102	2.58×10^{-5}

The genotypes of each phenotypic group for the top SNP (chr8, BICF2P361090) are presented in Table 6. Some groups consist of low sample numbers, and thus we cannot draw any definite conclusions ("head" and "L1"). Groups "healthy" and "L2" show the ratio of individuals with AA and CA genotypes close to 50%, while groups "L3" and "L4" show that most individuals carry the genotype AA. The CC genotype exhibits a comparatively lower frequency in all groups (Table 6).

Table 6. Genotypes for the top SNP (chr8, BICF2P361090) from the QGWAS for each of the six phenotypic categories, with percentages shown in parentheses. The highest proportions in each phenotype group are underlined.

Chr (SNP)	Genotype	Healthy (%)	Head (%)	L1 (%)	L2 (%)	L3 (%)	L4 (%)
chr8 (BICF2P361090)	AA	52 (47)	1 (33)	4 (67)	14 (50)	43 (88)	15 (79)
	CA	46 (41)	2 (67)	1 (17)	13 (46)	5 (10)	4 (21)
	CC	13 (12)	0	1 (17)	0	1 (2)	0
	missing	0	0	0	1 (4)	0	0
total		111	3	6	28	49	19

For the additional GWAS analyses, we found associations with aRFA onset before 2 years of age (Table 7, Figure S6) and level 4 aRFA (Table S5, Figure S7). The top SNP in both analyses was on chromosome 21 (BICF2G630640798) with raw P = 5.01×10^{-7} and P = 1.28×10^{-6}, respectively (Table 7, Figures S6, S7, and Table S5). Moreover, in the analysis of the early onset before 2 years of age (Table 7), the result shows a stronger association (P = 5.01×10^{-7}; Bonferroni cut-off = 5.8×10^{-7}) than the most significant SNP in the case/control GWAS (P = 1.08×10^{-6}; Table 3). The results of the GWAS analyses of individuals older than 6 years and level 2 aRFA showed no significant associations. The average genomic inflation factor for all four additional GWAS analyses was 1.02 (range 1.00–1.05).

Table 7. Additional analysis (age of onset before 2 years of age) GWAS results show chromosome (Chr), SNP name, position (bp), allele frequency, and raw *P*-value for the top twenty SNPs. One significant SNP was identified on chromosome 21. The interrupted line divides the significant association from the rest. The significance threshold based on the Bonferroni correction was set to 5.8×10^{-7}.

Chr	SNP name	Position (bp)	Allele Freq	*P*-value
21	BICF2G630640798	47,085,771	0.221	5.01×10^{-7}
23	BICF2S23432401	11,113,618	0.377	5.75×10^{-6}
37	TIGRP2P420015_rs8709645	20,114,103	0.262	6.48×10^{-6}
8	chr8_59707832	59,707,832	0.221	8.66×10^{-6}
37	BICF2G630131116	25,669,986	0.148	1.07×10^{-5}
15	BICF2G630419811	59,659,531	0.434	1.22×10^{-5}
23	BICF2P438054	11,110,146	0.352	1.30×10^{-5}
21	BICF2G630641744	46,513,869	0.254	1.60×10^{-5}
27	BICF2G630139626	42,94,734	0.205	1.92×10^{-5}
17	chr17_40427743	40,427,743	0.426	2.87×10^{-5}

21	BICF2S23427379	46,584,445	0.270	3.07×10^{-5}
20	BICF2P1328442	55,962,058	0.320	3.49×10^{-5}
27	BICF2P675588	34,378,821	0.295	3.70×10^{-5}
23	BICF2G630386401	13,368,971	0.459	4.20×10^{-5}
18	BICF2G630699395	34,387,737	0.484	4.30×10^{-5}
27	BICF2G630139599	4,253,386	0.180	4.40×10^{-5}
27	BICF2G630139609	4,266,185	0.180	4.40×10^{-5}
27	BICF2G630139630	4,299,688	0.180	4.40×10^{-5}
27	BICF2G630139642	4,318,805	0.180	4.40×10^{-5}
27	BICF2S23028384	4,247,215	0.180	4.40×10^{-5}

3.2.4. Haplotype Identification

To adequately extend the area on chromosomes where the candidate genes could be located, we conducted a haplotype analysis. Based on the results of the case/control GWAS we looked closely at the haplotype distribution on chromosomes 19, 8, 30, and 36. Table 8 shows the results for the most significant haplotypes and the most significant haplotypes containing the most significant SNPs (from the case/control GWAS). On chr19, 4,443 SNPs passed filtering, and the most significant haplotype, consisting of the motif ATGGTCAGGG ($P = 2.09 \times 10^{-11}$), was found in 84% of cases and 54% of controls. A single-base (A or G; $P = 2.03 \times 10^{-6}$) haplotype containing the top chr19 SNP from the case/control GWAS study was found, with the A-haplotype in 55% of cases and 77% of controls, and the G-haplotype in 45% of cases and 24% of controls. On chr8, 6,105 SNPs passed filtering and the most significant haplotype (AAG; $P = 7.31 \times 10^{-8}$) was found in 75% of cases and only 49% of controls. A haplotype containing the suggestive chr8 SNP from the case/control GWAS was found (GGG; $P = 2.25 \times 10^{-7}$) in 85% of cases and 62 % of controls. On chr30, 3,922 SNPs passed filtering and the most significant haplotype (GCGA; $P = 5.04 \times 10^{-6}$) was found in 16% of cases and 36% of controls. A haplotype containing the suggestive chr30 SNP from the case/control GWAS has the motif ATACAGGA ($P = 1.45 \times 10^{-5}$) and was found in 22% of cases and 41% of controls. On chr36, 2,746 SNPs passed filtering and the most significant haplotype (CC; $P = 3.5 \times 10^{-5}$) was found in 37% of cases and 19% of controls. This haplotype also contains the suggestive chr36 SNP from the case/control GWAS.

Table 8. Haplotypes for chromosomes were revealed by the case/control and quantitative GWAS and subsequent haplotype analysis. For each chromosome, we show the most significant haplotype and a haplotype containing the most significant or suggestive SNP for each chromosome (marked by *).

Chr	bp	Haplotype	% Cases	% Controls	*P*-value
8	43,341,287–43,356,221	AAG	75.0	49.4	7.31×10^{-8}
8*	43,463,820-43,494,231	GGG	85.0	62.4	2.25×10^{-7}
19	19,807,697-20,172,164	ATGGTCAGGG	84.4	53.9	2.09×10^{-11}
19*	47,856,573	A	54.7	76.5	2.03×10^{-6}
19*	47,856,573	G	45.3	23.5	2.03×10^{-6}
30	26,126,946-26,143,675	GCGA	15.8	35.5	5.04×10^{-6}
30*	26,245,545-26,328,881	ATACAGGA	21.5	41.3	1.45×10^{-5}
36*	28,573,704-28,584,717	CC	36.7	18.8	3.53×10^{-5}

3.2.5. Candidate Genes Identified by GWAS

Using the results of the different GWAS analyses with subsequently constructed LD plots (Figure S5) and the abovementioned haplotype analyses, we identified 144 potential candidate genes within a 2–4 Mb window using the most significant and several suggestive SNPs on each chromosome (11 genes on chr19, 61 genes on chr8, 60 genes on chr30, and 11 genes on chr36) (Figure S8 and Table S6). Given the nature of aRFA and the available scientific information regarding RFA and other non-inflammatory alopecic disorders in dogs, we focused mainly on genes associated with circadian rhythm and keratin metabolism. We identified eight genes that met these criteria (CSNK2A1, PIF1, RORA, TCF12, FUT8, ZFP36L1, RNF111, SNX22) [www.genecards.org; www.pathcards.genecards.org]. The mRNA expression of four out of the 144 GWAS candidate genes has been previously associated with different HC stages (telogen—GULP1, anagen—PCLAF, PIF1, TLN2) [119]. A spreadsheet summarizing all GWAS candidate genes is shown in Table S6.

3.2.6. Histopathological Phenotyping and Sample Selection

To identify a precise histological phenotype of aRFA, we examined skin biopsies of aRFA-affected and control dogs. The histological phenotype in all biopsies from the control dogs as well as samples of unaffected haired skin from alopecic dogs was histopathologically unremarkable (Figure 8A). HFs were predominantly in anagen, and

the inferior portion of the HFs extended deep into the panniculus. Few follicles were in telogen or kenogen. Sebaceous glands appeared normal, and the epidermis was unremarkable.

All biopsies from the affected skin of alopecic animals displayed similar features previously described in typical RFA cases (Figure 8B–F). Anagen follicles were absent. Infundibuli were moderately to severely dilated, sometimes appeared long, and were filled with abundant orthokeratotic keratin, which was laminar to compact and extended into the openings of the secondary follicles, resulting in a “witch’s feet”-like appearance (Figure 8E). The follicular parts proximal to the infundibula were shortened and limited to the dermis (Figure 8B–E). In some sections, rare telogen (Figure 8C) or kenogen (Figure 8F) follicles could be identified but often only the outer root sheath was visible and a definitive follicular stage could not be assigned (Figure 8B–D). Atrophic follicles were present in some biopsies (Figure 8F). A mild distortion of the HFs could be observed (Figures 8B–F). The sebaceous glands appeared multifocally prominent. The epidermis was mildly hyperplastic and covered by mild to moderate basket-weave, orthokeratotic keratin. Excessive pigmentation was not seen.

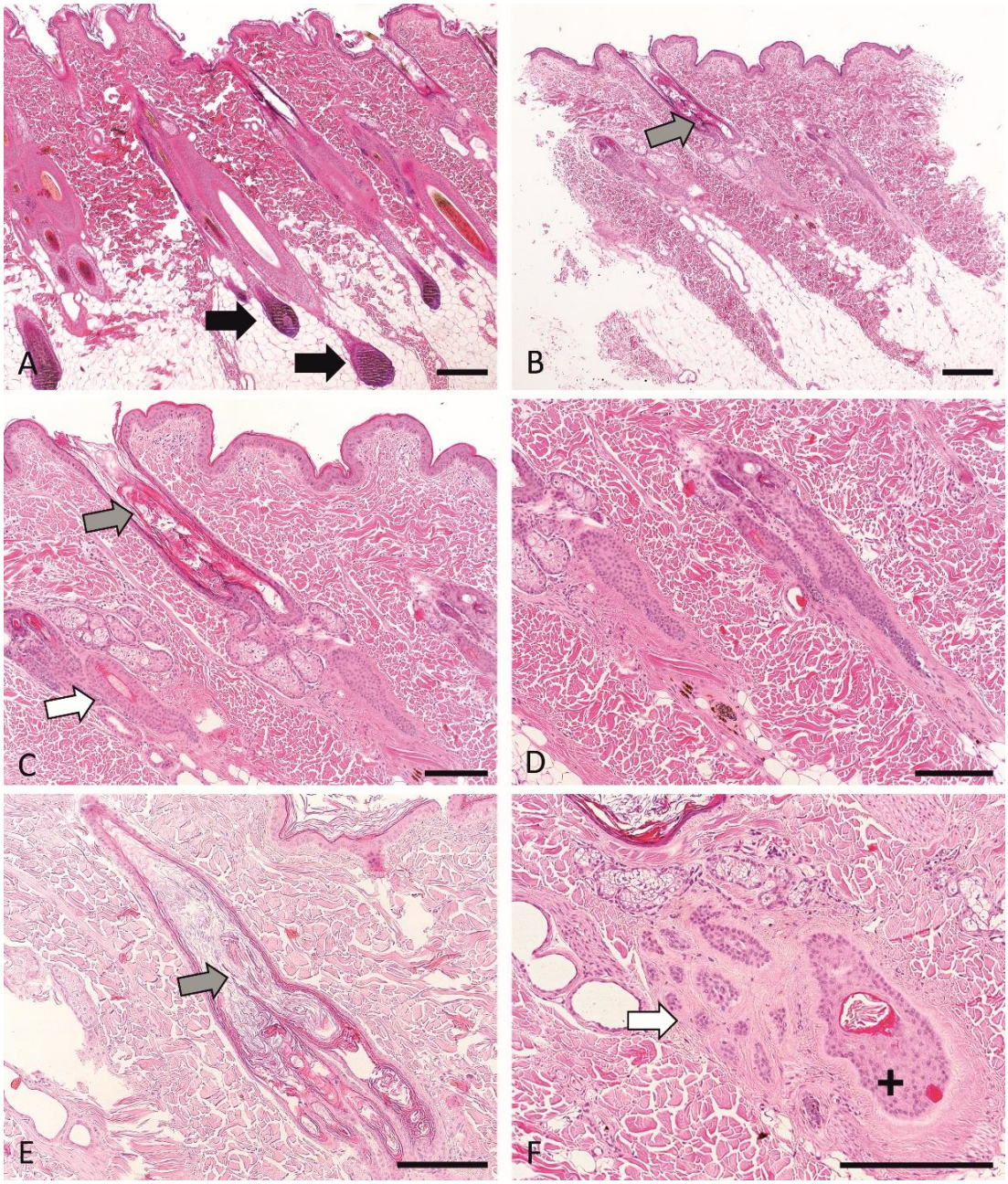


Figure 8. Histological representation of biopsy samples from control skin from unaffected dogs (**A**) and affected dogs **(B–F**). Note numerous anagen hair follicles in A identified by the presence of numerous hair bulbs (black arrows). In aRFA, infundibuli (gray arrows) are moderately to severely dilated (**B**, **C**, **E**) and are filled with abundant keratin, which extends into the openings of the secondary follicles, resulting in a "witch's feet"-like appearance (**E**). The follicular parts proximal to the infundibuli are shortened and limited to the dermis (**B**–E). A few telogen follicles (**C**, white arrow) or kenogen follicles (**F**, white arrow) can be identified. Follicular atrophy may be seen (F, black cross). A mild distortion of the HFs is observed (**B–F**). All samples are stained with hematoxylin and eosin (H&E) and the scale bars represent 200 microns.

3.2.7. RNA Sequencing Analysis

Single-end sequencing of the fifteen RNA libraries produced a mean number of 37 million (M) reads per sample on average (range: 31–43 M). The mean percentage of reads uniquely mapped to the genome was 90.19%, ranging from 87.50–91.59%. Among

those, 80.84% on average mapped to the annotated canine transcriptome (range: 75.65–86.25%), resulting in 26 M counts per sample on average.

To identify genes that were differentially expressed in alopecic and healthy skin, we conducted a transcriptome analysis. A PCA plot was constructed based on gene expression profiles (controls n = 6; normal skin of affected dogs n = 4, alopecic skin of affected dogs n = 5) and demonstrates distinct clustering of samples from control dogs and biopsies of unaffected skin from affected dogs compared to alopecic skin of dogs with aRFA (Figure 9). Based on these clear clustering results, we combined samples from control dogs and healthy skin samples from dogs affected by aRFA (n = 10) and compared those with samples of affected skin from dogs with aRFA (n = 5) for further analysis.

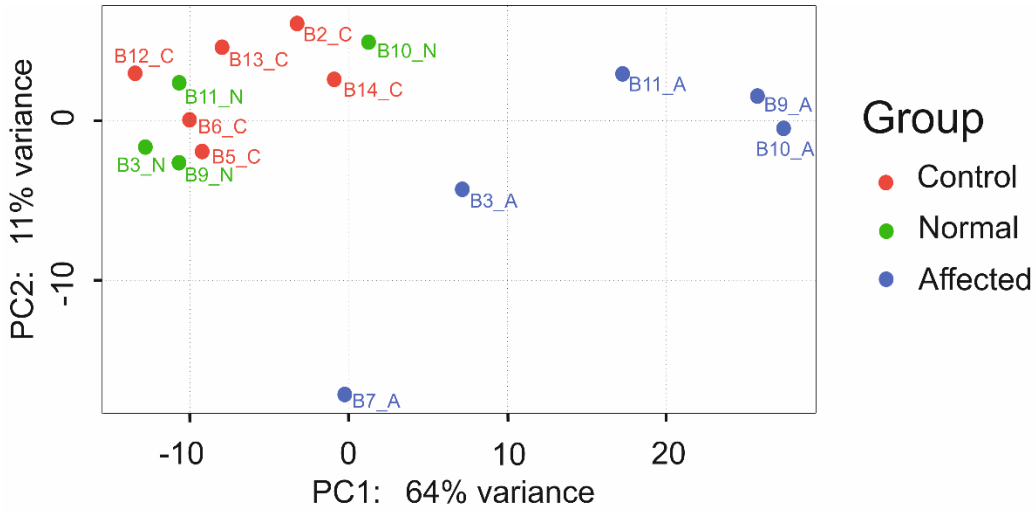


Figure 9. Principal component analysis (PCA) of samples demonstrating clustering based on expression profiles plotted against the two most variable components (PC1 and PC2). Samples from control animals (red) and normal skin from affected animals (green) cluster together, whereas samples from alopecic skin from affected animals (blue) are clearly separated from the clusters representing normal skin but show a higher inter-group variability.

We identified a total of 1435 DEGs with an adjusted P-value of < 0.01. Of these, 669 genes (46.6%) were downregulated, whereas 766 (53.4%) genes were upregulated in alopecic skin samples from affected dogs (Appendix 3). Of all deregulated genes, 135 were strongly upregulated with a log2fold change of at least 2, and 101 genes were strongly downregulated with a log2fold change of at least -2 (Appendix 3). Twenty-five of the DEGs has previously been associated with HF morphogenesis or HC in the literature (Table 9). With the exception of DLX5, LGR6, and NFATC2IP, all of the HC-associated genes were downregulated and most of them were associated with the WNT or SHH (Sonic Hedgehog) pathway. Only four genes could be considered strongly

downregulated, however, on the lower end of the scale (Log2FC ranging between −2.00 and −2.89; Table 9).

Table 9. Differentially expressed genes associated with a role in hair follicle (HF) morphogenesis and the hair cycle (HC) identified by transcriptome analysis comparing unaffected skin of control dogs and dogs affected with aRFA (n = 11) with alopecic skin of dogs with aRFA (n = 5).

Gene Symbol	Full Gene Name	Described Function	Signaling pathway	Log2FC
CTNNB1	catenin beta	promotes HF growth	WNT	−0.498
CUX1	Cutl1, cut like homeobox 1	inhibitor of HF differentiation	NOTCH	−0.895
DLX1	distal-less homeobox 1	HF cycling and differentiation	WNT	−2.120
DLX2	distal-less homeobox 2	HF cycling and differentiation	TGF-b	−1.852
DLX3	distal-less homeobox 3	HF cycling and differentiation	WNT	−1.154
DLX5	distal-less homeobox 5	HF cycling and differentiation	BMP	1.026
FGF5	fibroblast growth factor 5	catagen induction	FGF	−2.896
FOXE1	forkhead box E1	governs HF stem cell (SC) niche	SHH	−1.575
FOXN1	forkhead box N1	HF development, HS differentiation	WNT, BMP, SHH	−1.476
FZD2	frizzled class receptor 2	receptor WNT pathway	WNT	−0.948
FZD3	frizzled class receptor 3	receptor WNT pathway	WNT	−0.978
GLI2	GLI family zinc finger 2	HF SC related transcription factor	SHH	−0.927
HHIP	hedgehog interacting protein	HF organogenesis	SHH	−2.185
HOXC13	homeobox C13	HS differentiation	WNT	−1.734
JAG1	Jagged 1	HF maintenance	Notch	−0.668
LEF1	lymphoid enhancer binding factor 1	HS differentiation	WNT	−1.636
LGR4	leucine rich repeat containing G protein-coupled receptor 4	delays HC; inhibits activation of follicular SCs	WNT	−0.464
LGR5	leucine rich repeat containing G protein-coupled receptor 5	follicular SC marker; anagen initiation	WNT	−1.430

LGR6	leucine rich repeat containing G protein-coupled receptor 6	SC associated marker	WNT	1.145
LHX2	LIM homeobox 2	HF differentiation, SC associated marker	WNT	−1.377
MSX2	Msh homeobox 2	HS differentiation	BMP	−1.454
NCAM1	neural cell adhesion molecule 1	expressed in dermal papilla	FGF	−1.642
NFATC2IP	nuclear factor of activated T cells 2 interacting protein	aging of HF stem cells		0.332
SHH	Sonic hedgehog	HF development and cycling	SHH	−2.002
SMO	Smoothened	HF development and cycling	SHH	−0.858

An analysis of the deregulated genes showed that some (n = 12) were associated with vitamin D and steroid hormone metabolism; however, of these only HSD3B2 could be considered strongly upregulated (Table 10).

Table 10. Differentially expressed genes associated with either vitamin D or steroid hormone metabolism comparing unaffected skin of control dogs and dogs affected with aRFA (n = 11) and alopecic skin of dogs with aRFA (n = 5).

Gene Symbol	Full Gene Name	Function	Log2FC
CYP27B1	cytochrome P450 family 27 subfamily B member 1	activates vitamin D3	−1.650
CYP2R1	cytochrome P450 family 2 subfamily R1	major vitamin D25-hydroxylase	0.980
CYP39A1	Cytochrome P450 Family 39 Subfamily A Member 1	7-alpha hydroxylation of 24-hydroxycholesterol	−0.818
CYP51A1	cytochrome P450 family 51 subfamily A member 1	cholesterol biosynthesis	0.712
DHCR7	7-Dehydrocholesterol reductase	converts 7-dehydrocholesterol (substrate for vitamin D formation cholesterol)	0.719
ESR2	estrogen receptor 2	nuclear receptor, expressed in the HF in outer root sheath, dermal papilla, matrix cells, and in the bulge	−1.212
HSD17B2	17β-Hydroxysteroid dehydrogenase 2	inactivation of estrogens and androgens: converts	1.061

		estradiol to estrone, testosterone to an drostenedione, and androstenediol to DHEA; activates the weak progestogen 20α-hydroxyprogesterone into the potent progestogen progesterone	
HSD17B6	17β-Hydroxysteroid dehydrogenase 6	androgen catabolism: convert 3 alpha-adiol to dihydrotestosterone and androsterone to epi-androsterone.	0.762
HSD17B7	17β-Hydroxysteroid dehydrogenase 7	biosynthesis of estrogen and cholesterol	0.687
HSD3B2	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2	biosynthesis of all classes of hormonal steroids	2.406
RXRG	retinoid X receptor gamma	increases the transcriptional function of VDR	−1.132
VDR	vitamin D receptor	nuclear transcription factor, absence leads to defects in HF regeneration and alopecia	−1.211

3.2.8. Functional Classification of DEGs

The online Reactome pathway analysis revealed that amongst the genes downregulated in the skin biopsies, there is an overrepresentation of pathways involved in the organization and assembly of the extracellular compartment and signal transduction. For the latter specifically, 17 downregulated genes identified were involved in the SHH and WNT signaling pathways (Table 9). Pathway analysis of the upregulated genes identified that 251 genes (33%) were involved in metabolism generally, whereas 135 (18%) of the upregulated genes were specifically related to the metabolism of lipids. Among the most relevant pathways identified for up and downregulated genes, no common pathways were found (Tables S7 and S8, respectively).

3.2.9. Protein-protein Interaction Analysis

To discover functional connections between GWAS candidate genes and RNA-seq deregulated genes, we conducted a protein-protein interaction analysis in STRING. The GWAS candidate genes showed enriched protein-protein interactions. Specifically, they were predicted to be connected in six clusters of more than three proteins each (Figure S9), altogether comprising 96 interaction edges (significant enrichment of

interactions; observed N edges = 96, expected N edges = 20, P-value < 1.0×10^{-16}). We identified very limited overlap between the 144 GWAS candidate genes and the 1435 DEGs in the skin biopsies. Only 11 identical genes (SLC25A3, GTDC1, ARG2, PAPLN, RAD51B, RDH11, HACD3, LACTB, SNX1, TPM1, TRIP4) were identified in the GWAS study and RNA-seq experiment and their overlap were not significant (P-value = 0.61, Fisher's exact test; Table S9). Moreover, no functional connections between the 11 identical genes were found. Due to the lack of overlap between GWAS candidates and DEGs, we speculated that they may be linked at the level of functional processes. This would mean that genetic changes identified in the GWAS analysis resulted in gene expression shifts of the interacting protein partners. Thus, we sought evidence that GWAS candidate genes d with the DEGs, which would identify the molecular processes related to aRFA. We took advantage of protein interaction information from the STRING database, which provides an estimate of proteins' joint contributions to a shared function [116]. For each GWAS candidate, we searched for the presence of STRING interactors with proteins coded by DEGs. To focus only on genes with a likely stronger impact on aRFA, we limited our list of DEGs to those strongly deregulated in the skin biopsies, exceeding the Log2FC value of +/−2. Following this approach, we found that out of the 144 GWAS candidates, 40 were predicted to interact with at least one of the 236 strongly DEGs. In fact, thirteen interacted with more than one strongly DEG (Figure 10).

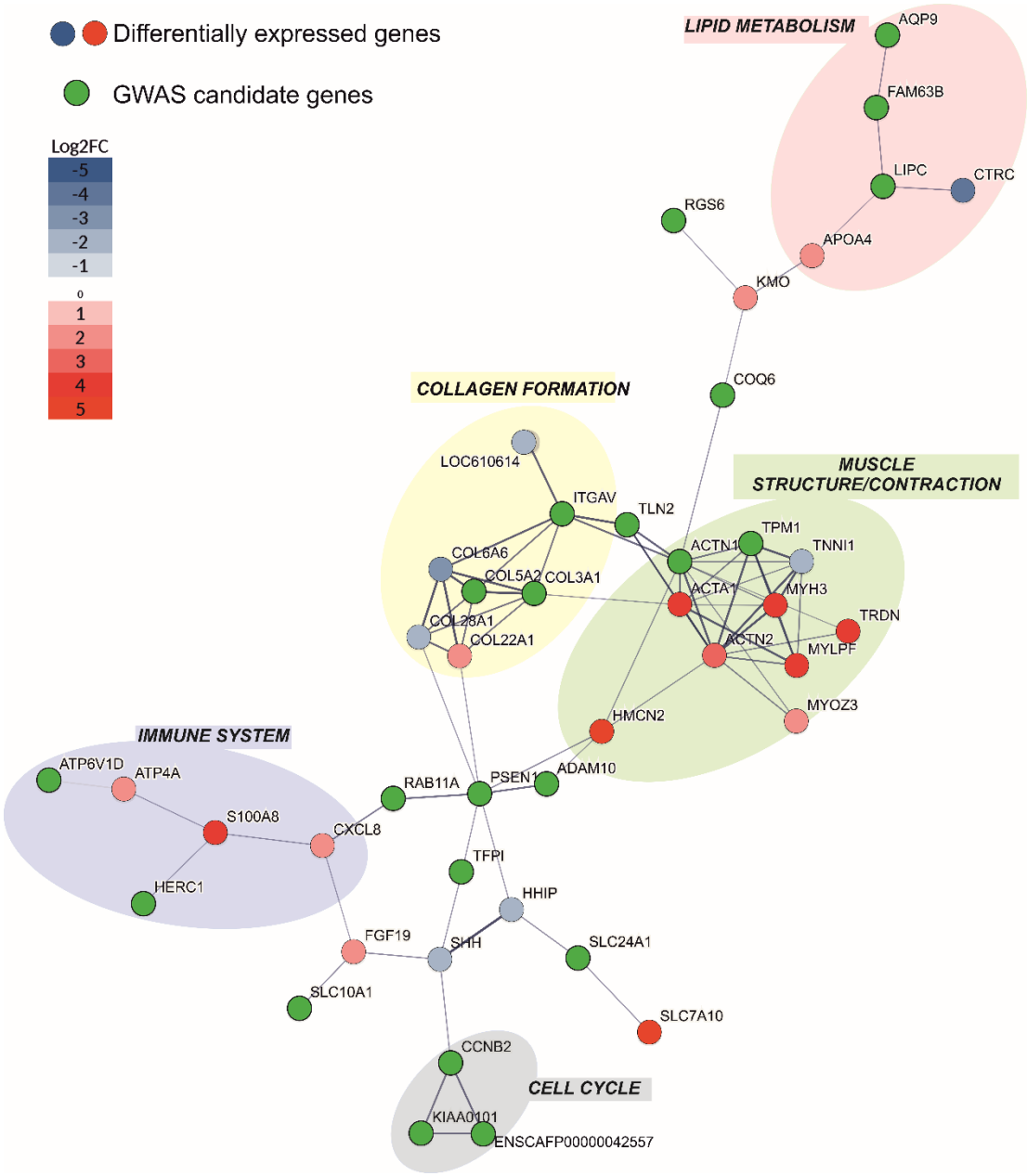


Figure 10. Interactions of GWAS candidate genes (green) and STRING-associated strongly differentially expressed genes colored by their level of expression. We used only medium confidence associations and higher (increasing thickness of lines connecting genes indicates greater confidence). Colorful bubbles represent the metabolic pathways common for each cluster of genes.

Several large STRING clusters were identified in this analysis (Figures 10 and S10). The largest of these clusters were centered on collagen formation, muscle structure/contraction, the immune system, and lipid metabolism. Some of these cohorts were already seen among the most significant functional pathways of deregulated genes (Table 9 and Table 10). Taken together, STRING analysis supports our hypothesis of distinct molecular functions being involved in the pathogenesis of aRFA.

3.3. Results – Additional analyses of health in CF (paper in prep.)

3.3.1. Longevity, cause of death - statistical evaluation

The median longevity of the CF breed identified from all the records was 11.24 years (Table 11). In the Czech Republic was the median longevity 11.43 years including dogs that died of an accident (Table 11). Without these animals was the median longevity 11.76 years. In abroad was the median longevity 11.19 years including animals that died of an accident and stolen/lost animals (Table 11). Without these animals was the median longevity 11.70 years. The mean longevity for the whole population (without "accident" records) was 11.74 years. The oldest dog in the Czech population lived up to 17.23 years, and the oldest dog in the whole CF population lived up to 18.25 years (Table 11).

The main type of housing in abroad was found to be inside the house (n = 39; 84.80 % of answered surveys) compared to the Czech Republic, where the main type of housing is an outside kennel (n = 56; 50.90 % of answered surveys) or combination of the kennel and indoors (n = 32; 29.10 % of answered surveys) (Figure S11). The dogs are held mostly in rural areas in both, the Czech Republic (n = 93; 86.10 % of answered surveys) and abroad (n = 27; 60 % of answered surveys; Figure S11).

The basic descriptive parameters for relationships between age and categories environment, housing, COD, sex, and origin are stated in Table 11.

Table 11. Table showing basic descriptive parameters of the CF population from survey data in connection to age. n – number of observations; % - percentile of observations; Mean – mean age; Median – median age; Min – minimal age; Max – maximal age; SD – standard deviation; SE – standard error of the mean; VC – coefficient of variation; Q1 – 25% quantile; Q3 – 75% quantile; First section – an environment where the dog lives; Second section – housing of the dog; Third section – cause of death; Fourth section – sex; Fifth section – assignment to Czech or abroad population

	n	**%**	**Mean**	**Median**	**Min**	**Max**	**SD**	**SE**	**VC**	**Q1**	**Q3**
Rural	120	78.43	11.25	11.65	0.44	17.23	3.45	0.32	30.69	9.27	13.99
Urban/suburban	33	21.57	11.72	12.53	4.41	16.00	2.85	0.50	24.37	10.47	13.47
Inside	61	39.10	11.84	12.49	2.15	18.25	3.10	0.40	26.22	10.11	13.66
Outside	61	39.10	11.05	11.71	0.44	17.23	3.95	0.51	35.75	9.08	14.17
Combination	34	21.79	11.26	11.48	7.34	16.02	2.51	0.43	22.35	9.13	13.41
Cancer	71	27.31	10.54	10.69	1.00	16.23	2.49	0.29	23.57	9.02	12.28
Other health problem	89	34.23	9.50	9.88	0.44	17.23	3.91	0.41	41.18	7.09	12.70
Accident	36	13.85	6.90	7.28	0.78	15.59	3.72	0.62	54.00	3.93	9.20
Naturally	36	13.85	13.63	14.01	9.70	18.25	1.91	0.32	14.02	12.38	14.88
Euthanasia-age	28	10.77	13.89	14.00	11.44	16.31	1.34	0.25	9.61	12.79	14.98
Male	182	57.23	10.37	10.85	0.78	18.25	3.71	0.28	35.79	8.15	13.29
Female	136	42.77	10.56	11.48	0.44	17.23	3.94	0.34	37.30	7.96	13.77
CZ	136	42.77	10.96	11.43	0.78	17.23	3.41	0.29	31.14	8.76	13.73
Abroad	182	57.23	10.07	11.19	0.44	18.25	4.04	0.30	40.12	7.62	13.10

Using main effect ANOVA, we tested the dependence of age and environment, housing, COD, sex, and origin. The dependence on age was found significant only for COD. The subsequent posthoc testing of COD was done using the Tukey HSD test. It was found that there is not a statistically significant difference between cancer and the "other health problem". Similarly, the difference between natural dying and euthanasia due to old age complications was found insignificant. The rest of the relationships have been found significant. All p-values are shown in Table 12.

Table 12. Post-hoc testing of COD categories. The significant p-values are printed in bold. The average age in each category is stated in brackets.

	cancer (10.9y)	**other health problem (10.7y)**	**accident (7.3y)**	**natural (13.4y)**	**euthanasia-old age (13.9y)**
cancer		0.998	**0.003**	**0.022**	**0.003**
other health problem	0.998		**0.006**	**0.011**	**0.001**
accident	**0.003**	**0.006**		**0.00002**	**0.00002**
natural	**0.022**	**0.011**	**0.00002**		0.980
euthanasia-old age	**0.003**	**0.001**	**0.00002**	0.980	

The basic descriptive parameters for relationships between inbreeding (Fx for 10 generations) and categories COD, sex, and origin are stated in Table 13. Minimal Fx was often found equal to 0.00, these individuals were often in the first generations after an outcross.

Table 13. Table showing basic descriptive parameters of the CF population from survey data in connection to the value of inbreeding (Fx) coefficient calculated for 10 generations. n – number of observations; % - percentile of observations; Mean – mean Fx; Median – median Fx; Min – minimal Fx; Max – maximal Fx; SD – standard deviation; SE – standard error of the mean; VC – coefficient of variation; Q1 – 25% quantile; Q3 – 75% quantile; First section – cause of death; Second section – sex; Third section – assignment to Czech or abroad population

	n	%	Mean	Median	Min	Max	SD	SE	VC	Q1	Q3
Cancer	69	28.16	5.76	5.19	0.00	15.87	3.77	0.45	65.57	2.33	8.20
Other health problem	84	34.29	6.14	5.06	0.00	20.77	4.33	0.47	70.49	2.34	9.81
Accident	33	13.47	7.07	7.01	0.00	20.77	4.40	0.77	62.27	4.74	8.36
Naturally	33	13.47	5.07	4.28	1.07	10.49	2.77	0.48	54.76	2.88	6.75
Euthanasia-age	26	10.61	5.24	3.75	0.00	14.73	4.37	0.86	83.36	2.28	8.36
Male	172	57.14	5.98	5.34	0.00	20.80	4.11	0.31	68.67	2.45	9.06
Female	129	42.86	6.27	5.38	0.00	20.77	4.28	0.38	68.38	2.78	9.48
CZ	124	41.20	4.94	4.48	0.00	15.87	3.02	0.27	61.09	2.52	6.75
Abroad	177	58.80	6.92	6.41	0.00	20.80	4.67	0.35	67.47	2.52	9.81

The dependence on Fx was found significant for origin (p = 0.0004), for COD and sex was found insignificant (p = 0.434 and p = 0.319, respectively). This means that individuals from abroad had a higher average Fx value (Table 13).

Records for COD show that in the Czech Republic (n = 116) the most common cause of death was cancer (n = 35; 30.2 %), "other health problem" (n = 34; 29.3 %), natural cause of death (n = 23; 19.5 %), accident (n = 14; 11.9 %), and euthanasia due to old age complications (n = 10; 8.5 %). In abroad was the most common cause of death from all answered surveys (n = 144) "other health problem" (n = 55; 38.2 %), followed by cancer (n = 36; 25 %), accident (n = 22; 15.3 %), euthanasia due to old age complications (n = 18; 12.5 %), and natural death (n = 13; 9 %). Graphical visualization is shown in Figure S11.

The most common COD from all answered surveys (n = 260) for the whole CF population was "other health problem" (34.23 %) closely followed by cancer (27.31 %), natural COD (13.85 %), accident (13.85 %), and euthanasia due to old age complications (10.77 %) (Table 11). The numbers of animals in each COD category in relation to sex and origin are shown in Figure S12.

When we look closely at the "other health problem" group, we found that in the Czech Republic (n = 34) was represented the most a movement impairment (n = 10), liver/kidney failure (n = 5), heart attack/heart problem (n = 5), infection (n = 5), digestion problem (n = 4), complications after surgery (n = 1), cysts (n = 1), mental problem (n = 1), and unspecified illness (n = 1). In abroad (n = 55) was the "other health problems" group represented by foreign object ingurgitation (n = 7), infection (n =6), movement impairment (n = 6), spinal damage/spondylosis (n = 5), seizures (n = 5), liver/kidney failure (n = 4), heart attack/heart problem (n = 2), breathing complications (n = 2), stroke (n = 1), digestive complications (n = 1), collapse (n = 1), and mental issue (n = 1). Graphical visualization is shown in Figure S13.

We examined the correlation between age and Fx using the Spearman rank correlation coefficient and the Pearson correlation coefficient. The correlation has been found insignificant (Spearman p = 0.096; Pearson p = 0.052). Similarly, the relationship between sex and COD, and between origin and COD has been found insignificant (Pearson chi-square test, p = 0.352 and p = 0.063, respectively).

3.3.2. Health screening, COI, haplotypes

We collected, in total, 276 Embark genotypes from individuals from the Czech Republic (n=191), New Zealand (n=2), and the USA (n=84). Additional 3 European individuals were tested for Degenerative Myelopathy (DM) only, and one additional individual was tested for DM and Hyperuricosuria, Hyperuricemia, and Urolithiasis (HUU), using a separated genetic test. We discovered 17 DM carriers and 5 carriers of HUU in the whole tested population. Eight DM carriers were detected in the Czech population, and nine in the US population. Two HUU carriers have been detected in the Czech population, two in New Zealand, and one in the US population.

The average value of COI is 14.3 % for the whole tested population. Maximal COI reached 25 %, and the lowest COI was 9 %.

In the whole CF population, there were found four maternal haplogroups (A1b; A1d; A1e; B), containing ten maternal haplotypes (A11a; A228; A250; A288; A458; A466; A361/409/611; A18/19/20/21/27/36/94/109; B1/13; B81). Paternal haplogroups were detected only two (A1a; D), containing five paternal haplotypes (H1a.8; H1a.17; H1a.18; H1a.8/32/43/44; H7.1/6/7). Looking at the Czech population only, there are represented all four maternal haplogroups containing eight maternal haplotypes (A228; A250; A288; A466; A361/409/611; A18/19/20/21/27/36/94/109; B1/13; B81).

4. Discussion

We have conducted research of genetic variability on an example of a small population-sized breed – the Cesky Fousek [47]. The values of genetic variability detected by our study (Appendix 1) can be considered sufficient considering the breed management and the number of breeding individuals. The value of F_{IS} for CF is one of the lowest in the study suggesting that line-breeding serves its purpose (Table 1). Even other values of genetic variability parameters were on the level of much larger population-sized breeds (Table S2). Two reasons for this observation could be 1) the recent out-crossing with GSP and DD (both in 2000), and 2) good genetic management of the breed population as a whole. The breed wardens select three potential males from which a female owner can select. Such a strong limitation in mate selection is uncommon in other companion animal breeds. Other factors, such as the occurrence of important traits (including hereditary diseases), play a role in the management decisions. Aside from alopecia, CF has a low prevalence of hereditary diseases in contrast to the situation found in many other breeds with a small population size [120].

The effect of line-breeding was not clearly visible within the present data. We did not test the internal breed structure further because the individual animal membership to a specific line is set according to the pedigrees. This may change according to the population management needs and can be different from the genetic origin. Some individuals are used in more than one line to increase genetic diversity in that additional line.

CF and DD breeds are phenotypically very similar and for a non-skilled person, it is often impossible to discriminate between these breeds. These two breeds were freely mixed until 1924 when the studbook of DD was closed. In the case of CF, the studbook was closed in 1960. Even though the history of CF is complicated and included genetic rescue from the DD and GSP, our study brings clear evidence that recently the genetic pool of CF is well delimited from these German breeds.

This work showed evidence that despite different registration systems for DD and GWP breeds since 1959, both breeds are still close genetically (F_{ST} = 0.036; Figures 4,5 and 6) although the appearance of the individuals can differ markedly (Figure S1). High genetic similarity between DD and GWP is related to a high level of admixture between

the breeds, as DD can be imported to North America and registered as GWP under the AKC or CKC, but the GWP can never be registered as DD (the German breed club is against). Obviously, the selection of different coat colors, which is usually under-laid by a limited number of loci [121], does not outbalance the effects of admixture at the genomic level.

Since CF has been used itself as an outcross in an American WPG population (first time in 1985), we evaluated the genetic variability of these "cross-breed" (BWPGCA) individuals as well. The reason for the initial outcross to CF in the WPG breed was a high level of inbreeding and occurrence of genetic diseases (hip and elbow dysplasia, eye disease, autoimmune thyroiditis) and diminishing hunting abilities [71]. From the results of hunting tests in the US and the personal communication with the members of BWPGCA (nowadays called CFNA), we can conclude that hunting abilities improved significantly. The BWPGCA individuals are more differentiated from the original WPG (F_{ST} = 0.135; Table 2) than from CF (F_{ST} = 0.030; Table 2). The position of the BWPGCA animals is not intermediate between CF and WPG but rather shifted toward CF (Figure 5), reflecting different proportions of particular parental breeds within the founding stock. This is a phenomenon described in other mixed breeds such as Czechoslovakian Wolfdogs [48]. Our results evaluated the level of inbreeding in WPG by a value (F_{IS} = 0.061; Table 1) higher than in a previous study where F_{IS} = - 0.027 [122]. This difference may be the result of different loci used by both studies. The inbreeding coefficient of the BWPGCA individuals lowered (F_{IS} = 0.002; Table 1) compared to the WPG (F_{IS} = 0.061; Table 1) so the CF outcross was a success in this matter as well.

Our results of survey data showed there is a significant difference between inbreeding levels of individuals from the Czech Republic and abroad. The abroad group was mostly represented by dogs from the US club (CFNA, former BWPGCA). This suggests, that the high inbreeding level of the original WPG stock was successfully lowered by the CF outcrosses, however, it is still not as low as in the Czech CF population. We see here a live example of a transition breeding when one breed (WPG) slowly changes into another (CF) due to continuous and numerous outcrosses to the CF breed. Nowadays, all CFNA individuals have more than 80 % of CF ancestry in their pedigrees which corresponds with our findings.

Our second study went deeper into the CF genome. We focused on the main health issue in the breed, alopecia (Appendix 2). The breed has been affected by alopecia for decades, and the situation used to be much worse than today. Sometimes the affection was so severe, that the dog had the coat left only on his head and legs. The owners did not know the condition is a heritable disease, thus, they were breeding also with those animals that showed signs of alopecia [72]. Given the breeding method when distant breeding of relatives is used, the alopecia became highly prevalent in the breed. Later was found that alopecia is a heritable disease and decided that the coat quality would be checked every spring in breeding individuals. KCHCF developed a system of evaluation of the severity of the disease containing five levels (L1-L5) [36]. Ideally, all affected individuals should be excluded from the breeding system. The problem in this case, however, was, that in such a small population could not be used extensive selection otherwise the number of breeding individuals would become too low. Thus, in 1980' the club decided to exclude only individuals affected by alopecia levels L2-L5. L1 individuals with affection on their ears were allowed to stay in the breeding system [72]. The limitations of that time did not allow scientists to study alopecia beyond a statistical evaluation of the number of affected offspring after affected vs. healthy parents [72]. Although the prevalence of the disease lowered compared to the situation about 50 years ago, there are still individuals suffering from alopecia. We have conducted a research of alopecia in CF (Appendix 2) using the GWAS method, based on a genomic comparison of healthy vs. affected individuals [36]. Alopecia (aRFA) in CF is a disease influenced by genetic factors. Pedigree analysis shows that aRFA is more prevalent in some families than in others (an example of such a family is shown in Figure 11). Dostál et al. [72,76] suggest that aRFA in CF is a recessive disease with incomplete penetrance. They conducted a simple statistical analysis of the offspring of parents that were affected, healthy, or a combination of both [72]. It is believed that the incomplete penetrance is dependent on environmental factors, such as housing and nutrition.

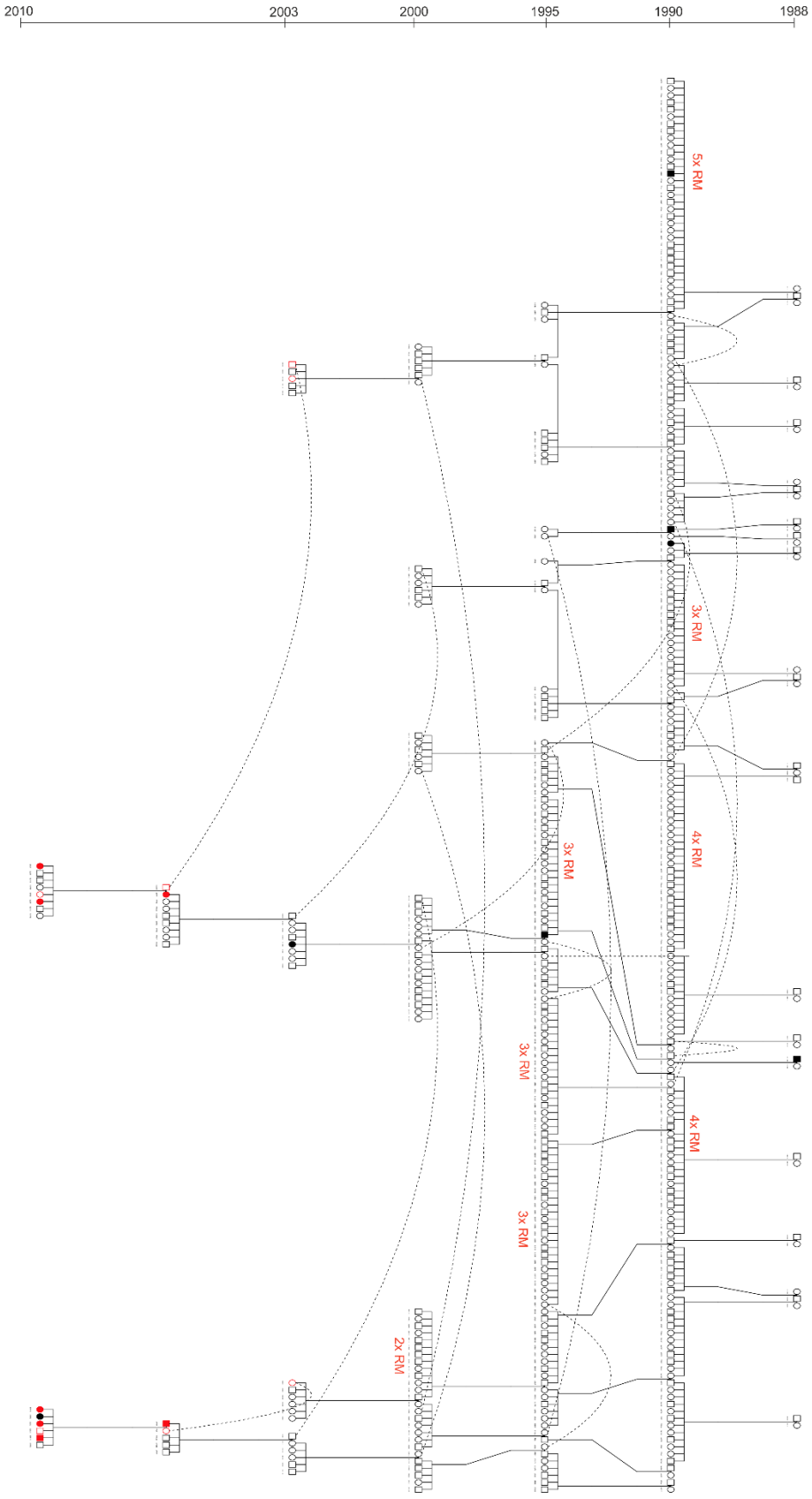


Figure 11. Five-generation pedigree of two aRFA affected litters. Full black squares and circles represent aRFA-affected individuals (square-male, circle-female); red color represents genotyped individuals. In red writing, there are marked several matings of the same parents (repeated mating – RM).

The cumulation of affected individuals in the most recent generation (Figure 11) is similar, for example, to autosomal recessive diseases Trapped Neutrophil Syndrome and Neuronal Ceroid Lipofuscinosis pattern in Border Collies [123]. However, establishing a reliable dominant or recessive heredity pattern of aRFA was not yet possible due to the high probability of missing records of alopecic individuals in earlier generations.

In this study, we identified two significant associations with aRFA in the Cesky Fousek (chr19 and chr21) and other suggestive associations on chromosomes 8, 36, and 30. The suggestive associations were used for the candidate genes' identification because these variants may sometimes help us to get more complete information about the connection of the genes to the phenotype [124]. The significance of the GWAS-identified variants in our study is comparable to other GWAS studies of complex genetic diseases in dogs (e.g., lymphoma, elbow dysplasia, mast cell tumor) [81], suggesting that aRFA likely has a polygenic inheritance. Even though we used a within-breed design, our dataset was rather small for a complex disease GWAS. Follow-up analysis using a larger sample size is needed to confirm these findings. Interestingly, the region identified in our study on chr19 maps to chr2 (144,837,140–147,020,527bp) in the human genome (hg38), and this region has been associated with male pattern baldness [125]. However, the significance of this association was relatively low ($p = 5.65 \times 10^{-10}$; 181 out of 287 associated regions), and thus there is a possibility that the overlap might have happened by chance.

In total, we identified 144 GWAS candidate genes based on significant and suggestive associations on chromosomes 19, 21, 8, 30, and 36. Genotype analysis for the chr19 variant (BICF2G630255452) did not reveal a clear pattern between cases and controls; nevertheless, most of the control individuals were of genotype AA while most of the cases were of genotype GG and GA, suggesting the G allele is associated with higher risk for aRFA. The frequency of genotypes on chr8 (BICF2P361090) identified by QGWAS suggests that the CC genotype is associated with a lower risk of aRFA occurrence. Proportionally, more individuals severely affected by the disease (level 3 aRFA and level 4 aRFA) were of genotype AA, while mildly affected (level 2 aRFA) and healthy individuals were of genotype CA. The genotype could be a contributing factor and, along with possible environmental factors, may influence the severity of the disease.

However, we have no evidence of environmental factors influencing aRFA yet. This matter is still needed to be explored.

There are several limitations to the current GWAS study. One of them is the uncertainty of the development of aRFA in individuals from the control group. aRFA may manifest later in life; therefore, some individuals may later be reclassified. Ideally, we would use only animals aged 10+ years; however, due to the small population size of the breed, this was not possible. We believe that the lack of an age threshold does not strongly affect the results of our study, because the average age of aRFA onset for our affected group was 3.9 years and out of 117 controls only five were slightly under this average age of manifestation. Another possible limitation is that the causative variants might be fixed in the population or at a high frequency; thus, the GWAS method would not detect them similarly to the obsessive-compulsive disorder in Doberman Pinchers [126]. In this case, the identified significant and suggestive associations may only be modifiers of the causative variants. We also need to consider that the Bonferroni cut-off for the case/control GWAS is rather high. There is a discussion about multiple testing corrections and which cut-off threshold to use to find truly significant results [127]. The convention for the Bonferroni cut-off values of very dense arrays of WGS datasets is $p = 5 \times 10^{-8}$. However, the Bonferroni correction is often considered too conservative and the authors either lower the threshold to reduce false-negative results [124,128] or use other means of cut-off setting such as the False Discovery Rate [129,130].

Another possible GWAS limitation might be, that not all SNPs identified by GWAS impact the function or expression of the nearest gene, even when the SNP is within the gene itself [131,132]. An example of such case is gene FTO which had long been identified as obesity-associated in human GWAS studies. Despite the most-associated haplotype appeared to localize to an intron of FTO, the subsequent functional analysis revealed that FTO itself had no direct impact on obesity. Rather, the causal variants modified expression of two other genes, IRX3 and IRX5, that then influenced the state of obesity [133]. Another example in humans may be the strong chr1 low-density lipoprotein cholesterol (LDL-C) association near genes CELSR2 and PSRC1. This association is actually due to a transcription factor binding site for SORT1 gene [134] which is directly connected to the LDL-C Quantitative Trait Locus [www.genecards.org].

In GWAS studies of complex diseases is often problematic identifying specific candidate genes because the associated variants frequently fall in non-coding regions [132]. These variants probably influence the disease by altering gene regulation like in case of bone mineral density in humans [135] but they are not directly causative. It is stated that one third of phenotype-associated SNPs are more than 10 Kb from the nearest gene (LD is typically <10 Kb) and 15% of them are over 100 Kb from the nearest gene. [132]. The candidate genes identified in our GWAS were mostly placed in the inter-gene regions and within 4 Mb from the top SNP. This range is considered suitable for within-breed GWAS studies.

Although there are several concerns about GWAS in general, it is still considered the best method for detecting associations between SNPs and hereditary diseases, which can lead to the identification of possible causative genes and variants [127]. To overcome the limitations of the current study, it is necessary to conduct future research with more individuals. Ideally, several hundred samples of both, affected and healthy individuals, and from multiple dog breeds are needed to validate our findings and pinpoint the specific variants that contribute to aRFA risk. Subsequently the whole-genome sequences would help to uncover specific mutations responsible for the disease.

Transcriptome analysis revealed 1435 deregulated genes and the vast majority of these genes are also present within microdissected anagen and/or telogen hair follicles (HFs) [119]. Anagen is a stage of active growth of the hair cycle (HC), and telogen is the resting phase of the HC (Figure 12). Only 43 (3%) of the deregulated genes in the alopecic skin biopsies of dogs with aRFA have not been identified in microdissected HF, suggesting that these genes are derived from the HF macroenvironment. The HF macroenvironment is gaining more and more attention and it is well known that the cyclical regeneration of the HF is not only controlled by factors derived from the follicular microenvironment but also from the dermal macroenvironment [136–139].

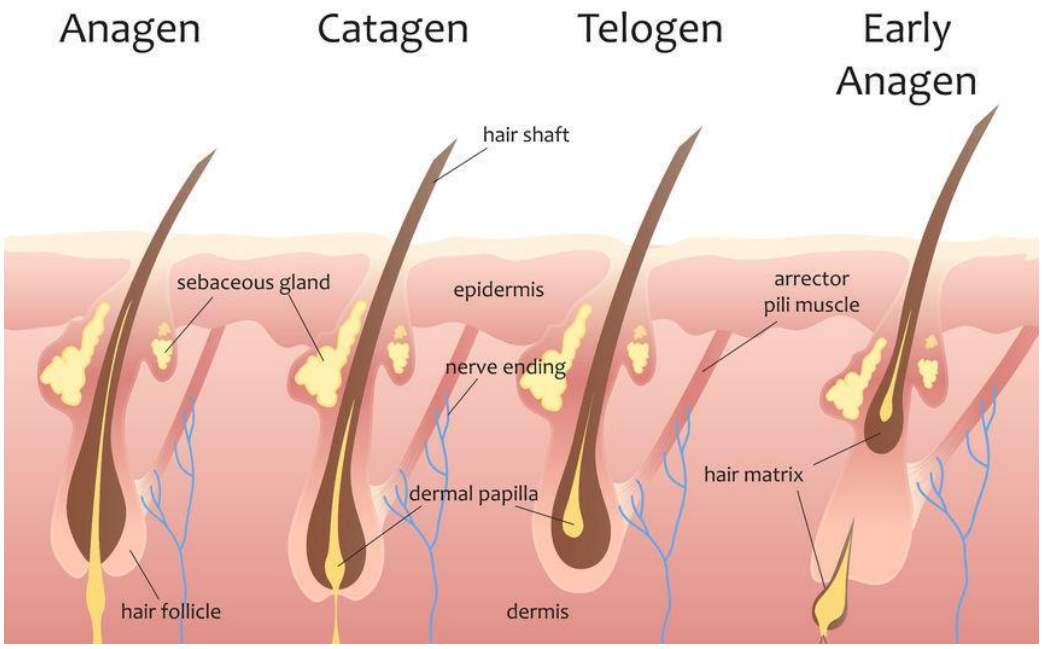


Figure 12. A simple illustration of the hair follicle and its surroundings. Shown are stages of the hair cycle (anagen – active growth, catagen – transition stage, telogen – resting stage), sebaceous glands, dermal papilla, and arrector pili muscles attached to the dermal papilla. The picture was taken from [https://smartgraft.com/hair-growth-cycle/].

Among the deregulated genes in our study, 25 (1.7 %) genes can be identified as being involved in HF development, specific HC stages, follicular stem cells (SCs), or the HC. Most of them are related to the WNT and SHH pathways, which are known to be important for anagen induction, promotion, and differentiation [139–144]. Recently, Alopecia X, another noninflammatory alopecic disorder with a presumed hereditary background, was also connected to altered WNT and SHH pathways [38]. Specifically, in our study, thirteen genes (CTNNB1, CUX1, DLX1, DLX2, DLX3, HOXC13, FOXN1, FZD2, FZD3, LEF1, LHX2, LGR4, and LGR5) encoding transcription factors or signaling molecules of the WNT signaling pathway were all downregulated in affected skin samples. It is of interest, the gene CTNNB1 is part of the same metabolic pathway as gene R-spondin-2 (RSPO2), which has been previously connected to the development of furnishings in wire-haired dogs [145], however, there has not been described any connection yet. Genes associated with the SHH pathway, including FOXE1, GLI1, HHIP, SHH, and SMO, were also downregulated. Among the genes involved in inhibiting anagen induction and in the BMP signaling pathway [144], only DLX5 was upregulated. Furthermore, MMP7, an antagonist of the WNT pathway, was also upregulated, indicating that there is likely an active inhibitory component of the WNT pathway

involved in the HC arrest observed in aRFA [146]. Interestingly, genes associated with the HF stem cell niche, namely GLI2, LGR5, LHX2, and FOXE1 are all downregulated suggesting that impaired SC function is associated with the development of aRFA [144,147,148]. It has been shown that HC is dependent on a fully functional SC compartment [38,149] and an altered SC compartment might be responsible for the extreme short follicles seen in histology. In conclusion, the findings of the RNA-seq experiments are compatible with the results of the histopathological examination showing a lack of anagen HFs and shortened follicles. The exact HC stage reflected by these shortened follicles cannot be assigned morphologically. Eventually, the downregulated genes associated with follicular SCs result in a completely dysfunctional HC that does not allow clear HC stages to be defined. This would also be supported by the histological observation of numerous HFs that have a dystrophic appearance. It is, however, still unclear whether the deregulated genes are the cause or the consequence of the HC arrest.

Twelve identical genes were identified in both the GWAS and the transcriptome analysis. While these concordant findings might imply that they represent the core genes involved in aRFA, we see two reasons that this is not necessarily the case. First, these genes were not predicted to interact, suggesting that they have roles in very different molecular processes. Second, although the 12 genes were classified as differentially expressed based on the expression significance, their actual expression shift was negligible (Log2FC ranging between 0.31 and 1.04), suggesting only a subtle impact of their differential expression on the organism.

In addition to the abovementioned DEGs associated with the WNT, SHH, and BMP signaling pathways 236 DEGs identified in the skin biopsies were predicted to interact with 40 genes of the GWAS study using the STRING database (Figure 5). They were associated with collagen formation, muscle structure/contraction, Lipid metabolism, and immune metabolic pathways (Figure 10). We identified significantly more interactions than expected in the network (enrichment p-value < 1.0×10^{-16}). In Figure 10 (and Figure S10), several regulators of different molecular processes are present, such as DNA repair (PCLAF aka KIAA0101), cell cycle (CCNB2), cellular and intracellular trafficking (SLC10A1, HERC1, RAB11A), cell signaling (ITGAV, RGS6, PSEN1), etc. Some of these regulators might be responsible for the altered function of the interacting DEGs.

Lipids have been implicated in three possible mechanisms for disrupting hair growth [150]. Two of the mechanisms are unlikely in aRFA but one mechanism suggested in this review, namely that an inherently altered lipid metabolism state may be linked to the HC by affecting signaling proteins involved in the SHH or WNT pathways, is a possibility [151]. This assumption is supported by the fact that several genes encoding for molecules involved in these pathways are deregulated in the skin biopsies of dogs with aRFA. If these signaling pathways are impaired, due to, for example, a sterol precursor accumulation, the induction, and promotion of the anagen HC phase are impossible, resulting in alopecia [152]. It was found that obesity had a negative impact on HF SCs and can cause hair thinning [153,154]. In our study, 30 out of 100 affected individuals (30 %) were identified as overweight, while only 10 out of 116 controls were overweight (11.6 %; Table S3). Obesity could be a contributing factor to aRFA in some individuals. Amongst other lipids, cholesterol is of particular importance for the skin. It is crucial for keratinocyte differentiation, has an important barrier function, and is a precursor for steroid hormone synthesis in the skin [155].

Interestingly, in the skin biopsies of dogs with aRFA seven deregulated genes are encoding proteins, mainly enzymes, involved in sex hormone or cholesterol biosynthesis. Cholesterol (7-Dehydrocholesterol) is also a precursor of vitamin D3 (cholecalciferol) under UV radiation [156]. Vitamin D3 is important for the skin. A mutation of Vitamin D receptors has been previously connected to Alopecia Totalis and a knockout of vitamin D receptors in mice stopped the initiation of the new HC [137,155,157–159]. In our study, we identified several downregulated genes associated with the vitamin D metabolism in affected skin samples (Table 10). Vitamin D has been shown to play an essential role in the biosynthesis of estradiol in mice and pigs [160]. It is well known that keratinocytes are the primary source of vitamin D and its active metabolite is processed in the skin, supporting local deregulation of the estrogen metabolism partially mediated by vitamin D [158]. Thus, it might be interesting to explore the role of cholesterol on the HC further to identify new drugs targeting the control of cholesterol in the skin. Interestingly, genes associated with sex hormone metabolism were also downregulated in the skin biopsies of dogs affected by aRFA. The degree to which sex hormone biosynthesis and metabolism, which involves the hormones and enzymes of the complex hypothalamic–pituitary–gonadal axis, is associated with vitamin D metabolism and is involved in the pathogenesis of aRFA remains to be further explored. A disrupted sex hormone metabolic pathway and

deregulated vitamin D metabolism have also been identified in another alopecic disorder with a most likely hereditary cause [38]. Future studies evaluating the nutritional and hormonal status of affected vs. control dogs would be helpful to gain more insights into the role played by these pathways.

The muscle structure/contraction metabolic pathway that was identified as another relevant pathway affecting aRFA might be associated with the arrector pili muscles (APM) (Figure 12). The APM has recently gained attention since it inserts close to the SC region of the HF, has been associated with impaired HF cycling [161] in humans, and is associated with impaired SCs function with age [162]. Conversely, the SCs of the HFs express genes facilitating the formation of tendons and ligaments and establishing a niche for smooth muscle myoblasts that create the APM [163,164]. The overexpression of these genes results in a poor vascular and nerve supply of the SC niche and contributes to SCs quiescence [163]. Our results show mostly a strong upregulation of genes in the muscle structure/contraction pathway. Moreover, genes encoding follicular SCs in the skin biopsies were downregulated further, indicating that in dogs with aRFA this pathogenetic mechanism may also be involved.

Disrupted immune system metabolism might be a contributing factor to aRFA occurrence. We identified genes involved in the immune system cluster (Figure 10) that are involved in glucocorticoid regulation, enzyme and ion cellular transport, and inflammatory response [www.genecards.org, accessed on 19 February 2022]. While the inflammation seen in the skin biopsies of two individuals is most likely caused by an impaired epidermal barrier and does not have a primary genetic cause, an altered glucocorticoid regulation, enzyme function, or ion cellular transport might be associated with the HC.

When we took a different approach and identified genes (out of the 144 GWAS candidates) connected to the preliminarily chosen metabolic pathways that could have a connection with aRFA, we discovered four genes controlling the circadian rhythm (RORA, PIF1, TCF12, CSNK2A1) and four keratin-associated genes (FUT8, ZFP36L1, RNF111, SNX22) (Table S6). The genes controlling the circadian rhythm might be associated with the seasonality of the disease. Besides circadian rhythm metabolism, melatonin metabolism has also been discussed as one of the causative factors of RFA [165]. In the protein-protein analysis of interacting GWAS genes and DEGs shown in

Figure 10, Kyneurine 3-Monoxygenase (KMO) and its paralog Coenzyme Q6 (COQ6) were identified. Interestingly, KMO is part of the nicotinamide adenine dinucleotide (NAD) biosynthesis II (from tryptophan) pathway and the tryptophan utilization super pathway and thus is directly connected to the melatonin degradation pathway [www.pathcards.genecards.com, accessed on 19 February 2022]. Although it has not been shown for dogs with aRFA, more than 50% of dogs with RFA respond well to melatonin treatment [165].

An effect visible in the pedigree above (Figure 11) is the phenomenon of repeated matings (RM) in CF. In the matings around the year 1990, is often seen even five RMs of the same parents. This breeding practice is nowadays banned with a few exceptions. The RM makes the Fx calculation a bit more difficult if done by a software algorithm because even though the two individuals are from different litters, they are in fact full siblings, but they have a different name so the software does not recognize them as such. The father of those five RM litters can be also used as an example of a popular sire overusing. The male sired in a total of 42 litters during his stud dog career (1993-1999) which equals seven litters per year. To reduce this breeding practice, the KCHCF established a limit of allowed breedings to four per male per year.

Our additional health analyses were focused more broadly on the health of the CF breed. Our findings revealed only two genetic diseases in the population – DM and HUU. DM is prevalent in many dog breeds [166,167], it is one of the most common disease variants in the modern dog breeds and the mixed breeds alike [73]. DM is a neurodegenerative disease with suspected similarities to human Amyotrophic Lateral Sclerosis or Lou Gehrig's disease [167,168]. It is a late-onset progressive disorder, the dogs start to show first signs of weakness of their hind legs around 8-10 years of age [168], and there is no cure, although some positive effect of curcumin has been observed [169]. The clinical signs appear in individuals homozygous for one specific mutation in the SOD1 gene. There exist one more mutation, however, it is present only in Bernese Mountain dogs [166]. Previously there have been detected five DM carriers in the CF breed in the US population [120] but there were no available data for the Czech CF population. One individual outside of the studied population in the Czech Republic has been previously diagnosed with DM by a veterinarian (probably recessive homozygote), suggesting his parents must have probably been at least carriers. The pedigree analysis

showed one more probable DM carrier, a female whose son was tested as a DM carrier and partner tested as healthy. This means the female is probably a DM carrier as well. In total, we detected 25 DM (~8.1 %) carriers and one affected individual in the whole CF population. The fact that there was already detected one recessive homozygote might mean that the frequency of the recessive DM alleles increases in the population and it could potentially mean another problem for the breed. However, some studies have shown that even if the individual is homozygous in the SOD1 mutated allele, it does not necessarily mean that it shows signs of the disease [166,170]. An example can be Welsh Corgi Pembroke (WCP) where even homozygous individuals older than 15 years have never developed signs of DM [166]. This fact suggests, that in some breeds might exist a modifying mechanism that influences the affection and age of onset of the disease [170]. Some breeders and breed clubs make a mistake and exclude all homozygous and sometimes even heterozygous animals from the breeding system [171,172]. But when the animals carrying the modifier are excluded, the preventing mechanism might be lost from the breeding system as well. It is unknown how would be the situation in the CF since there has never been any testing and monitoring. Anyway, it is unwise to exclude all animals carrying harmful alleles from the breeding system, especially if the breed is of small population size. In this case would be more effective to test the animals and use all of them in the breeding system, even the recessive homozygotes [166]. The breed wardens or breeders would just need to be careful in the composition of the breeding pairs and avoid breeding carriers together and generating more affected individuals. This way, the genes encoding hunting abilities and other valuable traits, including the potential modifiers, carried by the affected individuals would be allowed to pass to the next generation and the genetic variability would not decrease as significantly. One of the reported causes of death in CF was euthanasia due to movement impairment, usually hind legs movement (~5 % of all records). It is possible, that in this 5% of animals might be hidden additional recessive homozygotes of the SOD1 mutation. However, the average age of death of these animals in our dataset is 13 years, while in other breeds of similar size (Chesapeake Bay Retriever, Boxer) was found the age of death due to DM to be around 10.5 years [168]. This might suggest that either the problem in these animals was not DM but rather a different orthopedic disease, or the disease is not as progressive in CFs. Number of records in this group, however, is low (n = 16) which might have caused a bias in the age of death.

A disease detected for the first time in the CF population was HUU. HUU is a recessive Mendelian disorder caused by a mutation in the SLC2A9 gene and along with DM is one of the most common disease variants in both, mixed breeds and the modern dog breeds [73]. It causes an accumulation of uric acid in the urine and predisposes the affected individual to the formation of bladder and kidney stones [41]. HUU was found to be prevalent in Dalmatians in such a high frequency, that it has became fixed in the population and all individuals carried the HUU deleterious allele [41] until there was used an outcross to English Pointer to introduce the "wild" alleles back to the Dalmatian population [https://www.luadalmatians-world.com/enus/; accessed on 27.6.2022]. The deleterious allele probably came to the CF breed from a common ancestor. Since both Dalmatian and wire-haired pointing dogs are forming same clade [8], it is possible these breeds have interbred in the past, resulting in shared alleles. Nevertheless, the disease has been found in other breeds from different clades as well, suggesting the mutation comes from even further past, predating the creation of modern dog breeds similarly to DM [41]. In the CF were the three HUU identified individuals members of one family, and all were carriers. The club might recommend testing the individuals and monitoring the HUU prevalence, however, the danger for the population is relatively low.

One of the problems in the Czech Republic is that the breeders of CF are often people with trust issues toward genetics. It is hard for them to imagine how the mechanisms work and thus, they are very skeptical about testing their dogs with available genetic tests. This might be a remnant of the recent Czech(oslovakian) history when during the communist regime genetics has been considered a "bourgeois pseudoscience" and thus, the acceptance of genetics among public got delayed by approximately 17 years compared to non-Soviet countries [173–175]. In abroad the thinking is usually different and genetic testing is accepted more easily among breeders. Even in the Czech Republic, however, the situation is improving in this matter. Specifically in the CF, the setting of the breeding system (controlled breeding) does not make the breeders think deeply about their breeding choices. They simply ask the breed warden for males suitable for their female. It is not the breeders' fault, it is merely a consequence of the controlled breeding. In other breeds in the Czech Republic is the situation probably different since their breeding is not controlled or is controlled only moderately.

The number of samples we have tested at Embark array (over 250 individuals) allowed the company to adjust its algorithm of breed assignment for their whole dataset. Even though the outcross is used in some tested CF individuals, it depends on the type of breeding and a chance for how long will be the alleles prevalent in the descendants. The Embark test detected a DD background in one CF female, Percy, which corresponds with the DD outcross in the third generation of her pedigree (85.4 % of CF and 14.6 % of DD detected). Her sister, Poly, was detected with 13.2 % of "unresolved" ancestry. Possibilities of the ancestors according to Embark are DD, Weimaraner, or Brittany. Since her sister was correctly detected with DD, it is very probable, that the admixed ancestry in Poly is also DD with some possible addition of ancient alleles common for more breeds of pointing dogs. If calculated mathematically, the amount of the DD ancestry in these females should be 12.5 % so it is visible the Embark test is very close to this number, yet it differs slightly. However, in other individuals with an outcross in their pedigree the Embark test did not detect the GSP or DD ancestry at all, or vice versa, the Embark test detected a DD ancestry at a high level (20.9 %) in one female where is no record of such outcross in her pedigree. In this case, there might have been an unrecorded outcross used in her recent ancestors. The ancestry assignment using genotyping data is highly dependable on the reference population against which is the individual tested. If the number of animals in the reference population is low, the results might be inaccurate. However, this is not the case of Embark dataset where the number of samples in each breed is extensive. One limitation of the Embark dataset might be the origin of the samples. Since Embark is an American company, it is probable that the animals in the Embark database are mainly from the US. The genetic make-up of these animals might be slightly different from the European dogs, so a small bias might be caused by this fact. But this is not the case in the CF breed where most of the samples are from the Czech Republic or the American CFNA club.

Many breeders are against outcrosses because they feel it makes their breed "impure". As shown in our clustering analysis (Figure 5), though, despite the repeated blending of all wirehaired breeds in the past, the breeds are still well differentiated. The breed assignment through Embark test also shows that not every outcross is detectable in the population after a few generations. This is good news for the "purists" but it also shows, that a one-time outcross might be only a short-term help for the breed. Repeated

outcrosses are more effective against the increasing level of inbreeding in each generation [70].

The breed wardens in the KCHCF are people of knowledge, however, even they sometimes make a mistake. Maximal COI calculated from genomic data in the studied population reached up to 25 % which is equal to the breeding of two siblings. This is a very close level of inbreeding and such litter should have not been allowed to happen. In this case appeared two common ancestors, both in the 2nd (from mother's side) and 4th (from father's side) generation of the pedigree. Line-breeding is a great tool for containing as much genetic variability as possible. But if such matings are allowed, the inbreeding level in the lines rises quickly, and the lines would lose their purpose. Despite some mistakes, the mean COI (14.3 %) and 10 generations Fx (6.1 %) for the whole CF population, are adequate for the CF breeding method and population size. It was hypothesized that a level of inbreeding has a negative impact on the lifespan [30,176]. We have evaluated this hypothesis with the limited survey records and found that there is no influence of the inbreeding level on the age in CF. Although it was found the dogs that died naturally or were euthanized due to old age complications had the lowest mean Fx value (Table 13), the difference was insignificant (Spearman rank correlation coefficient $p = 0.096$; Pearson correlation coefficient $p = 0.052$). Since the Pearson coefficient nearly reached the significance threshold, it is possible, that more records would lead to more significant values. That would suggest that the level of inbreeding does affect the lifespan as previously described in other breeds [55], and it could be one of the signs of inbreeding depression.

One of the most common COD in the CF population was found to be cancer (27.3 %; Table 11). This cause has been found in different breeds of pointing dogs in a high occurrence as well (Weimaraner, Gordon Setter, Pointer) [53]. It was previously stated that with a higher inbreeding rate there is also a higher occurrence of cancer [177] and that larger animals are more susceptible to cancer [30]. CF is a middle-sized (by some authors considered a large) breed growing up to 66 cm in males, 62 cm in females, and the weight up to 35 kg. The cancer prevalence seems to be proportional to the CF body size. However, the cancer occurrence in the Czech CF population could be influenced by hereditary factors, level of inbreeding in the breed but possibly also by the fact, that the dogs are often working in crop fields, that are chemically treated repeatedly throughout

the year. The setting of the dog tests in the Czech Republic requires the dogs to be trained mainly during springtime when a lot of crops is growing and autumn when a new round of crops is being sowed and again need to be chemically treated. The dog then must work in this environment covered up to its shoulders, so for example the mammary glands are always in contact with the crops and chemicals. The mammary gland cancer is often seen in CF, however, there is no data yet on the prevalence of different types of cancer in CF.

In the abroad CF population was often the veterinary diagnosis more precise in case of the subcategories of "other health problem". We believe, the Czech people do not seek the proper diagnosis as thoroughly as breeders in abroad, although in the UK was also found, that only approximately 5 % of owners proceeded to autopsy to find the real COD [53]. The Czech people often euthanize the animal because it, for example, cannot walk anymore, but they do not seek the real COD. Such animals could have had spondylosis, DM, HD, or other, more rare COD, but the real cause remains unknown.

The median longevity of CF was found to be 11.76 in the Czech Republic and 11.7 in abroad (11.74 years for the whole CF population). This is comparable to other breeds of pointing dogs. In the UK was found the median longevity in Weimaraner is 10.3 and 11.1 years [53,178], in Pointer 11.4 and 12.4 years [53,178], in Irish Setter 11.1 and 12 years [53,178], in Gordon Setter 11.9 years [53], in German Wirehaired Pointer 10 years, in German Shorthaired Pointer 12 years, in English Setter 11.6 years, in Hungarian Vizsla 12.9 years [178].

The oldest recorded CF lived up to 18.25 years (Table 11), which is several years longer than the longest living recorded Pointer (16.6 and 16.4 years), Gordon Setter (14.8 and 16.3 years), or Irish Setter (12.91 and 15.17 years) [53,178]. In Weimaraner were recorded individuals below (17 and 15.5 years; [53,179]) and above (18.8 years; [178]) the oldest CF age. In CF, 25 % of dogs were older than 13.5 years (approximately 80 individuals) which shows that the ~18 years-old individual is not an outlier.

Unfortunately, in the Czech Republic does not exist a central database of health and longevity records from veterinarians as it does in other countries. The only option how to screen for health problems and longevity is through surveys directly from breeders. The main limitation in our survey study was the number of records and a possible bias caused by the type of respondents. Many records were taken from the CF database, but these records did not contain information about housing and environment,

sometimes even COD, thus, the results might be altered. Also, the respondents were more probably younger people, who are able to work on a computer. However, despite the low number of records, the results seem to correspond with the reality – most people in the Czech Republic are keeping their dogs in an outside kennel and mostly live in rural areas.

We are going to continue in the collection of available health-related data to obtain a more robust dataset, and thus, more accurate results in the future.

5. Conclusions

In this dissertation thesis, we reviewed the main problems in genetic variability evaluation in small populations of dogs. The loss of genetic variability is a problem in dog breeding in general. All breeds have gone through several bottlenecks during their evolution and development resulting in a strong decrease in genetic variability. This decrease continues even today. The increasing homozygosity of the dog breed populations is connected to a higher occurrence of recessive genetic diseases. Some of these diseases have an equivalent in humans as well, some do not. Either way, studying the mechanisms of genetic diseases in dogs is a valuable tool for discovering new treatments and detection methods directly for human disease equivalents or indirectly in similar human diseases.

Breeds with small populations are very challenging in terms of maintaining genetic variability. There are many factors at play that cause higher homozygosity in the population. However, if the breeding is managed properly by people with the necessary knowledge, it is possible to keep the breed viable with minimal loss of genetic variability. One example of a small population-sized breed, CF, we explored parameters of genetic variability that can be considered sufficient. Aside from the aRFA, CF can be considered a healthy breed despite its breeding method that might bring some genetic diseases to the surface. The only genetic diseases detected by our research were DM and HUU, and only carriers were found. The findings were brought to the attention of the breed club (KCHCF) and action was taken to solve the problem. We do not have enough information to make any radical steps, so we recommend testing breeding animals and monitoring the situation as well as collecting more health-related data in the future.

We have investigated the genetics and the differential gene expressions associated with alopecia (aRFA) in the Cesky Fousek using a unique combination of techniques - genome-wide association study on 216 individuals, RNA-seq experiments from skin biopsies of 11 dogs, and examined the histopathological phenotype of dogs with aRFA. This was the first complex genomic study of canine alopecia in dogs using such an extensive sample size. Histologically, we found that aRFA is similar to RFA and compatible with an impaired HC. The mRNA of genes associated with the initiation and promotion of the HC, as well as of genes encoding for follicular stem cell markers, were

mostly downregulated. These findings explain the lack of anagen follicles in the skin of affected individuals. In total, we identified 144 candidate genes from the GWAS analysis (including both the significant and suggestive associations) and 236 strongly deregulated genes from the RNA-seq analysis. Using the suggestive GWAS candidate genes we discovered four major metabolic pathways associated with aRFA - collagen formation, muscle structure/contraction, lipid metabolism, and the immune system. The findings from our study suggest that aRFA has a complex genetic inheritance that warrants further study.

Given the limitations of the GWAS analyses, further genetic studies involving independent and larger cohorts, including multiple breeds, are needed to validate our findings and pinpoint the specific variants that contribute to aRFA risk.

The median longevity in CF is comparable to other breeds of pointing dogs. One of the most common causes of death was found cancer and “other health problem”, however, more records are needed to verify our results and to re-evaluate a possible influence of the coefficient of inbreeding on the longevity. A central national veterinary database would be very useful to properly evaluate the most common cause of death and longevity in the Czech populations of dog breeds.

We conclude that CF is a suitable model organism for studying genetic variability since there is used an unusual type of breeding (a controlled line-breeding), outcrosses, and precautions in the breeding such as limit of breedings for males or ban of repeated matings. Our results imply the breeding management of the breed helps to maintain as much genetic diversity as possible. Such information can be used in a small population of domesticants as well as semi-captive species of animals.

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Supplementary material

Figures:

Figure S1: *Example of high differentiation in coat color in two breeds of the same historical origin -(a) Deutsch Drahthaar; (b) German Wirehaired Pointer.*

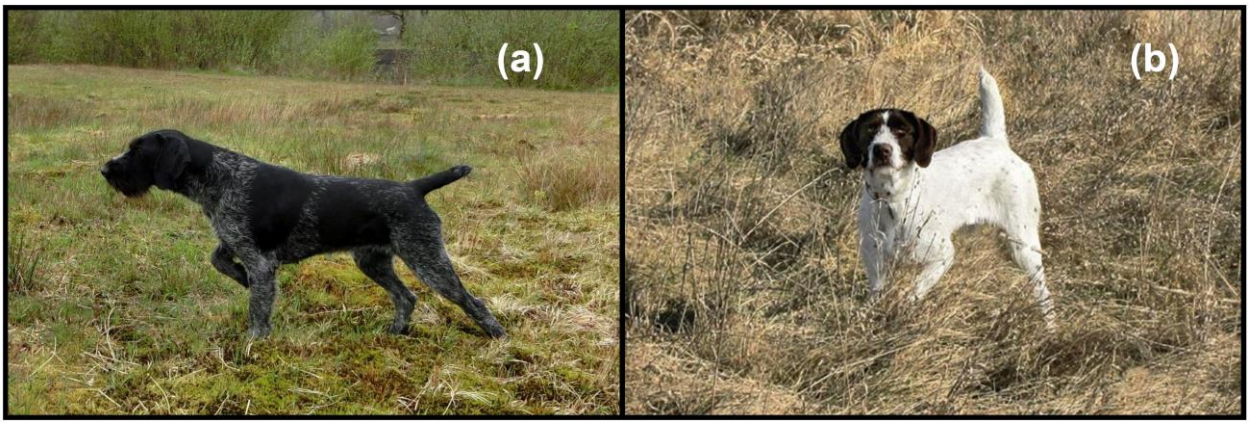
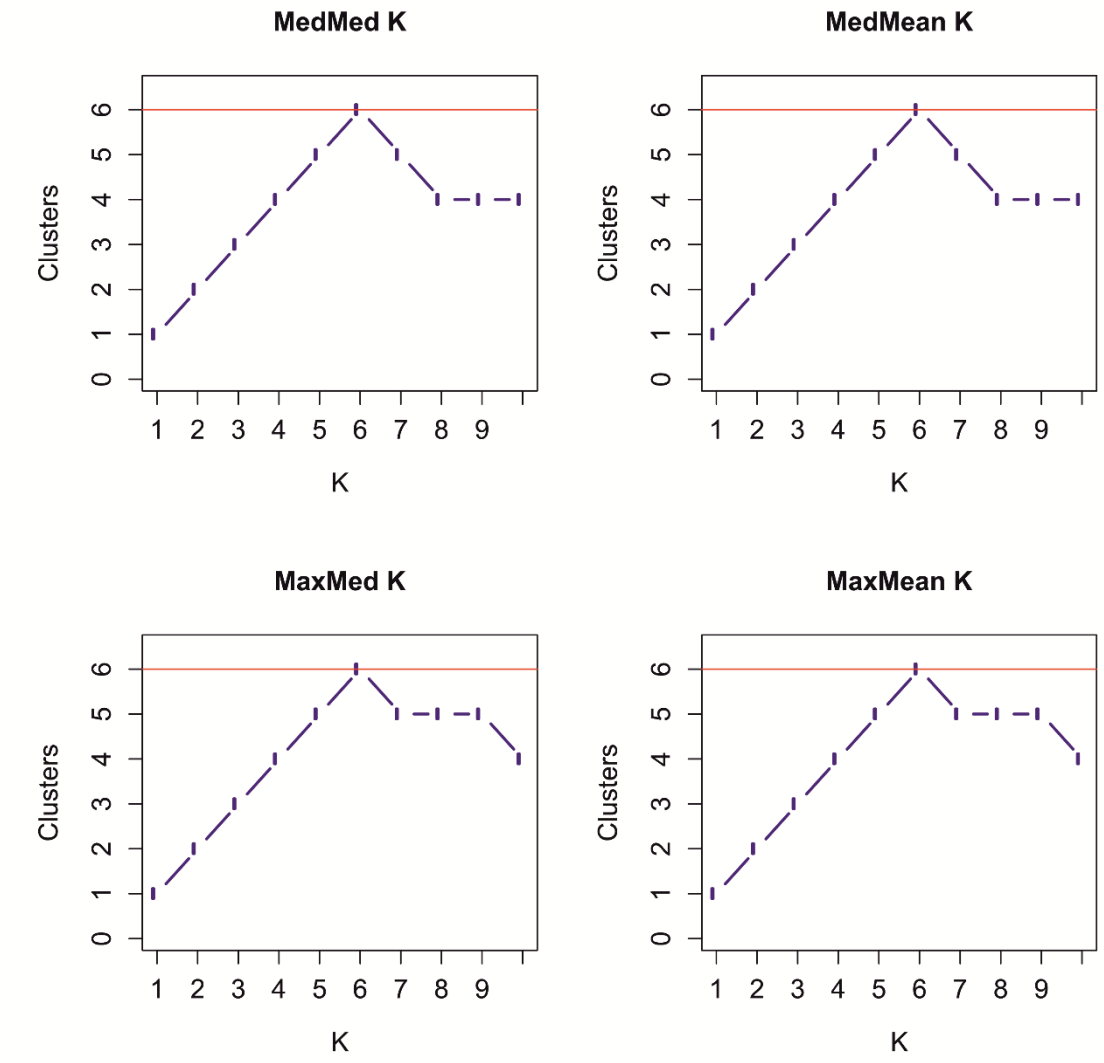


Figure S2: *Output from Structure Selector based on Bayesian clustering in Structure.*



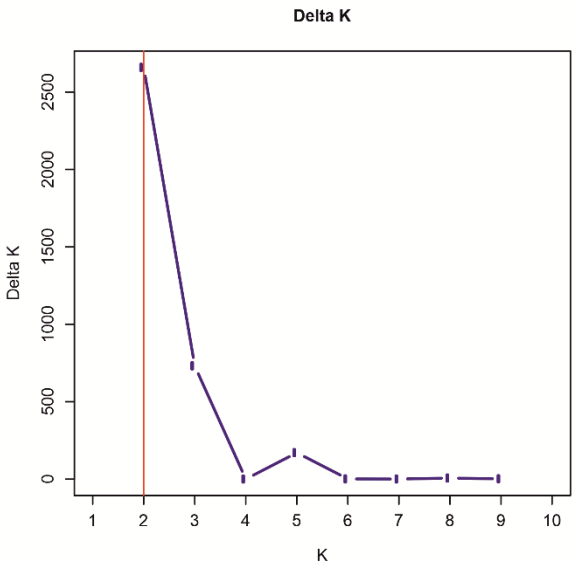


Figure S3: *Studied breeds visualized by Factorial Correspondence Analysis using different axes (1x3) than in Figure 3.*

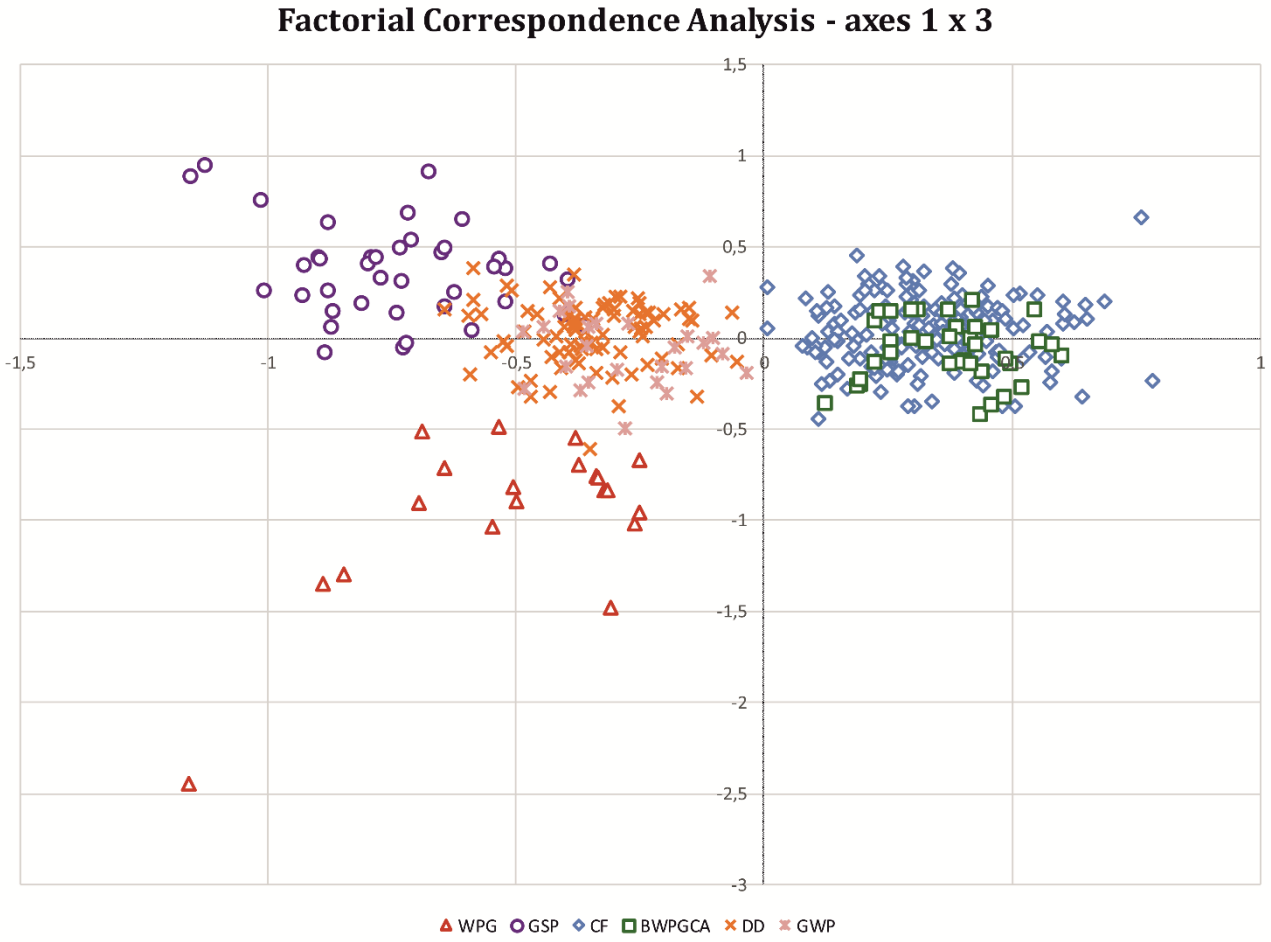
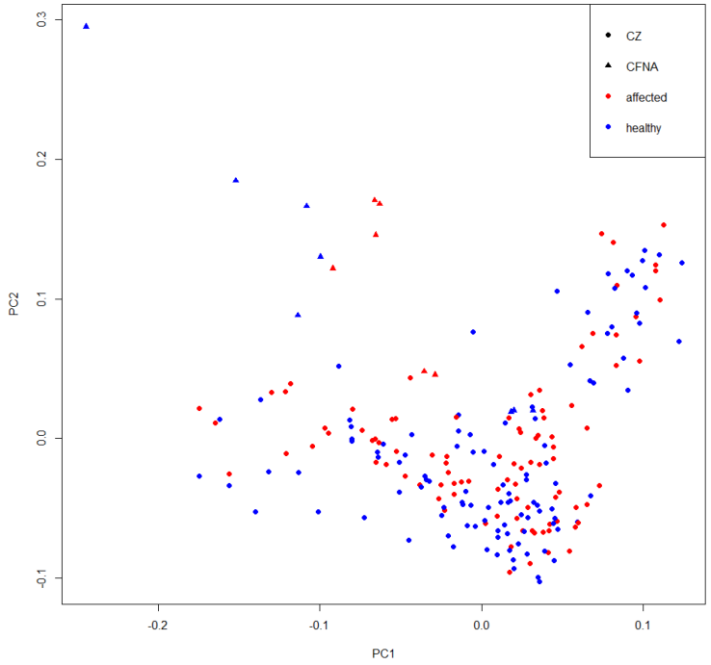


Figure S4: *Two PCA plots of all individuals collected for the study (n = 216). The PCA plot shows genetic distances between individuals. Figure (a) shows relationships between affected (red) and healthy (blue) individuals and their geographic origin (circular point - individuals from the Czech Republic; triangle point - individuals from the US Club CFNA). Note that individuals kept in the US but born in the Czech Republic were placed in the "CZ" group. Figure (b) shows relationships between three plates of samples genotyped in 2016 (plate 1), 2018 (plate 2), and 2019 (plate 3).*

(a)



(b)

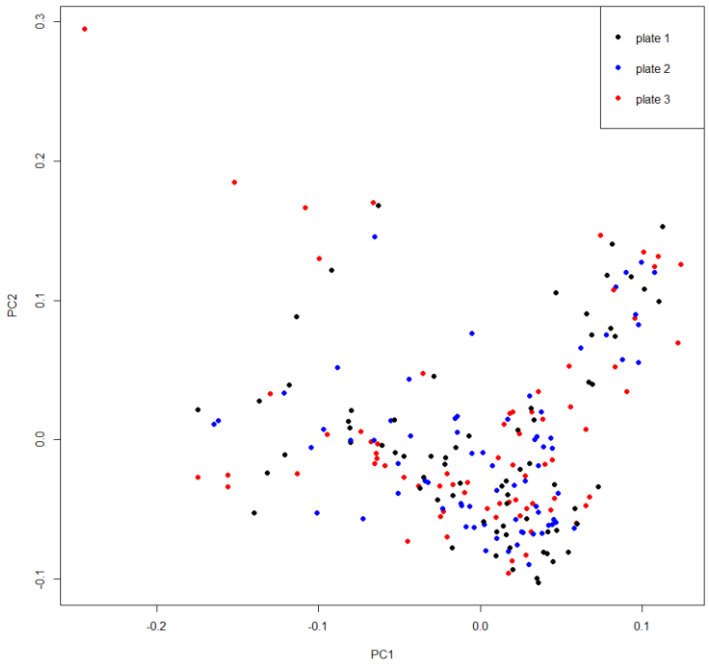


Figure S5: *Manhattan and Quantile-Quantile Plot of quantitative GWAS. The top SNP p-value does not reach the significance threshold (Bonferroni correction = 5.8x10-7; shown as a purple line), however, there were identified many SNPs on chr8 (colored in green) close to each other. We consider these associations suggestive. Lambda value shows that stratification correction worked well.*

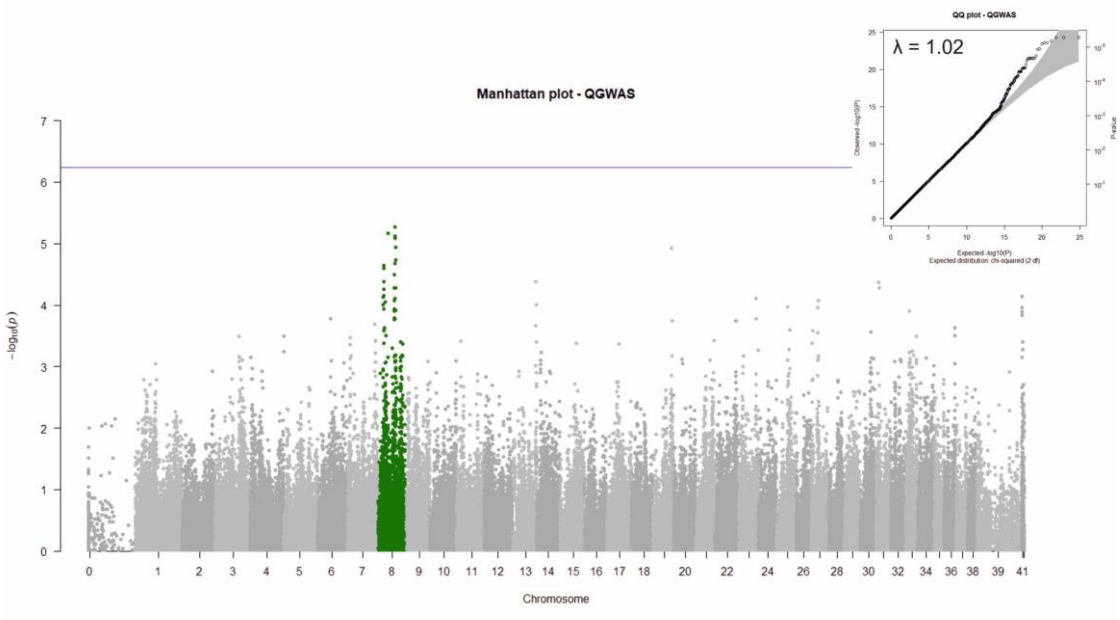


Figure S6: *Manhattan and Quantile-Quantile Plot of GWAS analysis of individuals affected up to two years of age. Identified variants could affect the time and/or severity of the affection. One variant can be considered significant (chr21, BICF2G630640798, raw P-value = 5.01x10^{-7}), it reaches the significance threshold (Bonferroni correction = 5.8x10^{-7}; shown as a purple line), and it lies in a gene ANO3. Lambda value shows that stratification correction worked well.*

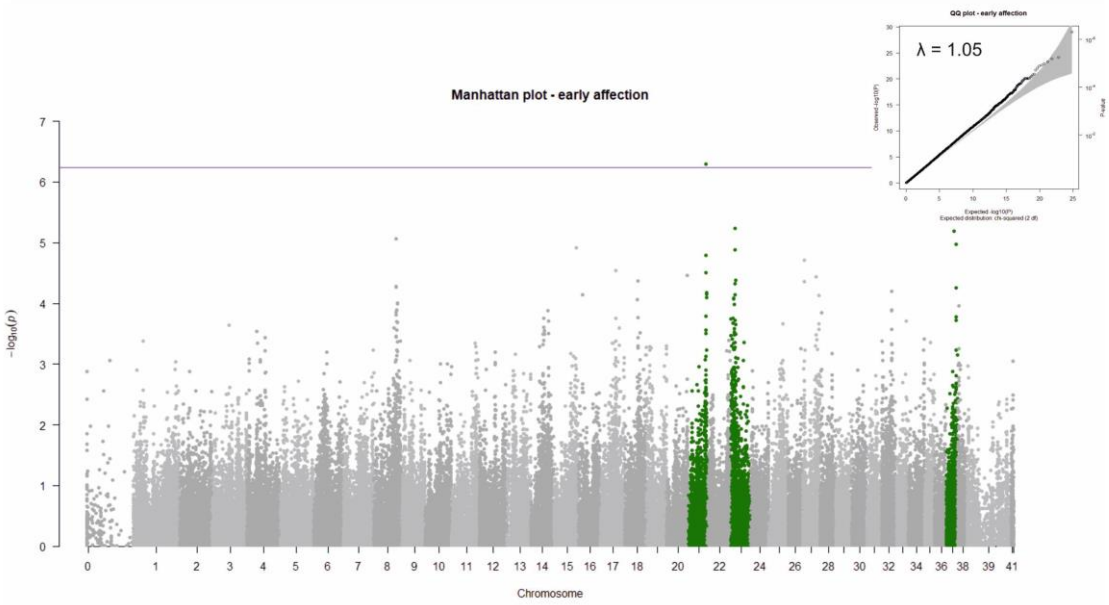


Figure S7: *Manhattan and Quantile-Quantile Plot of GWAS analysis of individuals with severe affection (level 4 aRFA). In green are colored chromosomes carrying the top three SNPs. The significance threshold was based on Bonferroni correction (5.8.x10-7; shown in a purple line). The top SNP does not reach the significance threshold, however, it is the same SNP as in the early onset GWAS. Lambda value shows that stratification correction worked well.*

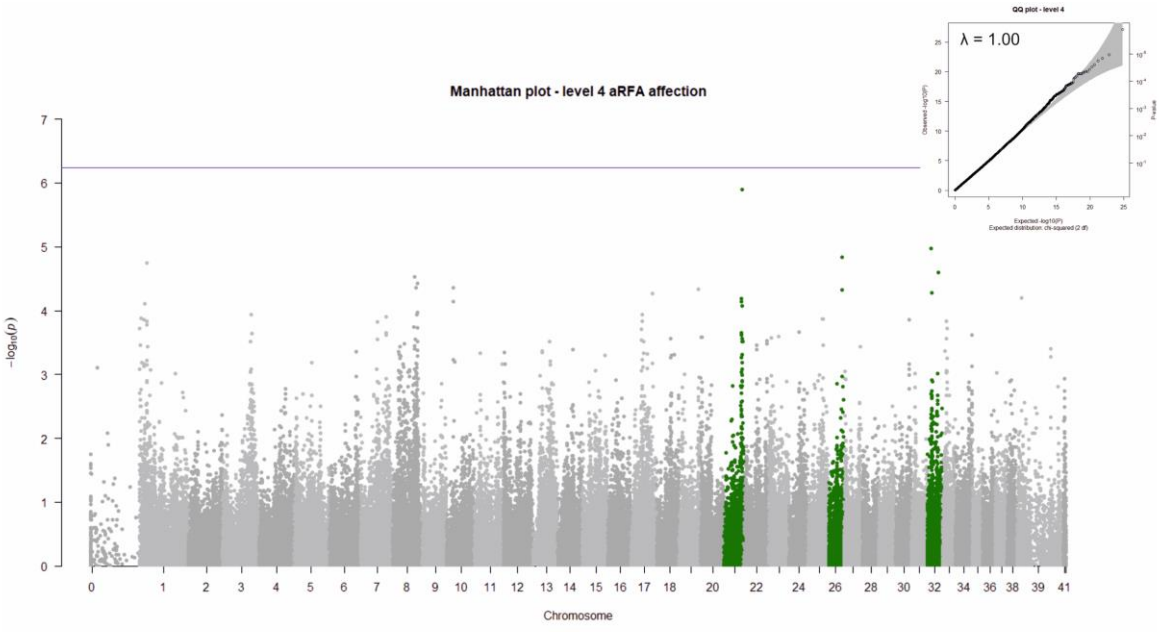


Figure S8: *Linkage disequilibrium (r2) plots for top SNP on chromosomes 19 and 8 from the case/control GWAS. The plots show LD among the top SNP on the chromosome and other SNPs within 2Mb or 4Mb (black-no LD; blue – weak LD, green –intermediate LD, yellow – moderate LD; red – strong LD).*

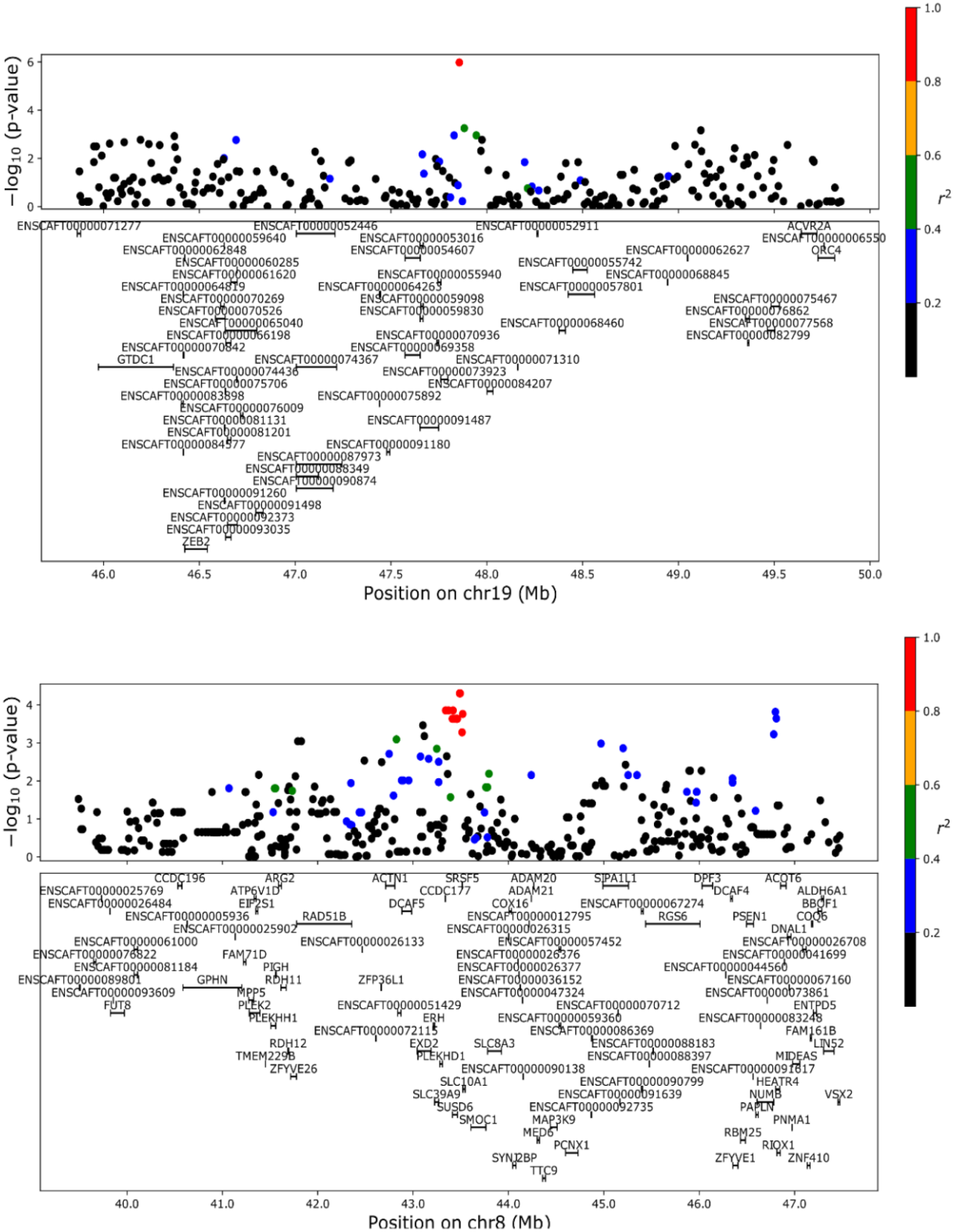


Figure S9: *Predicted protein-protein interactions among the 144 GWAS candidate genes' products. The GWAS gene candidates showed enriched protein-protein interactions, specifically, they were predicted to be connected in six clusters of more than three proteins. Each cluster is connected through the lines of different thicknesses, that represent the strength of evidence of the functional connection between genes. The color of each gene has not been changed from the STRING database output.*

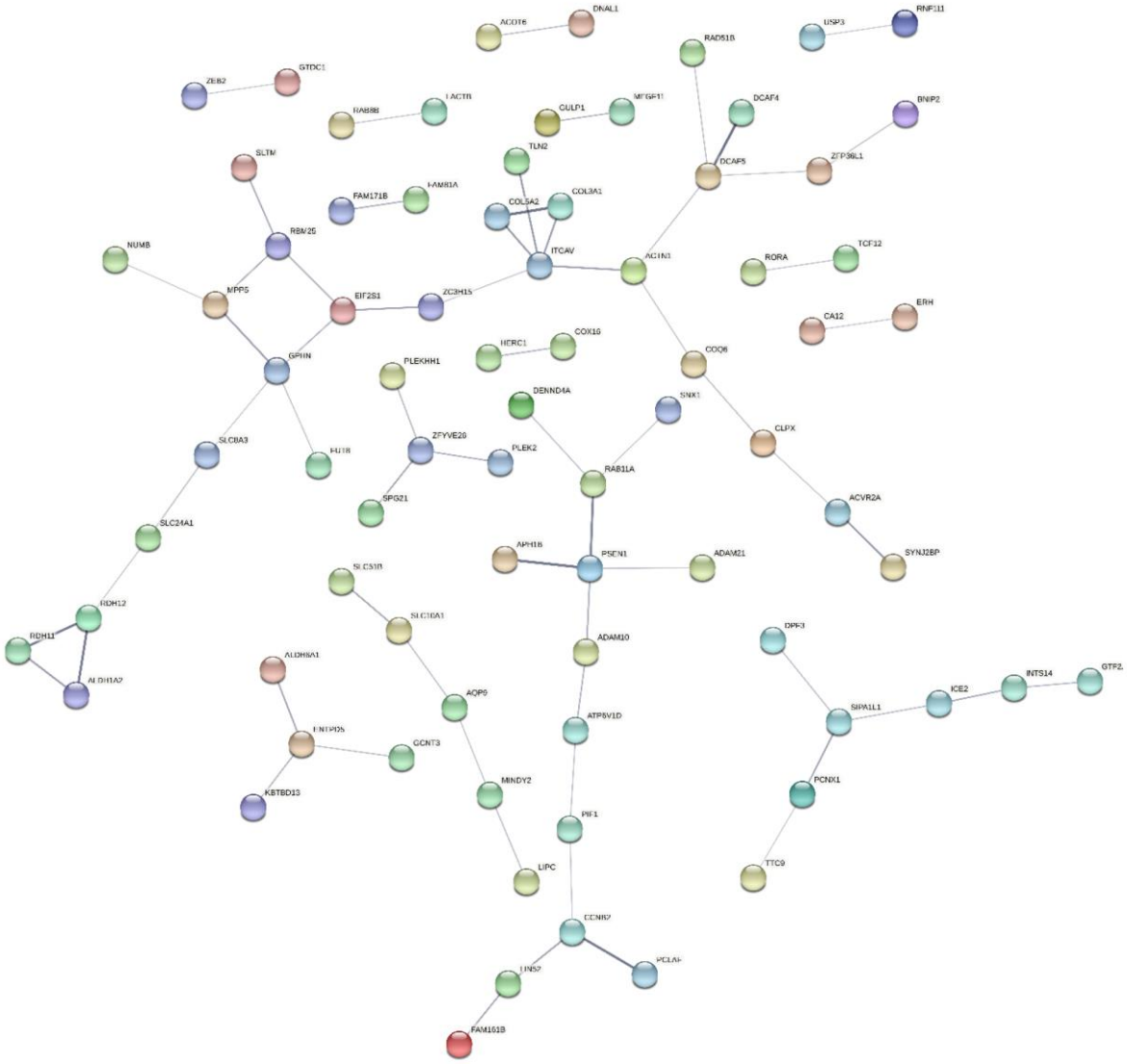


Figure S10: *Interactions of GWAS candidate genes (green) and STRING-associated strongly differentially expressed genes, colored by their level of expression. Only medium and higher confidence associations were used (increasing thickness of lines connecting genes indicates greater confidence). Colorful bubbles represent the metabolic pathways common for each cluster of genes. Shown are also couples and smaller clusters of interacting genes.*

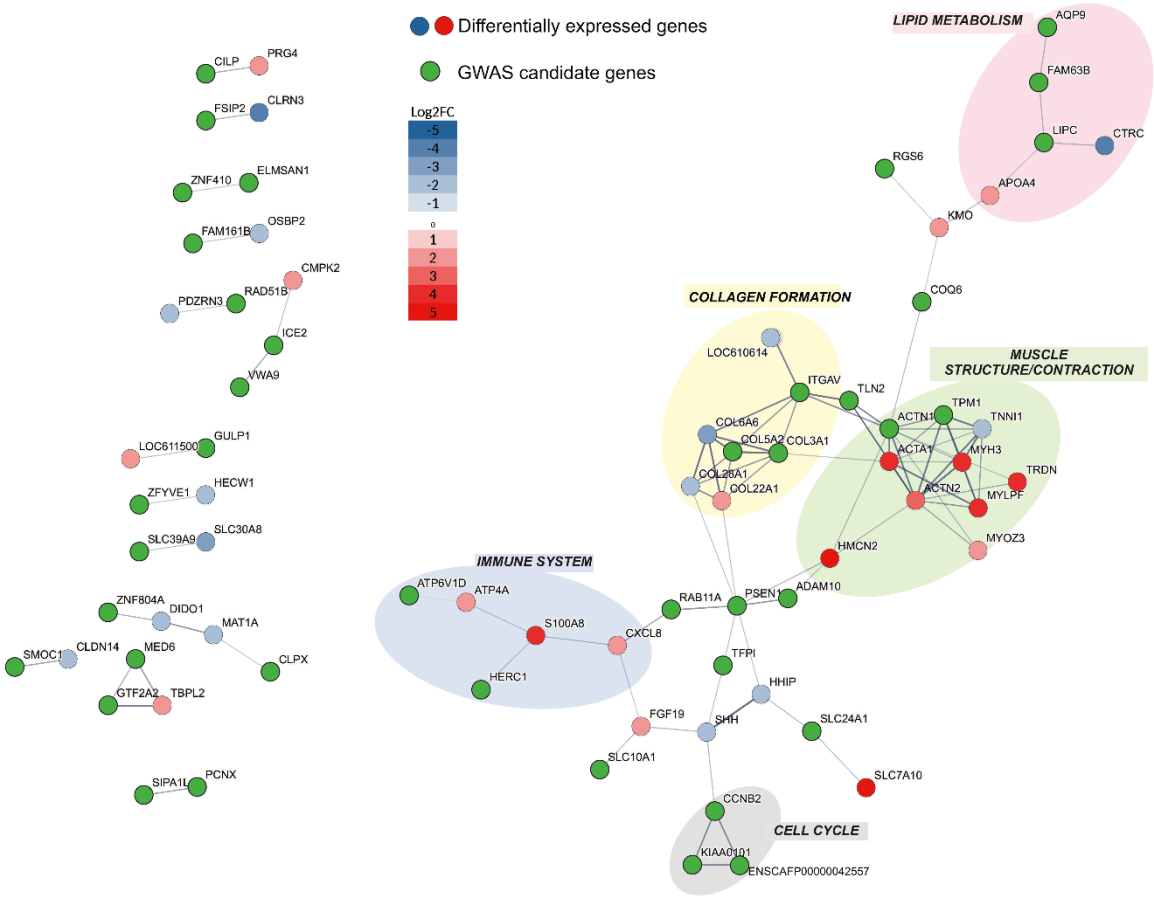


Figure S11: *Simple graphs showing numbers of survey records for housing of the dogs, the environment where they live, and the main cause of death in the Czech Republic and abroad.*

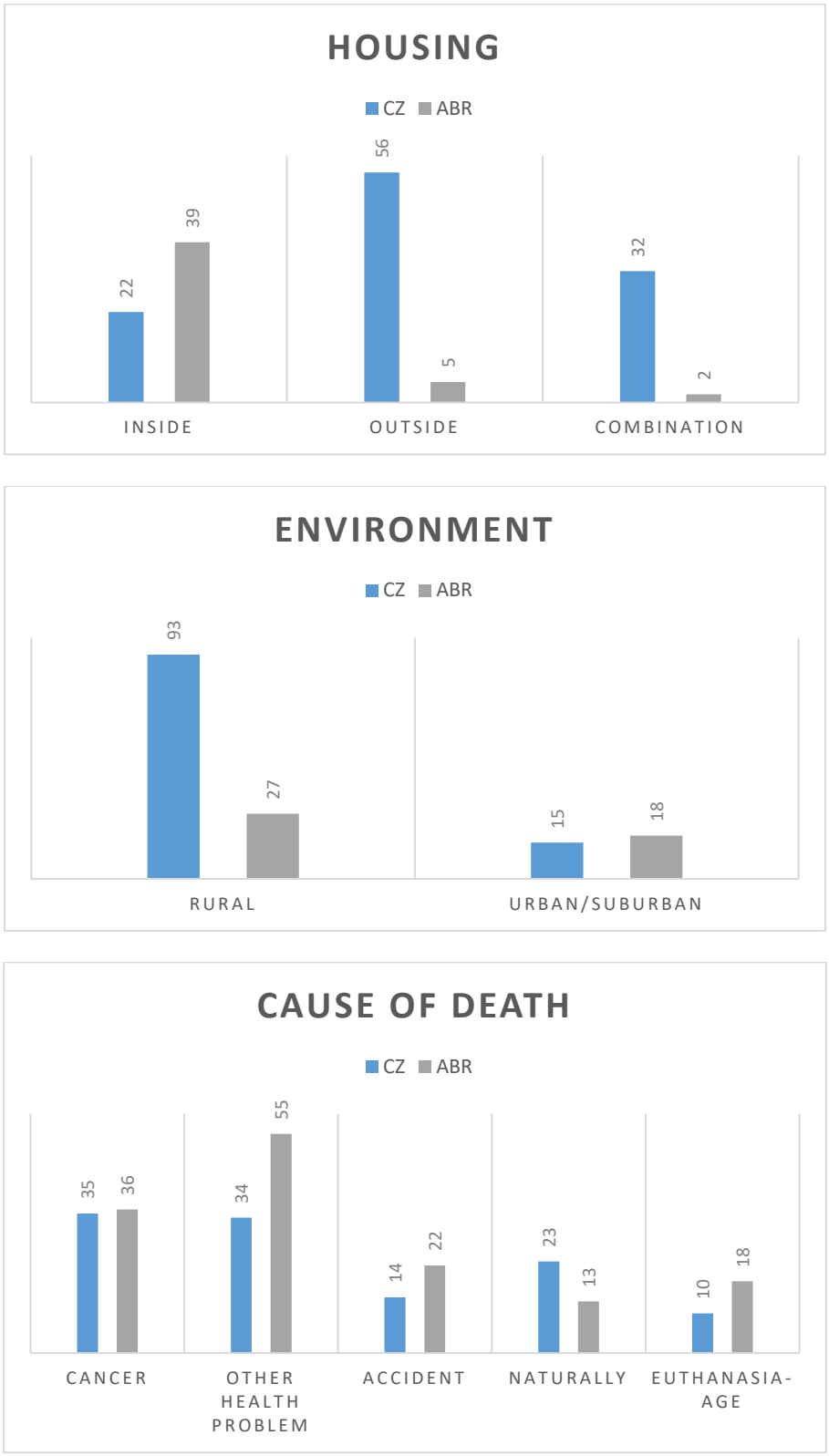
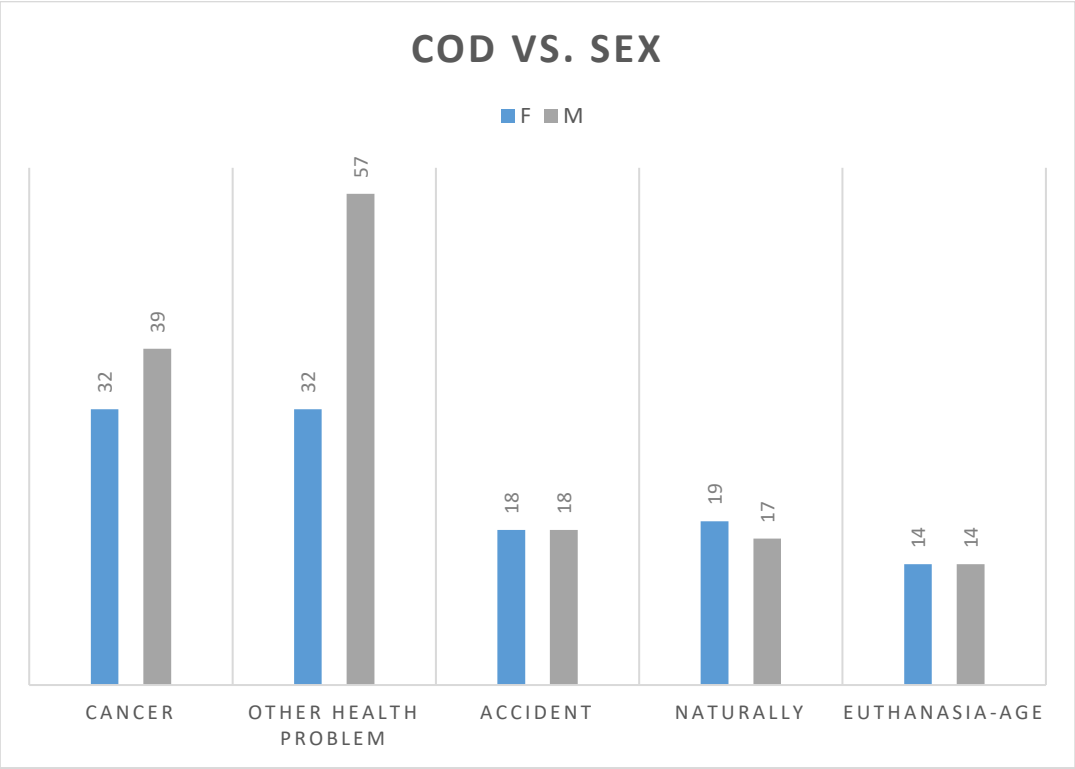


Figure S12: *Simple graphs showing numbers of survey records for each COD category in relation to sex, and origin.*



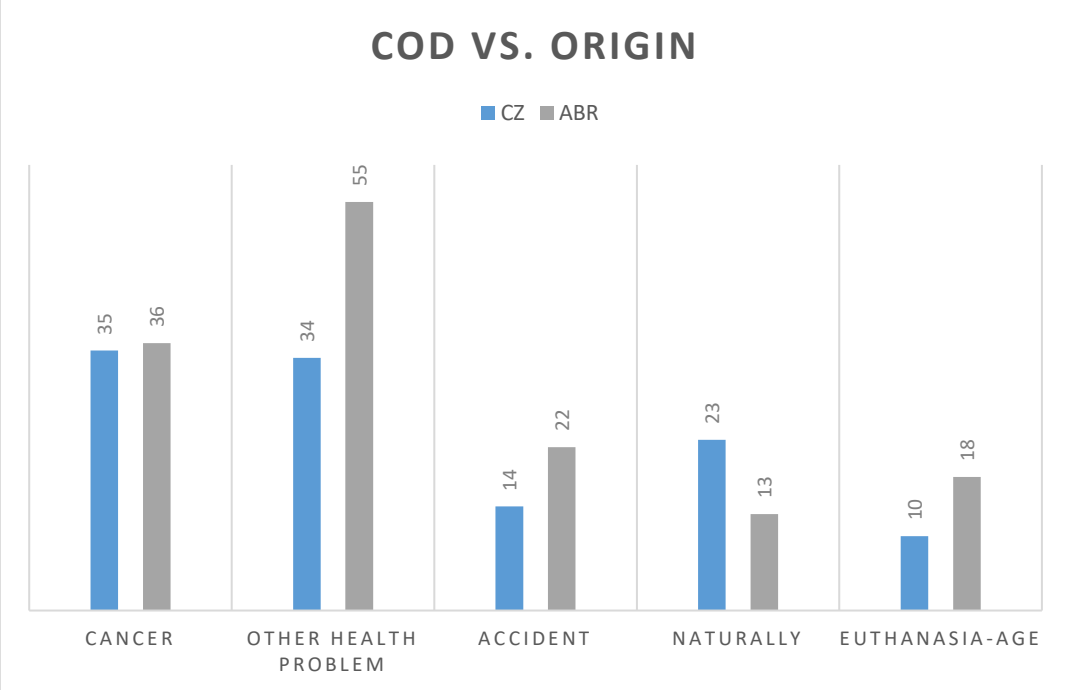
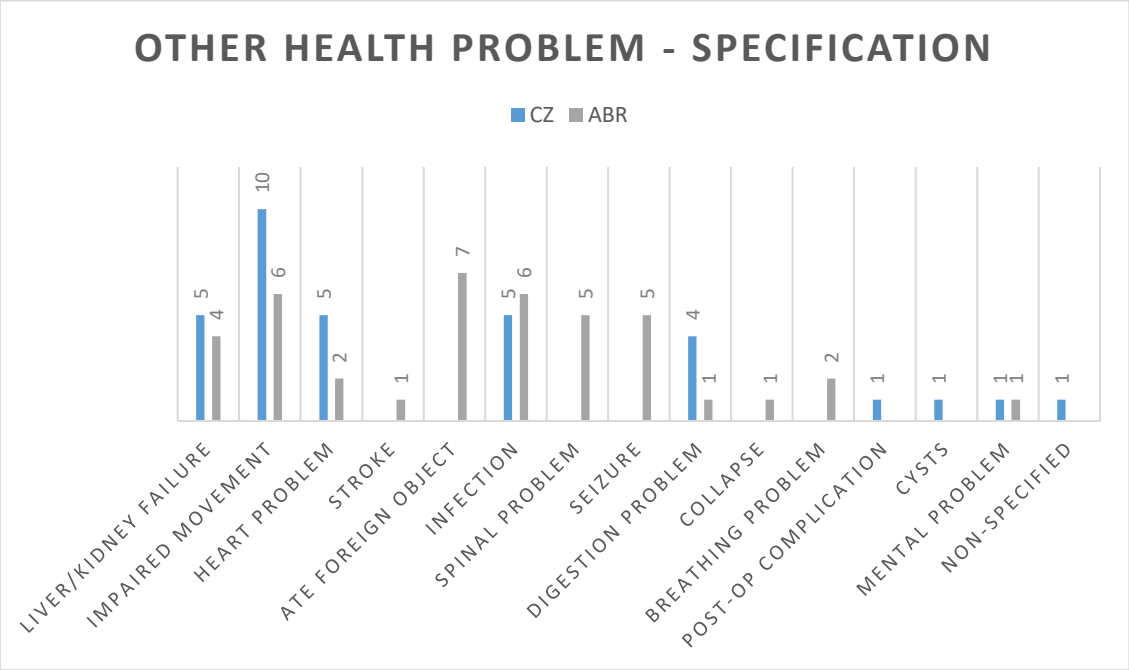


Figure S13: *Simple graph showing numbers of survey records for the "other health problem" group of COD. There are 15 specific subcategories divided according to the origin.*



Tables:

Table S1: *Genotypes and origin of the samples used for the study. Country = country of the sample origin; Pop = population; CVB = Cornell Veterinary Biobank; breeder = owner of the individual; authors = authors of this paper*

Sample	Pop	Country	Sample taken by	AHTk211		CXX279		REN169O18		INU055		REN54P11		INRA21		AHT137		REN169D01		AHTh260		AHTk253		INU005		INU030		FH2848		AHT121		FH2054		REN162C04		AHTh171		REN247M23	
1	CF	CZ	authors	89	89	117	127	157	167	203	203	228	236	94	98	146	150	208	216	245	247	289	289	129	131	144	150	230	242	95	103	157	173	202	204	217	225	272	274
2	CF	CZ	authors	85	87	115	125	161	161	203	203	228	236	100	102	130	150	212	216	245	245	289	289	129	129	144	146	230	230	101	107	153	173	202	204	225	225	272	280
3	CF	CZ	authors	85	89	127	127	165	167	199	203	234	236	94	94	146	150	212	216	245	245	289	289	111	131	148	150	238	240	101	109	157	161	202	206	219	225	270	272
4	CF	CZ	authors	87	89	115	123	157	163	203	203	228	236	92	94	148	152	216	216	245	245	287	289	125	131	146	150	230	230	101	101	153	161	202	204	219	225	274	280
5	CF	CZ	authors	87	87	115	127	159	163	203	203	236	236	98	102	146	150	216	216	245	251	289	289	125	127	144	144	240	240	95	101	153	157	202	202	225	225	272	274
6	CF	CZ	authors	87	89	115	125	157	165	203	203	228	236	100	102	130	148	212	216	245	249	289	289	129	131	144	148	230	240	101	101	153	153	202	204	219	225	274	280
7	CF	CZ	authors	89	89	127	127	161	167	201	203	234	234	96	102	148	148	212	212	237	251	289	289	111	111	144	144	238	240	109	109	165	173	202	202	225	233	274	274
8	CF	CZ	authors	85	89	127	129	159	161	203	203	228	228	94	96	130	150	212	216	245	247	289	293	125	129	150	150	238	242	109	109	153	157	204	206	219	225	274	280
9	CF	CZ	authors	87	87	115	127	159	163	203	203	236	236	98	102	146	150	216	216	245	251	289	289	125	127	144	144	240	240	95	101	153	157	202	202	225	225	272	274
10	CF	CZ	authors	89	93	117	127	167	167	203	203	234	234	94	102	130	148	212	212	245	251	289	289	111	125	144	144	230	240	109	109	153	165	202	204	233	233	272	274
11	CF	CZ	authors	89	93	115	119	157	169	203	203	228	228	94	96	130	150	212	216	245	251	289	291	131	131	144	150	230	240	95	101	157	173	200	202	219	225	280	280
12	CF	CZ	authors	85	89	117	127	157	159	201	203	234	236	94	98	130	146	208	212	245	247	289	289	129	131	144	150	238	238	101	103	157	177	202	202	225	237	280	280
13	CF	CZ	authors	93	93	119	127	157	159	201	203	234	236	92	98	146	150	216	216	245	245	289	289	125	125	144	144	240	240	103	107	173	173	202	202	225	225	270	270
14	CF	CZ	authors	87	89	115	115	157	161	203	203	236	236	94	100	148	150	216	216	245	249	289	289	129	131	146	148	230	238	101	101	153	173	202	202	225	225	280	280
15	CF	CZ	authors	87	89	127	127	159	161	201	203	228	234	94	94	144	150	212	212	245	247	289	293	129	131	150	150	234	240	95	95	161	173	204	210	233	237	272	280
16	CF	CZ	authors	85	87	115	127	161	167	201	203	228	228	92	102	130	148	208	216	245	245	289	293	131	131	144	150	240	246	95	109	157	177	202	206	219	225	270	272
17	CF	CZ	authors	89	89	127	129	159	167	201	201	234	238	102	102	130	130	212	216	245	245	289	289	125	131	144	144	240	240	101	109	157	157	202	202	219	225	272	274
18	CF	CZ	authors	89	93	127	127	161	161	201	201	234	238	92	98	130	148	216	216	247	251	289	289	131	131	150	152	242	242	95	107	161	177	200	202	219	239	272	274
19	CF	CZ	authors	89	93	119	127	159	169	203	203	234	236	92	94	148	150	212	212	245	247	289	289	129	129	144	150	230	240	95	109	161	177	202	204	219	237	280	280
20	CF	CZ	authors	89	93	115	117	167	169	203	203	234	236	94	94	148	150	212	212	245	245	289	289	125	129	144	150	240	240	95	101	157	165	202	202	225	233	270	280
21	CF	CZ	authors	85	85	119	127	157	159	201	203	234	236	94	94	146	148	212	212	245	247	289	289	125	131	144	144	238	240	103	109	157	173	202	202	219	225	270	270

22	CF	CZ	authors	89	89	127	127	161	163	203	203	228	238	92	94	144	148	216	216	245	247	287	287	125	125	144	144	230	230	95	107	157	173	202	210	225	237	270	280
23	CF	CZ	authors	89	89	123	127	165	169	203	203	228	238	102	102	130	150	216	216	247	247	289	293	125	131	144	150	240	240	95	101	157	173	202	204	219	219	272	280
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34	CF	CZ	authors	85	93	115	119	157	169	199	203	228	234	94	102	150	150	212	212	245	245	289	289	125	131	148	148	230	238	97	101	165	173	202	210	233	237	270	272
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52	CF	CZ	authors	85	89	115	127	159	167	199	203	228	236	94	94	130	150	212	212	247	249	289	289	111	125	144	150	240	244	101	109	173	173	202	202	225	227	274	280
53	CF	CZ	authors	87	89	115	125	165	165	199	203	228	236	102	102	130	148	212	212	245	247	289	289	129	131	144	150	230	230	101	101	153	157	202	204	225	237	272	280
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55	CF	CZ	authors	89	93	123	127	157	159	201	203	228	238	94	98	130	146	208	212	245	245	289	293	111	131	144	150	238	240	95	101	161	173	202	202	219	225	270	280
56	CF	CZ	authors	89	93	115	127	157	159	203	203	236	236	94	98	148	150	212	216	245	245	289	289	125	127	144	150	230	240	95	109	173	173	202	204	219	225	272	280
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58	CF	CZ	authors	87	93	115	117	163	165	203	203	228	236	92	94	146	150	212	212	245	245	289	289	111	131	148	150	230	230	95	101	157	173	202	208	225	225	274	280
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65	CF	CZ	authors	85	93	127	127	161	165	201	201	234	234	94	102	130	146	212	212	245	245	289	289	129	129	150	150	230	240	109	109	153	157	202	204	225	237	270	280
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68	CF	CZ	authors	89	89	123	123	163	167	201	203	228	238	102	102	130	152	198	216	247	251	289	289	125	131	144	150	238	240	109	109	157	161	202	202	225	225	274	280
69	CF	CZ	authors	85	89	117	127	157	167	201	203	228	236	96	98	130	150	212	216	245	245	289	289	111	131	150	152	240	240	95	101	153	157	204	210	225	227	270	280
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73	CF	CZ	authors	93	93	115	129	157	163	203	203	234	238	94	94	130	150	212	212	245	245	289	289	125	127	150	150	230	240	95	111	161	165	202	202	219	227	270	274

74	CF	CZ	authors	85	89	119	123	161	169	201	203	228	234	98	102	130	130	212	212	245	245	289	289	125	131	144	144	230	230	97	107	157	177	202	204	233	237	272	274
75	CF	CZ	authors	85	93	119	127	159	165	203	203	234	236	92	94	130	150	212	216	245	251	289	289	129	131	144	150	230	234	95	109	157	161	202	202	225	237	272	280
76	CF	CZ	authors	85	93	123	127	159	161	199	203	228	234	92	102	146	148	212	212	245	247	289	289	131	131	144	150	238	240	95	107	157	157	202	204	225	237	272	272
77	CF	NL	authors	89	89	127	127	163	167	201	203	228	238	94	94	146	150	212	216	239	245	287	289	125	131	144	144	230	230	101	107	173	173	202	202	225	237	272	280
78	CF	NL	authors	85	87	115	127	163	167	203	203	234	234	92	96	150	152	216	216	245	245	289	289	111	131	144	150	230	240	101	109	173	177	202	204	225	227	270	272
79	CF	NL	authors	85	89	127	127	157	167	203	203	228	238	94	102	144	146	212	216	239	251	287	289	125	131	144	144	230	238	95	107	173	173	202	202	219	237	274	280
80	CF	NL	authors	89	89	115	127	157	163	201	203	236	238	94	98	150	150	212	212	245	245	289	289	129	131	144	150	230	238	95	101	161	173	202	202	225	237	270	272
81	CF	NL	authors	85	89	127	127	159	169	203	203	234	234	102	102	150	150	204	216	245	245	289	289	131	131	144	144	240	240	105	105	161	161	202	202	225	233	274	276
82	CF	NL	authors	89	93	117	129	163	165	199	203	234	236	102	102	148	150	212	212	245	245	289	289	131	131	144	144	238	242	101	111	153	157	202	204	225	225	270	274
83	CF	NL	authors	89	93	115	123	157	161	201	203	228	234	92	96	130	146	212	212	245	245	289	289	111	125	144	150	238	240	95	109	157	177	202	202	225	237	272	274
84	CF	NL	authors	89	89	115	127	159	163	199	203	228	236	94	94	146	150	212	212	239	247	287	289	129	131	144	148	230	234	101	107	173	173	202	202	219	237	272	280
85	CF	NL	authors	89	89	115	127	157	165	201	203	236	236	94	98	148	150	212	216	245	251	289	289	125	129	148	148	238	238	101	111	153	173	202	202	225	225	274	280
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91	CF	NL	authors	87	89	127	127	159	161	203	203	234	234	94	102	148	150	208	216	243	245	289	293	125	131	144	150	230	238	107	107	153	161	202	202	219	219	274	274
92	CF	NL	authors	89	89	115	115	165	167	199	203	228	236	92	102	130	146	216	216	245	247	289	293	125	129	144	144	230	244	95	109	173	173	202	202	219	219	280	280
93	CF	NL	authors	85	89	123	127	167	169	201	203	234	236	94	102	130	148	208	216	245	251	289	293	111	125	150	150	238	240	101	109	161	177	202	208	219	225	270	274
94	CF	NL	authors	85	89	117	127	159	161	203	203	228	234	94	94	130	148	204	208	245	245	289	291	125	125	150	150	230	238	95	107	153	157	202	204	219	219	274	274
95	CF	NL	authors	85	89	117	127	161	167	203	203	234	234	94	102	148	152	204	204	237	245	283	289	125	129	144	148	230	236	95	107	157	157	202	202	221	225	274	274
96	CF	NL	authors	87	89	115	127	157	165	203	203	228	236	94	102	150	150	212	216	249	251	287	289	111	131	144	148	230	240	101	101	173	173	202	202	219	225	274	280
97	CF	NL	authors	85	89	115	127	163	165	203	203	236	238	94	94	146	150	216	216	245	247	287	289	125	131	144	148	230	234	101	109	173	173	202	202	219	237	280	280
98	CF	NL	authors	89	89	117	127	159	167	203	203	234	238	92	94	130	130	208	212	237	245	289	291	125	131	148	150	230	238	95	107	153	153	202	202	219	219	274	274
99	CF	NL	authors	85	89	123	127	167	167	199	203	234	236	96	102	130	146	216	216	245	251	289	293	111	125	144	150	238	244	109	109	157	161	202	208	225	225	270	274

100	CF	NL	authors	85	89	115	115	157	167	199	203	234	234	92	102	146	150	212	212	245	245	289	289	125	131	144	144	230	238	101	111	153	157	202	202	217	225	270	272
101	CF	NL	authors	85	89	115	127	159	167	201	203	236	238	94	94	146	146	212	216	239	247	287	293	129	129	144	144	230	230	101	107	173	173	202	202	219	237	272	280
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108	CF	NL	authors	87	89	115	127	163	167	203	203	234	238	94	94	144	150	208	216	247	251	289	293	131	131	144	150	230	230	103	109	173	173	202	202	225	237	270	280
109	CF	NL	authors	87	93	117	127	167	167	203	203	228	228	94	96	130	150	208	212	245	245	287	289	125	127	144	150	238	240	103	109	165	173	202	204	225	225	272	280
110	CF	NL	authors	85	89	115	127	157	161	203	203	236	236	94	94	130	148	206	212	245	247	289	293	125	125	144	150	230	242	95	103	157	173	204	204	219	225	270	274
111	CF	NL	authors	89	89	115	127	165	167	199	203	228	236	102	102	146	150	212	212	245	251	287	289	125	131	144	150	230	230	95	101	157	173	202	202	219	237	274	280
112	CF	NL	authors	85	89	127	127	165	167	203	203	228	236	92	102	130	146	212	212	245	245	289	289	125	131	144	144	230	240	101	111	153	173	204	204	227	237	274	280
113	CF	NL	authors	89	89	117	129	159	163	203	203	228	238	92	94	130	144	212	216	237	245	289	289	125	131	144	150	230	240	95	109	157	173	202	202	227	237	274	280
114	CF	NL	authors	89	89	127	127	161	167	199	203	234	236	96	96	130	148	208	212	237	245	287	289	125	125	144	150	238	244	109	109	173	177	206	208	219	225	270	274
115	CF	NL	authors	85	89	115	127	165	167	199	203	236	238	94	102	150	150	212	212	245	247	287	289	125	125	144	148	230	234	101	109	153	173	202	202	219	225	272	280
116	CF	NL	authors	89	89	115	127	161	161	203	203	234	234	92	94	130	130	212	216	245	245	291	291	125	131	150	150	230	238	95	107	157	173	202	202	219	219	274	274
117	CF	NL	authors	89	89	115	127	163	165	199	203	228	236	94	102	150	150	212	212	245	247	287	293	125	131	144	144	230	234	107	109	173	173	202	202	219	237	280	280
118	CF	NL	authors	89	89	115	117	157	161	203	203	236	236	94	98	146	150	216	216	245	247	289	293	127	129	148	150	238	240	101	101	153	173	202	206	225	237	280	280
119	CF	NL	authors	85	87	115	127	159	159	203	203	234	234	92	102	130	150	208	212	245	245	289	289	111	131	150	150	230	230	95	107	153	173	202	202	219	237	270	274
120	CF	NL	authors	87	89	115	127	157	165	199	203	228	236	94	102	146	150	216	216	245	251	289	289	129	131	144	148	230	238	95	107	153	173	202	202	237	237	274	280
121	CF	NL	authors	85	87	127	127	165	167	203	203	234	234	92	92	130	150	216	216	245	245	289	289	127	131	144	144	240	242	109	111	153	177	204	204	227	237	270	272
122	CF	NL	authors	87	89	115	115	157	163	203	203	234	236	92	94	148	150	216	216	245	245	289	289	111	129	146	150	238	240	101	101	173	177	202	202	225	225	270	280
123	CF	NL	authors	89	93	117	127	157	161	201	201	228	228	98	102	130	146	216	216	245	245	289	289	111	125	144	144	230	230	95	109	173	177	202	206	225	237	270	270
124	CF	NL	authors	85	87	115	127	157	167	203	203	234	236	94	96	150	152	216	216	245	245	289	289	111	131	144	146	230	240	101	101	153	177	202	204	225	227	270	280
125	CF	NL	authors	85	89	115	127	163	167	199	203	228	234	92	92	130	150	212	212	245	245	289	293	111	111	150	150	238	240	109	109	161	165	202	208	225	225	272	274

126	CF	CAN	breeder	89	93	115	127	157	167	203	203	234	236	94	96	130	150	212	216	245	245	287	289	125	125	144	148	230	240	95	95	153	157	202	204	225	227	274	274
127	CF	NZ	breeder	85	89	115	119	159	167	203	203	236	236	98	98	148	152	204	212	237	245	287	289	129	131	150	150	230	236	101	109	157	173	202	210	219	225	272	280
128	CF	CZ	authors	89	89	127	129	167	167	203	203	228	238	94	102	130	150	212	216	239	247	289	293	127	131	144	144	238	240	101	109	157	165	202	202	219	233	270	272
129	CF	CZ	authors	85	89	115	127	157	167	199	203	228	236	94	102	130	148	216	216	237	245	287	289	129	131	144	144	230	238	101	105	153	153	204	206	217	225	274	280
130	CF	CZ	authors	85	89	115	127	163	165	203	203	228	236	94	94	150	150	208	212	251	251	289	289	125	125	144	150	240	240	109	111	157	161	202	204	219	225	272	280
131	CF	CZ	authors	89	93	123	127	159	167	201	203	234	234	92	94	130	144	212	216	245	251	287	289	125	131	144	150	230	244	95	103	153	173	202	202	219	233	274	280
132	CF	CZ	authors	89	93	117	127	157	159	201	203	228	236	94	96	144	150	212	216	245	251	289	291	125	131	148	150	230	240	95	101	161	161	202	206	217	233	280	280
133	CF	CZ	authors	93	93	115	129	157	163	203	203	234	238	94	94	130	150	212	212	245	245	289	289	125	127	150	150	230	240	95	111	161	165	202	202	219	227	270	274
134	CF	CZ	authors	89	89	127	127	161	169	201	203	236	238	92	98	130	148	212	216	245	251	289	289	129	131	144	152	234	242	107	109	157	177	200	202	219	233	272	274
135	CF	USA	breeder	89	93	115	127	163	165	201	203	228	228	94	100	144	146	212	216	245	245	289	293	125	131	144	150	234	240	101	111	157	161	202	204	225	237	270	280
136	CF	PL	breeder	89	93	123	127	167	169	203	203	228	234	94	102	146	150	212	212	245	251	287	289	125	125	144	150	240	242	95	101	161	173	200	202	237	237	270	274
137	CF	B	breeder	85	89	127	127	159	169	199	201	234	236	98	102	150	150	216	216	245	251	287	289	125	131	144	150	230	240	101	105	161	161	202	202	225	233	270	280
138	CF	SK	breeder	85	89	123	127	159	169	201	203	228	238	94	94	130	150	212	216	245	247	287	289	131	131	144	146	230	238	95	111	157	157	202	202	219	233	280	280
139	CF	CZ	authors	87	89	115	127	159	167	199	203	236	236	94	102	130	150	212	212	245	245	289	293	127	129	148	150	238	242	95	101	173	173	202	202	217	225	274	280
140	CF	CZ	authors	89	89	115	127	161	167	201	203	234	236	94	102	148	150	212	212	245	251	291	291	125	125	144	150	238	240	95	109	153	165	202	206	219	237	270	280
141	CF	CZ	authors	89	89	115	115	157	167	201	203	236	238	92	102	148	150	212	212	247	251	291	291	111	125	144	150	240	240	101	109	153	165	202	206	219	237	280	280
142	CF	CZ	authors	89	93	123	127	163	165	203	203	228	234	92	94	146	150	212	212	245	251	287	289	131	131	144	150	240	240	107	111	157	173	204	208	225	225	272	274
143	CF	CZ	authors	85	87	115	127	157	163	201	203	228	236	94	98	144	148	216	216	245	249	287	289	129	131	144	148	230	238	101	101	153	173	202	208	225	237	272	280
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151	CF	CZ	authors	89	93	123	127	161	163	201	203	234	234	98	102	130	148	206	212	245	251	289	293	111	125	144	144	238	238	107	109	153	157	202	202	219	233	272	274

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154	CF	CZ	authors	85	87	115	119	159	161	201	203	234	234	94	102	150	150	212	216	245	251	289	289	129	131	144	150	240	240	109	109	153	173	202	202	219	219	274	280
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157	CF	CZ	authors	89	93	127	127	163	169	201	203	228	234	94	98	130	144	212	216	245	251	289	289	125	131	144	144	230	238	95	95	173	173	202	202	219	237	270	280
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168	CF	NL	authors	89	93	127	127	161	161	201	203	234	236	98	102	148	150	208	212	243	245	289	293	129	131	150	150	230	230	95	107	161	161	202	204	219	219	274	280
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171	CF	NL	authors	85	93	115	117	157	163	201	203	228	236	92	98	146	150	212	216	245	245	289	289	129	129	144	144	230	238	95	95	157	173	202	202	225	237	270	272
172	CF	NL	authors	87	93	115	127	163	163	201	203	234	236	92	96	146	150	216	216	245	251	287	289	111	131	144	150	230	240	95	101	153	161	202	204	225	237	270	274
173	CF	NL	authors	87	89	115	127	163	163	199	201	234	238	94	102	144	150	208	216	251	251	287	289	111	131	144	150	230	230	95	103	173	173	202	204	225	233	272	274
174	CF	NL	authors	87	89	115	127	161	165	201	203	228	236	92	94	150	150	208	216	245	251	287	289	111	131	144	148	230	238	101	109	153	173	202	202	225	237	274	280
175	CF	NL	authors	89	89	117	127	159	167	199	203	228	228	94	98	146	148	212	216	245	245	289	293	125	131	146	150	240	244	101	101	173	173	202	202	225	225	270	270
176	CF	NL	authors	87	93	117	127	167	167	203	203	228	228	94	96	130	150	208	212	245	245	287	289	125	127	144	150	238	240	103	109	165	173	202	204	225	225	272	280
177	CF	NL	authors	89	93	115	127	163	167	199	203	234	236	92	96	146	150	216	216	245	251	287	289	125	131	144	150	238	242	95	101	153	173	202	204	225	225	270	270

178	CF	NL	authors	85	87	115	127	161	167	203	203	234	236	92	94	148	152	216	216	245	245	289	289	111	131	146	150	230	240	101	109	173	173	202	204	225	225	272	280
179	CF	NL	authors	89	89	123	127	163	167	201	203	228	228	98	102	130	130	198	216	245	245	289	289	129	131	144	144	240	240	109	109	157	157	202	202	219	219	272	274
180	CF	NL	authors	85	87	117	127	165	167	203	203	234	234	102	102	130	152	212	216	245	245	287	289	131	131	150	150	230	240	103	109	153	173	202	202	225	227	270	272
181	CF	NL	authors	89	89	115	115	159	163	203	203	234	236	102	102	148	150	212	212	245	251	289	289	125	131	148	150	238	240	101	111	165	173	202	204	225	225	270	270
182	CF	NL	authors	87	93	119	127	159	161	201	203	234	234	94	98	148	150	212	216	237	243	289	289	129	131	144	150	238	240	95	107	153	161	202	202	219	237	274	274
183	CF	NL	authors	89	89	127	127	159	161	201	203	234	236	94	102	150	150	212	212	245	245	287	289	125	125	144	150	238	240	111	111	153	165	200	202	225	225	270	274
184	CF	NL	authors	87	89	123	127	163	167	203	203	228	228	94	94	150	150	212	212	245	245	289	293	125	129	146	150	238	240	95	109	157	157	202	204	219	233	272	280
185	CF	NL	authors	89	93	127	127	163	167	203	203	234	234	92	94	144	150	212	216	245	251	289	289	125	131	150	150	238	240	101	107	153	157	202	202	219	233	274	280
186	CF	NL	authors	89	89	117	127	159	161	203	203	228	234	94	94	130	148	208	212	245	245	289	291	125	125	150	150	230	238	95	107	153	157	202	204	219	219	274	274
187	CF	NL	authors	89	89	115	115	161	165	203	203	236	236	92	94	148	150	212	216	245	249	289	289	0	0	146	148	230	238	95	101	0	0	202	202	225	225	270	280
188	CF	NL	authors	87	89	115	115	163	167	199	201	234	234	94	102	146	148	208	212	245	245	289	289	125	131	144	146	238	240	101	111	157	157	202	202	219	225	270	280
189	CF	CZ	authors	89	93	115	127	159	167	199	203	228	238	94	102	150	150	212	216	243	243	293	293	131	131	144	150	234	240	101	109	153	165	202	202	219	225	270	272
190	CF	CZ	authors	87	93	119	127	157	167	203	203	228	228	94	94	150	150	212	212	245	249	289	289	125	125	148	150	238	238	0	0	165	173	202	204	219	225	272	272
191	CF	CZ	authors	93	93	115	115	161	165	203	203	234	238	94	94	130	150	216	216	245	247	289	289	125	131	144	148	230	234	97	109	161	173	202	202	219	225	270	270
192	CF	USA	breeder	85	89	115	127	159	165	203	203	228	234	92	96	144	150	212	212	247	247	289	293	111	131	144	150	230	240	101	105	161	161	202	206	225	237	274	280
193	CF	CZ	authors	89	89	127	127	161	163	203	203	228	234	102	102	148	148	212	212	237	247	289	289	111	125	144	148	230	230	103	109	157	173	202	202	225	225	274	274
194	BWPGCA	USA	CVB	89	93	115	127	159	169	203	203	228	236	102	102	148	150	212	212	245	245	287	289	125	125	144	148	230	244	95	101	153	153	202	206	225	237	272	274
195	BWPGCA	USA	CVB	85	93	127	127	159	159	201	203	236	236	98	102	146	148	212	212	245	245	289	289	125	129	144	150	240	240	95	103	173	173	202	202	225	225	270	272
196	BWPGCA	USA	CVB	85	93	123	123	157	159	203	203	234	236	102	102	150	150	212	212	245	245	287	287	127	129	144	152	230	240	95	101	173	173	202	206	219	225	272	274
197	BWPGCA	USA	CVB	87	89	119	127	157	161	203	203	228	236	94	102	148	150	206	216	245	245	287	289	125	125	148	150	230	230	95	101	153	157	202	206	225	225	274	274
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204	BWPGCA	USA	breeder	89	89	115	115	159	169	199	203	224	236	98	102	146	150	212	212	237	245	289	293	125	129	144	144	230	240	95	111	169	173	202	206	225	225	270	274
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212	BWPGCA	USA	breeder	85	87	115	123	157	157	203	203	234	236	94	102	150	150	212	212	245	251	287	287	127	131	144	150	230	240	95	107	157	173	202	206	219	225	272	274
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220	BWPGCA	USA	breeder	89	93	115	127	163	167	199	201	234	236	92	94	146	150	216	216	245	251	287	287	125	129	144	150	238	240	95	111	153	173	202	202	225	225	270	270
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280	DD	GE	breeder	89	89	117	119	159	165	197	199	228	228	92	94	146	150	212	216	245	247	287	293	125	125	148	150	238	240	111	113	165	177	202	202	225	233	270	270
281	DD	GE	breeder	89	89	117	129	159	165	201	203	224	228	94	94	130	150	216	216	243	247	289	293	125	131	148	150	240	244	101	113	157	165	202	210	219	219	270	274

282	DD	GE	breeder	85	89	117	119	165	167	203	203	228	228	94	94	146	150	216	216	239	245	287	293	125	125	148	150	230	244	103	107	157	165	202	206	219	225	270	270
283	DD	GE	breeder	87	89	117	119	159	165	201	203	224	228	92	92	148	148	212	214	245	245	289	293	111	125	144	148	230	238	101	111	165	173	200	202	219	225	270	270
284	DD	GE	breeder	89	89	117	123	161	163	201	201	228	236	92	94	146	148	212	216	239	253	289	293	131	133	144	144	238	238	111	113	165	165	202	202	219	219	270	274
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287	DD	GE	breeder	89	89	123	127	163	163	201	201	228	236	92	92	130	146	212	216	239	245	287	293	125	129	144	144	230	238	107	111	165	165	202	204	219	219	270	274
288	DD	GE	breeder	87	89	123	129	161	163	201	203	228	228	92	94	146	146	212	216	239	245	287	293	111	131	144	144	238	240	111	111	165	165	202	202	219	219	270	274
289	DD	GE	breeder	89	93	117	123	163	163	201	203	234	236	92	92	146	150	208	212	245	247	289	291	125	127	148	148	230	244	107	107	157	165	202	202	219	233	270	274
290	DD	GE	breeder	89	89	123	123	163	165	201	201	228	228	92	94	146	146	212	212	239	253	287	289	111	125	144	144	230	238	109	111	165	165	202	202	219	219	270	274
291	DD	GE	breeder	87	89	123	129	163	163	201	203	228	236	92	92	146	146	212	216	245	253	289	293	125	131	144	144	238	238	109	111	165	165	202	202	219	219	270	274
292	DD	GE	breeder	87	89	117	117	161	161	201	201	228	236	92	94	144	146	198	212	239	247	289	293	111	131	148	150	230	230	111	111	149	165	202	206	219	225	270	274
293	DD	GE	breeder	89	93	123	123	161	163	201	203	228	228	92	92	146	146	212	216	239	245	287	289	131	131	144	144	238	240	109	111	165	165	202	202	219	219	270	272
294	DD	USA	breeder	89	89	117	129	163	163	201	207	228	238	94	94	146	146	216	216	239	245	287	289	111	125	148	150	230	244	105	107	157	177	202	202	219	219	270	270
295	DD	USA	breeder	89	89	125	127	161	161	201	201	238	238	94	94	146	150	212	216	245	245	289	293	111	125	148	150	240	244	107	109	153	165	202	204	219	233	272	272
296	DD	USA	breeder	89	89	119	129	161	163	203	203	234	238	92	94	146	146	212	216	239	239	289	289	111	125	148	150	230	244	107	107	153	157	202	202	219	233	270	272
297	DD	USA	breeder	89	93	125	127	161	165	201	201	228	238	94	94	150	150	212	216	239	245	289	293	111	111	148	150	238	244	107	109	157	165	202	204	219	233	272	274
298	DD	USA	breeder	89	89	125	129	157	165	201	203	228	236	94	94	146	146	210	212	245	245	289	293	111	125	144	144	230	238	107	109	157	177	200	202	233	233	270	270
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300	DD	GE	breeder	89	93	115	117	159	165	197	203	228	236	92	94	146	146	216	216	245	245	289	291	125	131	144	148	230	240	109	109	153	165	202	206	219	233	270	270
301	DD	GE	breeder	89	93	117	129	157	161	203	203	228	234	92	94	146	150	212	212	239	239	289	291	125	127	144	148	230	244	107	113	165	165	202	206	219	219	272	272
302	DD	GE	breeder	89	93	125	125	161	163	201	203	234	236	94	94	148	150	198	216	239	245	289	291	125	125	144	150	230	238	107	107	157	165	202	202	219	219	272	274
303	DD	GE	breeder	89	89	117	117	159	165	201	201	236	236	92	94	146	150	212	216	245	245	293	293	125	131	148	150	238	240	109	111	165	165	202	206	219	219	274	274
304	DD	GE	breeder	89	93	115	127	159	165	201	203	234	236	94	102	144	150	212	212	239	245	287	289	125	125	150	150	240	244	107	111	165	165	202	206	219	229	272	272
305	DD	SK	breeder	85	87	117	125	159	163	201	201	228	234	98	98	130	144	212	216	239	245	293	293	125	125	148	150	230	244	107	111	165	165	202	202	225	225	270	274
306	DD	SK	breeder	85	93	115	117	163	163	201	203	234	234	94	98	130	148	216	216	245	245	287	293	125	125	148	150	244	244	109	111	165	177	202	202	225	225	272	274
307	DD	SK	breeder	87	93	117	117	163	165	199	203	228	234	94	98	144	148	212	216	239	245	289	293	125	125	148	150	240	244	107	111	165	165	202	202	225	225	270	272

308	DD	CZ	authors	85	89	117	125	163	163	201	203	228	236	92	94	146	150	216	216	245	245	287	291	111	133	144	150	238	240	101	105	157	165	206	206	219	233	270	272
309	DD	USA	breeder	89	93	117	117	163	165	203	203	234	236	92	98	144	146	216	216	239	247	291	293	125	125	148	148	238	238	107	107	149	165	202	202	219	219	270	272
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316	DD	GE	breeder	89	89	117	127	163	163	203	203	236	236	92	94	146	148	214	216	239	239	287	293	125	129	148	150	240	244	107	111	165	165	202	204	219	233	274	274
317	DD	GE	breeder	89	89	117	127	159	159	201	203	228	234	94	98	130	148	212	216	239	239	289	289	125	125	150	150	238	240	111	113	165	165	202	208	219	225	270	272
318	DD	GE	breeder	89	89	117	117	157	163	203	203	234	236	92	94	130	150	212	214	239	245	293	293	111	129	148	148	240	244	103	107	153	157	202	210	219	225	274	274
319	DD	GE	breeder	89	89	117	119	161	163	201	203	224	236	94	94	130	148	214	216	239	245	287	291	111	129	148	150	230	230	107	109	153	153	202	206	219	219	272	274
320	DD	GE	breeder	89	93	117	129	159	161	203	203	234	236	94	94	146	148	210	212	245	245	291	293	111	125	144	148	238	240	105	109	165	165	202	210	219	233	272	274
321	DD	GE	breeder	89	89	117	127	159	163	201	203	236	236	92	94	146	148	214	216	245	245	287	293	125	129	144	148	240	244	107	111	153	165	204	206	233	233	270	274
322	DD	CZ	authors	89	89	123	129	163	165	199	203	224	228	94	98	130	150	212	216	245	245	287	293	111	129	148	148	238	244	103	111	157	165	206	210	219	219	270	272
323	DD	CAN	breeder	87	93	117	117	163	165	201	203	234	236	94	98	146	150	198	212	245	245	287	289	111	127	148	148	230	230	105	107	165	165	202	206	219	219	270	272
324	DD	CAN	breeder	89	93	127	129	163	165	203	207	224	228	94	98	130	150	212	216	247	247	293	293	125	127	148	148	230	240	101	107	149	157	202	202	219	219	270	274
325	DD	CAN	breeder	89	89	125	125	159	163	201	203	228	228	94	98	146	146	212	212	245	245	289	291	125	125	148	150	230	238	107	111	153	165	202	208	219	219	274	274
326	DD	CAN	breeder	87	89	119	127	163	167	203	207	228	228	94	98	150	150	208	212	239	239	287	293	111	127	148	148	230	238	107	109	157	157	202	208	219	225	270	274
327	DD	ESP	breeder	93	93	117	125	157	161	201	203	228	228	92	92	148	148	214	216	245	253	289	293	125	127	144	150	240	246	109	113	157	165	202	206	219	219	270	274
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334	DD	SWE	breeder	89	89	117	117	163	165	201	201	228	234	94	94	146	150	208	216	239	245	289	293	125	131	144	144	238	240	107	111	153	165	202	202	219	219	270	272
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336	DD	SWE	breeder	87	89	117	127	159	165	201	203	228	234	94	94	144	148	198	216	245	245	293	293	111	111	148	150	230	244	107	111	165	165	202	202	219	219	270	272
337	DD	CZ	authors	89	93	117	117	163	165	201	201	228	236	94	94	146	146	212	212	245	253	289	291	125	125	148	152	240	246	103	109	153	165	202	202	219	221	270	270
338	DD	USA	breeder	89	89	117	123	161	163	199	201	234	236	94	94	148	150	216	216	239	245	289	291	127	131	144	148	244	244	105	109	157	165	202	206	219	233	270	272
339	GWP	NOR	breeder	89	89	119	125	165	165	203	205	228	236	94	98	130	150	212	216	245	247	289	293	125	125	144	150	240	244	107	109	157	177	204	206	223	225	274	274
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361	GWP	NOR	breeder	85	87	117	129	163	165	203	203	228	228	98	98	132	146	212	212	239	245	289	289	111	125	148	148	240	244	103	107	153	153	202	210	233	233	272	274
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384	GSP	CZ	authors	89	89	117	125	159	159	201	201	234	236	92	100	146	146	212	216	245	247	291	297	125	125	148	148	234	240	105	107	157	161	202	202	219	225	270	272
385	GSP	CZ	authors	89	89	123	127	159	165	201	203	228	234	94	102	130	146	208	210	247	251	289	289	111	125	144	148	234	234	105	105	157	165	202	208	225	237	270	270

386	GSP	CZ	authors	89	89	115	123	159	165	203	207	228	236	94	102	148	150	216	216	245	247	289	291	131	133	144	144	230	242	107	111	153	169	198	202	225	225	270	270
387	GSP	CZ	authors	89	89	123	125	157	165	201	201	224	228	94	94	146	150	208	208	245	251	285	289	111	125	148	148	230	234	105	113	165	165	202	202	225	225	270	270
388	GSP	CZ	authors	89	93	115	123	159	165	203	203	228	228	94	102	146	148	208	216	247	253	291	291	111	127	144	150	230	230	107	109	157	161	202	202	225	229	270	270
389	GSP	CZ	authors	89	89	123	123	159	159	203	203	234	236	94	100	146	146	212	216	245	247	285	289	125	133	148	148	234	240	105	107	153	169	202	202	225	237	270	272
390	GSP	CZ	authors	87	89	123	129	159	165	201	207	228	238	94	100	146	148	216	216	245	247	287	291	111	127	144	148	230	234	107	107	157	169	202	202	225	225	270	270
391	GSP	CZ	authors	89	93	117	125	159	165	203	203	228	238	102	102	146	148	206	212	245	247	287	289	111	131	144	150	230	230	105	111	165	169	202	202	219	225	270	270
392	GSP	CZ	authors	87	89	123	125	159	165	199	203	236	242	100	102	146	150	212	216	245	247	293	297	111	133	144	148	230	242	105	105	153	169	202	206	225	237	270	270
393	GSP	CZ	authors	89	89	117	123	165	165	203	203	228	228	98	102	148	150	198	216	247	249	291	291	125	125	144	148	230	234	103	107	165	177	202	202	225	237	270	270
394	GSP	CZ	authors	89	89	115	123	159	165	201	203	228	234	88	94	144	150	198	210	245	247	289	291	111	133	144	144	230	230	107	109	157	169	202	206	219	237	270	276
395	GSP	CZ	authors	89	89	123	125	159	165	199	201	224	238	92	102	144	150	198	208	245	247	289	291	111	125	144	144	234	246	105	107	153	169	202	206	225	237	270	270
396	GSP	CZ	authors	89	89	117	127	159	159	203	207	234	236	102	102	144	146	210	212	239	245	291	291	125	133	144	148	230	230	105	111	157	157	202	202	237	237	270	270
397	GSP	CZ	authors	89	89	117	117	159	159	199	203	228	228	94	98	132	146	210	216	247	253	289	289	125	125	144	144	230	246	105	105	169	169	202	206	219	225	270	270
398	GSP	CZ	authors	89	89	117	129	159	165	201	207	234	234	94	102	146	148	198	198	245	251	285	287	125	125	144	150	230	234	105	111	153	169	202	202	225	225	270	270
399	GSP	CZ	authors	87	89	123	123	159	165	203	207	234	236	92	100	146	148	198	210	245	253	287	287	125	125	148	148	230	242	109	111	153	157	202	202	225	237	270	276
400	GSP	CZ	authors	89	89	117	125	159	165	201	207	228	238	98	100	146	148	208	212	249	253	289	291	111	125	144	152	240	246	103	105	157	165	202	208	225	225	270	270
401	GSP	CZ	authors	89	89	115	125	161	167	203	203	228	238	94	102	146	148	216	216	245	247	285	291	123	123	148	148	234	234	101	109	157	169	202	202	219	225	270	270
402	GSP	NZ	breeder	87	89	117	123	165	167	203	207	228	236	94	102	132	144	210	216	245	249	289	297	125	133	148	148	234	240	111	111	169	169	202	202	219	225	270	270
403	GSP	NZ	breeder	89	89	117	123	159	159	201	203	234	242	94	98	132	150	208	212	245	251	285	289	111	125	148	148	234	246	105	105	157	157	202	202	219	225	270	270
404	GSP	SWE	breeder	85	93	123	125	159	165	201	203	228	228	92	102	146	146	206	206	241	245	287	289	125	131	150	150	234	246	105	105	165	169	202	202	225	225	270	270
405	GSP	SWE	breeder	89	89	117	125	165	165	201	207	228	236	102	102	146	148	206	210	251	251	285	289	125	133	148	148	234	234	105	105	157	169	202	204	225	233	270	270

Table S2: *Information about hunting breeds gathered from different scientific papers.*

Breed	n	H_E	Ho	Na	F_{IS}	Ar	Author
German Wirehaired Pointer	50	0.71	0.69	6.94	nd	nd	(DeNise et al., 2004)
	11	0.73	0.72	5.10	nd	nd	(Pedersen et al., 2013)
German Shorthaired Pointer	148	0.69	0.68	7.35	nd	nd	(DeNise et al., 2004)
	30	0.70	0.69	nd	0.018	5.4	(Leroy et al., 2009)
	36	0.72	0.71	6.2	nd	nd	(Pedersen et al., 2013)
Wirehaired Pointing Griffon	33	0.57	0.62	4.94	nd	nd	(DeNise et al., 2004)
	27	0.69	0.71	nd	-0.027	5.0	(Leroy et al., 2009)
Pointer	50	0.68	nd	nd	nd	nd	(Parra et al., 2008)
	20	0.62	0.57	nd	0.080	4.8	(Leroy et al., 2009)
	78	0.64	0.60	5.82	nd	nd	(DeNise et al., 2004)
English Setter	66	0.62	nd	nd	nd	nd	(Parra et al., 2008)
	20	0.66	0.64	nd	0.027	5.1	(Leroy et al., 2009)
	125	0.48	0.45	5.29	nd	nd	(DeNise et al., 2004)
English Setter (show)	36	0.46	0.43	3.7	nd	nd	(Pedersen et al., 2013)
English Setter (field)	13	0.66	0.69	4.7	nd	nd	(Pedersen et al., 2013)
Irish Red White Setter	30	0.7	0.65	nd	0.069	5.3	(Leroy et al., 2009)
Irish Setter	132	0.65	0.63	6.12	nd	nd	(DeNise et al., 2004)
	17	0.63	0.62	4.8	nd	nd	(Pedersen et al., 2013)
Gordon Setter	149	0.57	0.55	5.18	nd	nd	(DeNise et al., 2004)
Vizsla	116	0.68	0.67	6.00	nd	nd	(DeNise et al., 2004)
Weimaraner	24	0.64	0.66	nd	-0.033	4.3	(Leroy et al., 2009)
	36	0.61	nd	nd	nd	nd	(Irion et al., 2003)
	50	0.72	0.65	6.90	0.059	nd	(Streitberger et al., 2012)
	88	0.54	0.54	5.24	nd	nd	(DeNise et al., 2004)
Labrador Retriever	16	0.56	0.54	nd	0.042	3.3	(Björnerfeldt et al., 2008)
	22	0.60	0.58	nd	0.021	4.4	(Leroy et al., 2009)
	50	0.48	0.35	3.30	nd	nd	(Zajc et al., 1997)
	52	0.66	0.65	5.76	nd	nd	(DeNise et al., 2004)
	44	0.64	nd	nd	nd	nd	(Irion et al., 2003)
Bracco Italiano	72	0.64	0.59	6.43	0.060	nd	(Ciampolini et al., 2011)
Brittany Spaniel	16	0.66	nd	nd	nd	nd	(Parra et al., 2008)
	44	0.66	nd	nd	nd	nd	(Irion et al., 2003)
	64	0.64	0.64	5.65	nd	nd	(DeNise et al., 2004)
	30	0.68	0.71	nd	-0.032	5.0	(Leroy et al., 2009)
	72	0.65	0.63	6.50	nd	nd	(Pedersen et al., 2013)
Dachshunds	632	0.74	0.67	7.60	0.047	nd	(Přibáňová et al., 2009)
	130	0.72	0.64	7.88	nd	nd	(DeNise et al., 2004)
Hanover Hound	92	0.66	0.67	6.44	-0.012	nd	(Lüpke and Distl, 2005)

Table S3: *List of sampled animals for GWAS study. The table contains the number of samples; date of birth (DOB); information about aRFA affection (severity L2 and more; Y/N); severity of the affection ("0" - healthy individual; "L1" – affection on ears; "L2" – affection on body sides up to 10x10 cm; "L3" – affection on body sides up to 10x25 cm, affection of body sides up to cm; "L4" – affection on body sides up to 10x40 cm; "head" – affection only on the head); the age of aRFA onset (in years). Information about body condition was gathered using surveys - "O" - overweight, "JR" - just right, "NA" - information not available.*

Sample number	DOB	Dog ID	aRFA	aRFA affection severity	aRFA onset (years)	Sex	Body condition
1	06.04.2004	15618	Y	L2	8	M	O
2	15.04.2007	15619	N	0		M	JR
3	26.04.2014	15625	N	0		F	NA
4	20.05.2007	15622	N	0		F	JR
5	20.05.2007	15621	N	0		F	JR
6	09.05.2013	15623	N	0		F	JR
7	24.10.2006	15624	N	0		F	JR
8	25.04.2009	cf_8	N	0		M	O
9	22.07.2006	16165	Y	L3	2	F	JR
10	02.03.2010	16166	Y	L4	4	M	JR
11	04.07.2009	16167	Y	L2	2	F	JR
12	23.02.2007	16168	Y	L3	2	F	JR
13	24.05.2007	16169	Y	L2	7	F	O
14	08.11.2011	16170	N	0		M	O
15	26.04.2010	16171	Y	L3	3	F	JR
16	02.04.2007	16172	Y	L3	4	F	O
17	09.03.2014	16173	Y	L3	1	F	JR
18	22.05.2014	16174	N	0		F	JR
19	28.12.2006	16175	N	0		M	JR
20	28.12.2006	16176	N	0		M	JR
21	18.01.2009	16177	N	0		M	JR
22	25.01.2013	16178	N	0		M	JR
23	03.01.2010	cf_23	Y	Head	4	M	JR
24	01.12.2010	16180	N	0		M	JR
25	28.01.2012	16181	N	0		M	JR
26	23.01.2009	16182	N	0		M	JR
27	06.05.2012	16183	N	0		M	JR

28	07.06.2012	16184	N	0		M	JR
29	20.02.2007	16185	N	0		M	JR
30	31.05.2013	16186	N	0		M	NA
31	20.11.2006	cf_31	N	L1		M	NA
32	18.05.2010	cf_32	N	L1		M	JR
33	24.04.2011	16189	N	0		M	JR
34	24.05.2009	16190	N	0		F	JR
35	17.02.2009	16191	N	0		F	JR
36	12.07.2013	16192	N	0		F	O
37	30.07.2010	16193	N	0		F	JR
38	01.12.2010	cf_38	N	0		F	JR
39	19.03.2012	cf_39	N	0		F	JR
40	13.04.2012	cf_40	N	0		F	JR
41	15.02.2013	cf_41	N	0		F	JR
42	01.08.2014	cf_42	N	0		F	NA
43	19.04.2011	cf_43	N	0		M	JR
44	19.07.2006	16200	Y	L2	NA	F	JR
45	10.06.2011	cf_45	N	0		F	NA
46	03.05.2011	cf_46	N	0		F	JR
47	07.01.2014	cf_47	N	0		F	JR
48	24.04.2008	16248	N	0		M	JR
49	06.05.2014	cf_49	N	0		F	JR
50	14.02.2014	cf_50	N	0		F	JR
51	10.03.2011	cf_51	Y	L2	8	F	JR
52	13.05.2003	16252	N	0		M	O
53	23.02.2007	16253	N	0		M	JR
54	14.03.2012	cf_54	N	0		M	JR
55	21.04.2013	cf_55	N	0		F	JR
56	21.07.2011	cf_56	N	0		M	JR
57	25.12.2010	cf_57	N	0		F	JR
58	22.01.2011	cf_58	N	0		F	JR
59	24.08.2010	cf_59	N	0		M	JR
60	08.04.2006	16260	N	0		M	JR
61	11.07.2010	cf_61	N	0		M	JR
62	09.08.2015	cf_62	N	0		F	JR
63	31.12.2008	16263	N	0		M	JR
64	07.03.2008	cf_64	N	0		M	JR
65	07.04.2011	cf_65	Y	L2	8	F	JR
66	28.05.2007	16266	N	0		M	JR
67	19.07.2010	cf_67	N	0		F	JR
68	27.08.2009	16268	N	0		M	JR

69	27.04.2012	18185	Y	L2	4	F	JR
70	06.03.2008	cf_70	N	0		F	O
71	11.09.2006	18187	Y	L2	NA	F	JR
72	28.04.2014	cf_72	N	0		M	JR
73	19.03.2013	cf_73	N	0		F	JR
74	08.03.2014	cf_74	N	0		M	JR
75	01.01.2008	cf_75	N	0		F	O
76	16.10.2012	cf_76	Y	L2	6	F	JR
77	01.01.2008	18193	N	L1		M	JR
78	09.03.2014	cf_78	N	0		F	NA
79	19.04.2011	18195	Y	L2	5	F	JR
80	03.02.2007	18196	N	0		M	JR
81	27.02.2014	cf_81	N	0		F	JR
82	15.05.2010	cf_82	N	0		F	JR
83	21.05.2015	cf_83	N	0		F	JR
84	19.03.2015	cf_84	N	0		F	JR
85	01.02.2015	cf_85	N	0		M	JR
86	01.05.2011	18203	N	0		F	JR
87	20.08.2010	cf_88	N	0		F	JR
88	25.04.2007	18205	N	0		M	JR
89	15.04.2013	cf_90	N	0		M	JR
90	10.03.2012	cf_91	N	0		M	O
91	28.01.2006	18208	Y	L2	NA	F	O
92	13.04.2015	cf_93	N	0		F	JR
93	14.07.2005	18210	Y	L3	NA	F	JR
94	03.01.2008	18211	N	0		F	JR
95	16.01.2015	cf_96	N	0		F	JR
96	11.05.2007	18213	Y	L4	4	F	JR
97	07.04.2015	cf_98	N	0		M	JR
98	14.06.2005	18215	Y	L3	3	F	JR
99	10.08.2010	cf_100	N	0		F	JR
100	27.07.2013	cf_101	N	0		M	JR
101	28.04.2007	18218	Y	L2	8	F	JR
102	16.04.2004	18219	N	0		F	JR
103	22.01.2011	cf_104	N	0		F	JR
104	24.03.2014	cf_105	N	0		F	O
105	25.04.2009	18222	Y	L3	4	M	JR
106	20.04.2011	cf_107	N	0		M	NA
107	02.02.2007	cf_108	N	L1	7	M	NA
108	15.02.2003	18225	Y	L4	8	F	NA
109	22.06.2009	18226	Y	L4	5	M	O

110	24.09.2009	18227	N	0		F	JR
111	05.09.2008	cf_112	N	L1		F	JR
112	01.05.2015	cf_113	N	0		F	JR
113	25.04.2009	18230	Y	L2	6	F	JR
114	08.04.2000	6021	Y	L2	NA	F	O
115	16.04.2011	7916	Y	L4	3	F	JR
116	13.07.2009	21249	Y	L3	3	F	O
117	10.04.2014	20855	Y	L2	3	M	JR
118	10.04.2014	20856	Y	L2	2	M	JR
119	14.09.2010	21251	Y	L2	6	F	O
120	06.09.2007	21252	Y	L2	5	F	JR
121	04.08.2011	21253	Y	L2	6	F	JR
122	18.05.2010	21254	Y	L3	5	F	JR
123	23.02.2011	21255	Y	L4	6	F	O
124	26.01.2009	21256	Y	L3	4	M	O
125	15.04.2012	21257	Y	L3	5	F	O
126	07.08.2006	21258	Y	L4	3	F	JR
127	03.04.2014	21259	Y	L3	1	F	O
128	06.04.2012	21260	Y	L3	2	F	JR
129	18.02.2008	21261	Y	L4	1	F	O
130	20.01.2010	21262	Y	L3	2	F	O
131	01.02.2008	21263	Y	L3	3	M	JR
132	20.02.2010	21264	Y	L3	4	F	JR
133	01.01.2011	21265	Y	L3	6	F	JR
134	05.09.2011	21266	Y	L2	2	F	JR
135	25.06.2014	21267	Y	L3	2	F	JR
136	03.03.2005	21268	Y	L4	3	M	JR
137	25.06.2014	21269	Y	L3	1	F	O
138	11.03.2007	21270	Y	L3	5	F	JR
139	08.03.2009	21271	Y	L3	1	F	O
140	18.04.2009	21272	Y	L2	7	F	JR
141	15.02.2009	21273	Y	L3	NA	F	JR
142	09.07.2004	21274	Y	L3	8	M	JR
143	31.07.2009	21275	Y	L2	3	F	JR
144	11.03.2007	21276	Y	L2	NA	M	O
145	15.09.2005	22724	Y	L4	6	F	JR
146	25.04.2009	22722	Y	L3	4	F	JR
147	28.07.2009	cf_149	Y	Head	3	M	JR
148	22.04.2010	21410	Y	L3	3	F	O
149	08.04.2006	21412	Y	L3	2	F	O
150	27.05.2013	21413	Y	L3	4	F	JR

151	08.03.2010	23466	Y	L3	NA	F	NA
152	22.03.2010	23467	N	0		F	JR
153	19.03.2016	23468	Y	L4	2	F	JR
154	15.03.2009	23469	Y	L3	8	F	JR
155	22.01.2012	23470	Y	L3	NA	F	JR
156	07.07.2006	6592	Y	L4	2	F	JR
157	07.07.2006	6032	Y	L3	4	F	JR
158	03.03.2002	6218	N	0		F	JR
159	21.01.2001	6061	Y	L3	6	F	JR
160	02.01.2011	cf_159	Y	L3	4	F	JR
161	24.08.2010	21408	Y	L3	4	F	O
162	06.04.2014	21411	Y	L3	1	F	NA
163	26.03.2008	24725	Y	L2	4	F	O
164	18.07.2014	24726	Y	L4	1,5	F	JR
165	19.03.2015	24727	Y	L4	3	F	JR
166	01.08.2013	24728	Y	L3	5	F	JR
167	26.04.2010	cf_166	Y	L4	5	F	O
168	03.01.2010	cf_167	N	0		F	O
169	31.12.2008	cf_168	Y	L3	6	F	O
170	01.03.2014	cf_169	Y	L3	3	F	JR
171	09.12.2015	cf_170	Y	L3	1	M	JR
172	18.12.2011	cf_171	Y	L3	2	F	JR
173	17.08.2008	cf_172	Y	L3	1	F	JR
174	03.01.2010	cf_173	Y	L3	NA	F	NA
175	06.04.2007	cf_174	Y	L3	8	F	JR
176	22.01.2012	cf_175	Y	Head	3	M	JR
177	25.05.2010	cf_176	Y	L3	NA	M	NA
178	30.05.2007	cf_177	Y	L3	2	F	JR
179	10.09.2012	cf_178	Y	L3	2	F	JR
180	06.05.2006	cf_179	Y	L4	6	F	O
181	30.05.2010	cf_180	Y	L3	5	M	JR
182	24.04.2011	cf_181	Y	L4	2	F	O
183	09.07.2015	cf_182	Y	L2	3	F	JR
184	05.07.2007	cf_183	Y	L4	2	M	O
185	18.07.2009	cf_184	Y	L2	4	F	O
186	24.01.2013	cf_185	N	L1	3	F	JR
187	18.05.2010	cf_186	Y	L3	1	F	O
188	19.05.2010	cf_187	N	0		F	O
189	26.01.2017	cf_188	N	0		F	JR
190	08.06.2013	cf_189	Y	L2	4	F	O
191	07.04.2009	cf_190	Y	L4	3	F	O

192	23.06.2006	5469	N	0		F	JR
193	27.02.2014	12037	Y	L2	4	F	JR
194	08.11.2017	24805	N	0		M	JR
195	18.08.2017	22814	N	0		F	NA
196	20.02.2018	23952	N	0		M	JR
197	19.03.2018	24342	N	0		M	NA
198	19.03.2018	24344	N	0		M	NA
199	01.03.2015	14707	N	0		F	JR
200	14.08.2008	6220	N	0		F	JR
201	03.04.2015	14854	N	0		F	JR
202	29.03.2014	20151	N	0		M	NA
203	11.11.2015	18103	N	0		M	NA
204	23.05.2018	24902	N	0		F	NA
205	07.07.2016	19059	N	0		F	JR
206	10.01.2016	16427	N	0		M	JR
207	22.03.2018	24787	N	0		M	NA
208	21.04.2017	25264	N	0		M	JR
209	15.02.2005	cf_Anja	N	0		F	JR
210	14.05.2013	cf_Bo	N	0		M	JR
211	15.05.2014	cf_Brita	N	0		F	NA
212	14.04.2015	cf_Murphy	N	0		M	JR
213	07.03.2015	cf_Argo	N	0		M	JR
214	02.06.2015	cf_Rika	N	0		F	JR
215	21.05.2015	cf_Adele	N	0		F	JR
216	31.12.2015	cf_Alma	N	0		F	NA

Table S4: *Summary of patient data from animals included in the study for transcriptome analysis. Animals were included or excluded from RNA sequencing analysis based on the presence of consistent histological features and clinical picture.*

Dog Number	Group	Age at time of biopsy	Sex
B2	Control	6 y 2 mo	F
B5	Control	10 y 5 mo	M
B6	Control	7 y 7 mo	F
B12	Control	7 y 9 mo	F
B13	Control	3 y 2 mo	F
B14	Control	1 y 4 mo	F
B7	Alopecia	9 y 2 mo	F
B9	Alopecia	10 y 11 mo	F
B10	Alopecia	3 y 11 mo	F
B11	Alopecia	1 y 11 mo	F
B3	Alopecia	9 y 4 mo	F

Table S5: *Results of one of the additional GWAS analyses for two groups of individuals - healthy individuals of age 10+ and alopecic individuals with severe affection (level 4 aRFA). The top SNP on chromosome 21 is the same SNP as in the analysis of early affection (Table 5), however, the significance level is not met.*

Chr	SNP name	Position (bp)	Allele Freq	P-value
21	BICF2G630640798	47085771	0.204	1.28x10-06
32	BICF2P976235	8550630	0.463	1.07x10-05
26	BICF2P946509	33145128	0.093	1.46x10-05
1	BICF2G630715088	19568314	0.426	1.77x10-05
32	BICF2P907131	27577699	0.065	2.52x10-05
8	BICF2P1386913	55523835	0.370	2.97x10-05
8	BICF2S23448109	63200694	0.102	3.71x10-05
10	BICF2G630476932	18071694	0.213	4.37x10-05
8	chr8_59707832	59707832	0.204	4.40x10-05
19	BICF2G630254010	49287352	0.259	4.62x10-05
26	BICF2P1246793	32942963	0.176	4.72x10-05
32	BICF2P1149618	10657030	0.296	5.29x10-05
32	BICF2P213903	10649129	0.296	5.29x10-05
32	TIGRP2P378950_rs8534752	10680407	0.296	5.29x10-05
32	chr32_10656790	10656790	0.296	5.29x10-05
17	TIGRP2P235215_rs8492674	54162595	0.426	5.36x10-05
39	BICF2G630535234	19332927	0.157	6.38x10-05
21	BICF2P300797	45020370	0.463	6.57x10-05
21	BICF2P597850	45432234	0.194	7.15x10-05
10	BICF2S23763624	18015742	0.194	7.24x10-05

Table S6: *List of all genes identified by GWAS analysis and haplotype analysis. Stated are the known functions for each candidate gene (from www.genecards.org) and a metabolic super pathway the gene is a part of (from pathcards.genecards.org). In yellow are colored genes controlling keratin metabolism, in orange are colored genes connected to circadian rhythm.*

Chromosome	Gene code	Protein	Known function	SuperPathway (highest overlap)
19	**ACVR2A**	Activin A Receptor Type A2	Mediator of signaling	Signaling by NODAL
19	**ENSCAFT00000006550 (SLC25A3**)	Solute Carrier Family 25 Member 3	Transport activity from the cytosol to mitochondria	C-MYB transcription factor network
19	**GTDC1**	Glycosyltransferase Like Domain Containing 1	Transferase activity	
19	**ORC4**	Origin Recognition Complex Subunit 4	DNA replication, cell cycle	CDK-mediated phosphorylation and removal of Cdc6
19	**ZEB2**	Zinc Finger E-Box Binding Homeobox 2	Transcriptional inhibitor	TGF-beta Receptor Signaling Pathway
19	**GALNT16**	Polypeptide N-Acetylgalactosaminyltransferase 16	Catalyzes the initial reaction in O-linked oligosaccharide biosynthesis, the transfer of an N-acetyl-D-galactosamine residue to a serine or threonine residue on the protein receptor	Metabolism of proteins
19	**LOC100856294**	rho GTPase-activating protein 20-like		
19	**LOC111091126**	uncharacterized		
19	**ATP5PD**	ATP Synthase Peripheral Stalk Subunit D	ATP synthesis	Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins
19	**PRDM5**	PR/SET Domain 5	Transcription factor, cell differentiation	
19	**NDNF**	Neuron Derived Neurotrophic Factor	Secretory protein - various cellular processes	
8	**ACTN1**	Actinin Alpha 1	Anchors actin to a variety of intracellular structures; bundling protein	Cell junction organization

8	**ADAM20**	ADAM Metallopeptidase Domain 20	Involved in sperm maturation and/or fertilization	Reproduction
8	**ACOT6**	Acyl-CoA Thioesterase 6	Regulation of lipid metabolism	Fatty Acyl-CoA Biosynthesis
8	**ADAM21**	ADAM Metallopeptidase Domain 21	Fertilization, muscle development, neurogenesis	Reproduction
8	**ALDH6A1**	Aldehyde Dehydrogenase 6 Family Member A1	Valine and pyrimidine metabolism; binds fatty acyl-CoA	Valine degradation
8	**ARG2**	Arginase 2	Regulation of extra-urea cycle, regulation of immune response	CDK-mediated phosphorylation and removal of Cdc6
8	**ATP6V1D**	ATPase H+ Transporting V1 Subunit D	Transport processes in the vacuolar system	RET signaling
8	**BBOF1**	Basal Body Orientation Factor 1	Aligns and maintains cilia orientation in response to flow	
8	**CCDC177**	Coiled-Coil Domain Containing 177		
8	**CCDC196**	Coiled-Coil Domain Containing 196		
8	**COQ6**	Coenzyme Q6	Mitochondrial electron transport, antioxidant	Metabolism
8	**COX16**	Cytochrome C Oxidase Assembly Factor	Mitochondrial respiratory chain complex	Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins
8	**DCAF4**	DDB1 And CUL4 Associated Factor 4	Substrate receptor for ubiquitin-protein ligase complex	
8	**DCAF5**	DDB1 And CUL4 Associated Factor 5	Substrate receptor for ubiquitin-protein ligase complex	
8	**DNAL1**	Dynein Axonemal Light Chain 1	Generates force for cilia mobility	
8	**DPF3**	Double PHD Fingers 3	An essential role in heart and skeletal muscle	
8	**EIF2S1**	Eukaryotic Translation Initiation Factor 2 Subunit Alpha	Protein synthesis	Peptide chain elongation
8	**ENSCAFT00000026484** (uncharacterized protein)			

8	**ENTPD5**	Ectonucleoside Triphosphate diphosphohydrolase 5	Metabolism of nucleotides	Purine metabolism
8	**ERH**	ERH mRNA Splicing And Mitosis Factor	Cell cycle; skin disease - ectodermal dysplasia	
8	**EXD2**	Exonuclease 3' - 5' Domain Containing 2	Various cellular proceses	
8	**FAM161B**	FAM 161 Centrosomal Protein B		
8	**FA71D**	Golgi Associated RAB2 Interactor Family Member 2		
8	**FUT8**	Fucosyltranferase 8	Keratin metabolism	Transport to the Golgi and subsequent modification
8	**GPHN**	Gephyrin	Microtubule-associated protein; plays a role in inhibitory synapses	Metabolism of water-soluble vitamins and cofactors
8	**HEATR4**	HEAT Repeat Containing 4		
8	**LIN52**	Lin-52 DREAM MuvB Core Complex Component	Regulation of PLK1 Activity at G2/M Transition and Cell cycle	Cell Cycle, Mitotic
8	**MAP3K9**	Mitogen-Activated Protein Kinase Kinase Kinase 9	Cellular responses evoked by changes in the environment; signal transduction	TGF-Beta Pathway
8	**MED6**	Mediator Complex Subunit 6	RNA transcription, gene expression	Regulation of lipid metabolism by Peroxisome proliferator-activated receptor alpha (PPARalpha)
8	**MIDEAS**	Mitotic Deacetylase Associated SANT Domain Protein		
8	**MPP5**	Membrane Palmitoylated Protein 5	Tight junction biogenesis; cell polarity in epithelial cells	Cell junction organization
8	**NUMB**	NUMB Endocytic Adaptor Protein	Neurogenesis	Signaling by NOTCH1
8	**PAPLN**	Papilin, Proteoglycan Like Sulfated Glycoprotein	Peptidase activity	
8	**PCNX1**	Pecanex1	Developmental processes	
8	**PIGH**	Phosphatidylinositol Glycan Anchor Biosynthesis Class H	GPI biosynthesis	Metabolism of proteins
8	**PLEK2**	Pleckstrin 2	Cytoskeletal arrangement	

8	**PLEKHD1**	Pleckstrin Homology And Coiled-Coil Domain Containing D1		
8	**PLEKHH1**	Pleckstrin Homology, MyTH4, And FERM Domain Containing H1		
8	**PNMA1**	PNMA Family Member 1	Neuron- and testis- specific protein	
8	**RAD51B**	RAD51 Paralog B	DNA break repair	Homologous DNA Pairing and Strand Exchange
8	**PSEN1**	Presenilin 1	Various cellular processes	Innate Immune Systém
8	**RBM25**	RNA Binding Motif Protein 25	Regulator of alternative pre-mRNA splicing; Involved in apoptotic cell death	
8	**RDH11**	Retinol Dehydrogenase 11	Retinal reductase	Metabolism of fat-soluble vitamins
8	**RDH12**	Retinol Dehydrogenase 12	Retinal reductase	Signaling by GPCR
8	**RGS6**	Regulator Of G Protein Signaling 6	Regulates G protein-coupled receptor signaling cascades	Chaperonin-mediated protein folding
8	**RIOX1**	Ribosomal Oxygenase 1	Ribosome biogenesis; central role in histone code	Chromatin Regulation/Acetylation
8	**SIPA1L1**	Signal Induced Proliferation Associated 1 Like 1	Reorganization of the actin cytoskeleton	Protein-protein interaction at synapses
8	**SLC10A1**	Solute Carrier Family 10 Member 1	Hepatic sodium/bile acid uptake system	Synthesis of bile acids and bile salts
8	**SLC39A9**	Solute Carrier Family 39 Member 9	Zinc-influx transporter	Metal ion SLC transporters
8	**SLC8A3**	Solute Carrier Family 8 Member A3	Member of the sodium/calcium exchanger	Cardiac conduction
8	**SMOC1**	SPARC Related Modular Calcium Binding 1	Essential roles in both, eye and limb development	
8	**SRSF5**	Serine And Arginine Rich Splicing Factor 5	Plays a role in constitutive splicing	Cleavage of Growing Transcript in the Termination Region
8	**SUSD6**	Sushi Domain Containing 6	Growth-suppressive activity and cell death	

8	**SYNJ2BP**	Synaptojanin 2 Binding Protein	Regulates endocytosis of activin type 2 receptor kinases; signal transduction	
8	**SNORD141A and B**	Small Nucleolar RNA, C/D Box 141A		
8	**TMEM229B**	Transmembrane Protein 229B		
8	**TTC9**	Tetratricopeptide Repeat Domain 9	Cancer cell invasion and metastasis; Ichthyosis	
8	**VSX2**	Visual System Homeobox 2	Transcriptional regulator; retinal development	
8	**ZFP36L1**	ZFP36 Zing Finger Protein Like 1	Various cellular processes; keratin metabolism	CDK-mediated phosphorylation and removal of Cdc6
8	**ZFYVE26**	Zinc Finger FYVE-Type Containing 1	DNA break repair	Cytoskeletal signaling
8	**ZNF410**	Zinc Finger Protein 410	Activates transcription of matrix-remodeling genes such as MMP1 during fibroblast senescence	
30	**ADAM10**	ADAM Metallopeptidase Domain 10	Cleaves many proteins	Signaling by NOTCH1
30	**ALDH1A2**	Aldehyde Dehydrogenase 1 Family Member A2	Vit A metabolism	Signaling by Retinoic Acid
30	**ANXA2**	Annexin A2	Regulation of cellular growth and signal transduction pathways (affinity to calcium	Innate Immune Systém
30	**APH1B**	Aph-1 Homolog B, Gamma-Secretase Subunit	Catalyzes the intramembrane cleavage of integral proteins such as Notch receptors and APP (amyloid-beta precursor protein)	Signaling by NOTCH1
30	**ANKDD1A**	Ankyrin Repeat And Death Domain Containing 1A		
30	**AQP9**	Aquaporin 9	Forms a water channel with a broad specificity; Also permeable glycerol and urea	Aquaporin-mediated transport
30	**BNIP2**	BCL2 Interacting Protein 2	Cell death suppression	Myogenesis
30	**CA12**	Carbonic Anhydrase 12	Zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide	Metabolism
30	**CCNB2**	Cyclin B2	Essential for the control of the cell cycle at the G2/M (mitosis) transition	Mitotic Prometaphase

30	**CGNL1**	Cingulin Like 1	Involved in anchoring the apical junctional complex, especially tight junctions, to actin-based cytoskeletons	
30	**CIAO2A**	Cytosolic Iron-Sulfur Assembly Component 2A	Component of the cytosolic iron-sulfur protein assembly (CIA) complex, a multiprotein complex that mediates the incorporation of the iron-sulfur cluster into extramitochondrial Fe/S proteins	
30	**CILP**	Cartilage Intermediate Layer Protein	Cartilage and joint development	RET signaling
30	**CSNK1G1**	Casein Kinase 1 Gamma 1	Involved in many cellular processes including DNA repair, cell division, nuclear localization, and membrane transport	Nanog in Mammalian ESC Pluripotency
30	**CSNK2A1**	Casein Kinase 2 Alpha 1	Involved in various cellular processes, including cell cycle control, apoptosis, and circadian rhythm	Mitotic Prometaphase
30	**CLPX**	Caseinolytic Mitochondrial Matrix Peptidase Chaperone Subunit X	ATP-dependent protein in mitochondria; protein degradation	
30	**DAPK2**	Death Associated Protein Kinase 2	Calcium/calmodulin-dependent serine/threonine kinase involved in multiple cellular signaling pathways that trigger cell survival, apoptosis, and autophagy	CDK-mediated phosphorylation and removal of Cdc6
30	**DENND4A**	DENN Domain Containing 4A	Exchange factor for guanine nucleotides	Vesicle-mediated transport
30	**DPP8**	Dipeptidyl Peptidase 8	May play a role in T-cell activation and immune function; inhibitor of pyroptosis	
30	**ENSCAFT00000025834 (MYZAP)**	Myocardial Zonula Adherens Protein	Cellular signaling	
30	**ENSCAFT00000026921 (FBXL22)**	F-Box And Leucine-Rich Repeat Protein 22	Promotor of ubiquitination	Class I MHC mediated antigen processing and presentation
30	**ENSCAFT00000059804 (PCLAF)**	PCNA Clamp Associated Factor	Regulator of DNA repair during DNA replication	Translesion synthesis by Y family DNA polymerases bypasses lesions on DNA template

30	**FAM81A**	Family With Sequence Similarity 81 Member A		
30	**FOXB1**	Forkhead Box B1		
30	**GCNT3**	Lucosaminyl (N-Acetyl) Transferase 3, Mucin Type	Glycosyltransferase that can synthesize all known mucin beta 6 N-acetylglucosaminides	Metabolism of proteins
30	**GTF2A2**	General Transcription Factor IIA Subunit 2	Plays an important role in transcriptional activation	Assembly of RNA Polymerase-II Initiation Complex
30	**HACD3**	3-Hydroxyacyl-CoA Dehydratase 3	Catalyzator of the fatty acids elongation cycle	Fatty Acyl-CoA Biosynthesis
30	**HERC1**	HECT And RLD Domain Containing E3 Ubiquitin Protein Ligase Family Member 1	Involved in membrane transport processes	Class I MHC mediated antigen processing and presentation
30	**ICE2**	Interactor Of Little Elongation Complex ELL Subunit 2	Component of the little elongation complex (LEC), a complex required to regulate small nuclear RNA (snRNA) gene transcription	Gene Expression
30	**IGDCC3**	Immunoglobulin Superfamily DCC Subclass Member 3		
30	**IGDCC4**	Immunoglobulin Superfamily DCC Subclass Member 4		
30	**INTS14**	Integrator Complex Subunit 14	RNA transcription, gene expression	Gene Expression
30	**KBTBD13**	Kelch Repeat And BTB Domain Containing 13	Transcription regulation, ion channel tetramerization and gating, protein ubiquitination or degradation, and cytoskeleton regulation	Class I MHC mediated antigen processing and presentation
30	**LACTB**	Lactamase Beta	Mitochondrial serine protease that acts as a regulator of mitochondrial lipid metabolism	
30	**LIPC**	Lipase C, Hepatic Type	Hepatic triglyceride lipase, which is expressed in liver	Statin pathway
30	**MEGF11**	Multiple EGF Like Domains 11	Retina development; stress reaction (indirectly)	

30	**MINDY2**	MINDY Lysine 48 Deubiquitinase 2	Regulatory role at the level of protein turnover	
30	**MTFMT**	Mitochondrial Methionyl-TRNA Formyltransferase	Protein encoded by this nuclear gene localizes to the mitochondrion, where it catalyzes the formylation of methionyl-tRNA	Mitochondrial translation
30	**MYO1E**	Myosin IE	Myosins are actin-based motor molecules with ATPase activity and serve in intracellular movements	Actin Nucleation by ARP-WASP Complex
30	**OAZ2**	Ornithine Decarboxylase Antizyme 2	Plays a role in cell growth and proliferation by regulating intracellular polyamines	CDK-mediated phosphorylation and removal of Cdc6
30	**PDCD7**	Programmed Cell Death 7	Promotes apoptosis when overexpressed	mRNA Splicing - Major Pathway
30	**PIF1**	PIF1 5'-To-3' DNA Helicase	Required for the maintenance of both mitochondrial and nuclear genome stability; DNA-dependent adenosine triphosphate (ATP)-metabolizing enzyme; Pathway s TIMELESS (circadian rhythm)	
30	**PLEKHO2**	Pleckstrin Homology Domain Containing O2		Innate Immune Systém
30	**RAB11A**	RAB11A, Member RAS Oncogene Family	Associated with both constitutive and regulated secretory pathways, and may be involved in protein transport; Rab are key regulators of intracellular membrane trafficking	Vesicle-mediated transport
30	**RAB8B**	RAB8B, Member RAS Oncogene Family	Key regulator of intracellular membrane trafficking	Vesicle-mediated transport
30	**RNF111**	Ring Finger Protein 111	Enhancer of gene transcription; associated with KRTAP5-9 and EDARADD (keratin metabolism)	Class I MHC mediated antigen processing and presentation
30	**RORA**	RAR Related Orphan Receptor A	Transcriptional regulation of some genes involved in circadian rhythm, member of the NR1 subfamily of nuclear hormone receptor; Key regulator of embryonic development, cellular differentiation,	Cytokine Signaling in Immune Systém

			immunity, circadian rhythm as well as lipid, steroid, xenobiotics, and glucose metabolism	
30	**SLC24A1**	Solute Carrier Family 24 Member 1	A critical component of the visual transduction cascade, controlling the calcium concentration of outer segments during light and darkness	Signaling by GPCR
30	**SLC51B**	Solute Carrier Family 51 Subunit Beta	Intestinal basolateral transporter responsible for bile acid export from enterocytes into portal blood	Drug Induction of Bile Acid Pathway
30	**SLTM**	SAFB Like Transcription Modulator	When overexpressed, acts as a general inhibitor of transcription that eventually leads to apoptosis	
30	**SNX1**	Sorting Nexin 1	Regulates the cell-surface expression of epidermal growth factor receptor	
30	**SNX22**	Sorting Nexin 22	Plays a role in intracellular trafficking; Epithelioid Trophoblastic Tumor (gene associated is KRT18); Interaction with CSNK1A1 (keratin) and CSNK1E (circadian rhythm)	
30	**SPG21**	SPG21 Abhydrolase Domain Containing, Maspardin	May play a role as a negative regulatory factor in CD4-dependent T-cell activation	
30	**TCF12**	Transcription Factor 12	Involved in the initiation of neuronal differentiation; activates transcription; interaction with ID2 and CREBBP (circadian rhythm)	Myogenesis
30	**TLN2**	Talin 2	Significant role in the assembly of actin filaments	Integrin Pathway
30	**TPM1**	Tropomyosin 1	Binds to actin filaments in muscle and non-muscle cells	Striated Muscle Contraction
30	**TRIP4**	Thyroid Hormone Receptor Interactor 4	Plays a role in thyroid hormone receptor and estrogen receptor transactivation; Also involved in androgen receptor transactivation;	

30	**USP3**	Ubiquitin Specific Peptidase 3	Associates with the chromatin	Deubiquitination
30	**UBAP1L**	Ubiquitin Associated Protein 1 Like		
30	**VPS13C**	Vacuolar Protein Sorting 13 Homolog C	Necessary for proper mitochondrial function and maintenance of mitochondrial transmembrane potential	
30	**ZNF609**	Zinc Finger Protein 609	Transcription factor	
36	**CALCRL**	Calcitonin Receptor Like Receptor		
36	**COL3A1**	Collagen Type III Alpha 1 Chain	Collagen metabolism - extensible connective tissue	Collagen formation
36	**COL5A2**	Collagen Type V Alpha 2 Chain	Collagen metabolism - fibrillar collagen	Collagen formation
36	**FAM171B**	Family With Sequence Similarity 171 Member B		
36	**FSIP2**	Fibrous Sheath Interacting Protein 2	Associated with the sperm fibrous sheath	
36	**GULP1**	GULP PTB Domain Containing Engulfment Adaptor 1	Phagocytosis of apoptotic cells	Arf6 signaling events
36	**ITGAV**	Integrin Subunit Alpha V	Various cellular processes	Integrin Pathway
36	**TFPI**	Tissue Factor Pathway Inhibitor	Regulates the tissue factor (TF)-a dependent pathway of blood coagulation	Formation of Fibrin Clot (Clotting Cascade)
36	**ZC3H15**	Zinc Finger CCCH-Type Containing 15	Stimulates DRG1 GTPase activity likely by increasing the affinity for the potassium ions	
36	**ZNF804A**	Zinc Finger Protein 804A	Zinc finger binding protein	
36	**ZSWIM2**	Zinc Finger SWIM-Type Containing 2	E3 ubiquitin-protein ligase involved in the regulation of Fas-, DR3- and DR4-mediated apoptosis	
21	**ANO3**	Anoctamin 3	calcium-dependent phospholipid scramblase activity; may inhibit pain signaling; potassium channel regulator	Ion channel transport

Table S7: *Top ten most relevant pathways identified using Reactome pathway analysis on downregulated genes.*

Pathway name	Entities				Reactions	
	found	ratio	P-value	FDR	found	ratio
Gap junction assembly	10/41	0.003	5.53x10-4	0.425	16/16	0.001
Extracellular matrix organization	39/329	0.023	5.81x10-4	0.425	125/318	0.025
Oligomerization of connexins into connexons	3/3	2.08x10-4	0.001	0.425	3/3	2.40x10-4
Transport of connexins along the secretory pathway	3/3	2.08x10-4	0.001	0.425	2/2	1.60x10-4
Gap junction trafficking and regulation	11/56	0.004	0.002	0.496	24/24	0.002
GLI proteins bind promoters of Hh responsive genes to promote transcription	4/8	5.54x10-4	0.002	0.544	4/4	3.20x10-4
Gap junction trafficking	10/52	0.004	0.003	0.653	20/20	0.002
Signaling by Hedgehog	21/168	0.012	0.006	0.72	71/82	0.007
RHO GTPases activate PAKs	6/27	0.002	0.01	0.72	14/15	0.001
Hedgehog 'off' state	16/124	0.009	0.011	0.72	26/32	0.003

Table S8: *Top thirteen most relevant pathways identified using Reactome pathway analysis on upregulated genes.*

Pathway name	Entities				Reactions	
	found	ratio	P-value	FDR	found	ratio
Activation of gene expression by SREBF (SREBP)	26/70	0.005	1.87x10-9	2.74x10-6	24/42	0.003
Striated Muscle Contraction	19/40	0.003	6.28x10-9	4.14x10-6	4/4	3.20x10-4
Regulation of cholesterol biosynthesis by SREBP (SREBF)	26/86	0.006	1.06x10-7	4.66x10-5	24/52	0.004
Cholesterol biosynthesis	19/72	0.005	3.30x10-5	0.011	27/32	0.003
Muscle contraction	41/256	0.018	2.48x10-4	0.066	22/41	0.003
Metabolism of steroids	47/322	0.022	6.75x10-4	0.149	87/235	0.019
Interferon alpha/beta signaling	30/184	0.013	0.001	0.227	3/20	0.002
PPARA activates gene expression	27/174	0.012	0.004	0.654	41/41	0.003
Regulation of lipid metabolism by PPARalpha	27/176	0.012	0.005	0.67	42/44	0.004
Synthesis of very long-chain fatty acyl-CoAs	10/51	0.004	0.016	0.867	8/12	9.61x10-4
Class I peroxisomal membrane protein import	5/20	0.001	0.033	0.867	6/6	4.80x10-4
Cholesterol biosynthesis via lathosterol	4/14	9.70x10-4	0.036	0.867	4/4	3.20x10-4
Cholesterol biosynthesis via desmosterol	4/14	9.70x10-4	0.036	0.867	4/4	3.20x10-4

Table S9: Fisher's exact test of eleven overlapping genes from GWAS study and RNA-seq study. The overlap was found non-significant.

V1 V2	**p-value**
ARG2	1.039044225
HACD3	1.017925863
PAPLN	0.780319225
TPM1	0.723505465
LACTB	0.558208956
RAD51B	0.497676468
SLC25A3	0.489588717
RDH11	0.478515289
GTDC1	0.459480995
SNX1	0.3261547
TRIP4	0.311368462

Appendices

List of the Appendices:

Appendix 1:

Title: Tracing genetic resurrection of pointing dog breeds: Cesky Fousek as both survivor and rescuer.

Authors: Silvie Neradilová, Laurie Connell, Pavel Hulva, Barbora Černá Bolfíková. Journal: PLoS ONE 14(8): e0221418.

Appendix 2:

Title: Genomic and Transcriptomic Characterization of Atypical Recurrent Flank Alopecia in the Cesky Fousek.

Authors: Silvie Neradilová, Alexandria M. Schauer, Jessica J. Hayward, Magdalena A. T. Brunner, Magdalena Bohutínská, Vidhya Jagannathan, Laurie B. Connell, Adam R. Boyko, Monika M. Welle, Barbora Černá Bolfíková.

Journal: Genes 2022, 13, 650.

Appendix 3:

Results of differential expression analysis investigating biopsies of alopecic skin of dogs with aRFA and control biopsies. Significantly differentially expressed genes sorted by Log2 Fold Change; BaseMean, mean of normalized read counts across all samples; LfcSE, standard error of the log2FoldChange; Stat, the log2FoldChange divided by lfcSE are presented. In red are marked strongly downregulated genes (n = 101) while in green are marked strongly upregulated genes (n = 135).