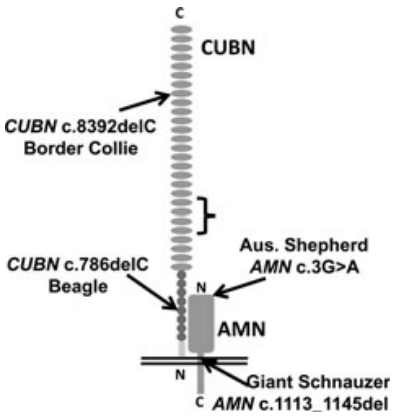
UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

Veterinary Master Thesis, 30 ECTS

Desirée Pii R. L. Mortensen

Imerslund-Gräsbeck in the Danish Beagle

Estimation of allele frequency in the Danish Beagle Dog Population.



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Front page picture from: http://www.101dogbreeds.com/pocket-beagle.asp and Fyfe *et al.*, 2014

Abstract

Selective cobalamin malabsorption, known as the Imerslund-Gräsbeck Syndrome, is an autosomal recessively inherited disease in the Beagle breed. It is caused by a deletion mutation in exon 8 area of the CUBN gene, and prohibits IF-cobalamin complex in binding to, and undergoing endocytosis by the cubam receptor complex. This causes various symptoms of malthriving, such as reduced growth and activity level, as early as 4 months of age.
Dogs that are homozygote for the mutant allele present in the clinic with the before mentioned symptoms, but also proteinuria, non-regenerative anaemia, and absolute neutropenia. Dogs being heterozygote does not show signs of disease, and has normal growth.

The aim of this study was to estimate a realistic allele frequency of the mutant CUBN allele, in the Danish Beagle population. This thesis consists of a theoretical part, summing up the recent knowledge about IGS in dogs, and a practical part. In the practical part, samples from a total of 63 healthy Beagles were collected to investigate allele frequency in the population. Genotyping was performed by the use of restriction enzyme digestion of PCR amplicons. Enough DNA for genotyping was obtained from 42 of the 63 dogs in total.

It was estimated, that the allele frequency among non-related dogs in the collected samples was 0,107, and the frequency estimated from the entire group of sampled dogs was 0,143.
This is interpreted as a relatively low frequency, and it is concluded, that under controlled circumstances, one should be able to eliminate the disease from the Danish population through a carefully planned breeding strategy.
It is important to note, that this sample size is based on dogs that the owners volunteered to the project, and were not collected randomly. The results can therefore not be readily extrapolated to the Danish population, as this the genetic variation is not accounted for. It will require further investigations in the future, to make a more precise estimation.

Indholdsfortegnelse

Acknowledgements

The preparation for this Master Thesis was done during the summer 2015. The work process was started in September 2015, and finished in January 2016.
The aim of this thesis was to estimate a realistic allele frequency of the CUBN mutation, which is known to be the cause of Imerslund-Gräbeck, in the Danish Beagle population. It also gives an update on the clinical features of the disease, giving an indication of what to keep in mind when presented with a misthriving juvenile Beagle in the clinic.
The thesis is meant for veterinary use, but also for owners and breeders of Beagles, in the planning of future mating within the Beagle breed.

This thesis has been made possible by the financial support from DKK, for which I am eternally grateful.
I am forever thankful, for the help and support with contacting owners, and collecting DNA samples, that Anna Sofie Gothen and the Beagle Club has provided. Without this help, this thesis could not have been made. Thank you.
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I owe a very special thanks to the laboratory staff at the Department of Veterinary and Clinical Science, section of Animal Genetics, Bioinformatics and Breeding under Copenhagen University, Faculty of Health and Medical Sciences. Thanks to Charlotte, Jennifer and Tina for all the help, the understanding and patience – Thank you for holding my hand all the way through the lab work.
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Frederiksberg, January 14th 2016

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Desirée Pii R. L. Mortensen

Introduction

The physicians Olga Imerslund and Ralph Gräsbeck first described the Imerslund-Gräsbeck Syndrome in humans. The syndrome is caused by a genetic mutation that makes the patient unable to absorb vitamin B_{12} through the gastrointestinal tract, which affects important intracellular metabolic processes [1].

The syndrome was described in multiple dog breeds since late 1980's, and several studies were conducted, showing that the breeds Giant Schnauzer, Border Collies and Beagles were predisposed for the disease as early as 4 months of age.
By using a candidate gene strategy, it was discovered that IGS in Beagles is caused by a single base cytosine deletion mutation in the exon 8 area on the CUBN gene [2]. In other breeds, the deletion mutation is located elsewhere, but the resulting symptoms and treatment is the same for all breeds. The mutant CUBN allele is passed on by simple autosomal recessive inheritance, if either one parent is affected, or both are heterozygote carriers [3].

Recently, a study was conducted in Germany to establish the prevalence of carrier Beagles, so that reservations could be made for the future breeding to avoid the birth of puppies expressing the genetic mutation causing malabsorption of vitamin B12. It was discovered that roughly 20% of the German Beagle population carried the mutation with a 75% risk of passing it on to their offspring; the puppies having 25% risk expressing the mutation and becoming sick when reaching juvenile state [4].
In Germany, two of the dogs enrolled in the study were of Danish descend, and this is why this thesis has been made. The purpose is to establish the allele frequency in Denmark, and discuss whether the Danish Beagle Club needs to implement guidelines to maintain or bring down the prevalence of carriers and diseased puppies.

Theoretical background

Absorption of vitamin B_{12} in the dog

In the normal healthy dog, absorption of vitamin B_{12} (cobalamin), is carried out using a complex cubam receptor to transport the vitamin across the intestinal villi. The receptor complex is made up by two components, cubilin (CUBN) receptors and amnionless (AMN) proteins [5]. These two components are co-expressed in a wide variety of tissue throughout the organism.

The cubilin receptor is the main receptor involved in the absorption of cobalamin. Cubilin is expressed in polarized epithelia, at the apical membrane of the intermicrovillar surfaces [6].
A study of cubilin's expression in vitro has previously shown that the highest expression of cubilin receptors with ligand binding activity is found 30-40 cm proximal to the ileocolic junction, which correlates to the results documented in studies of the site of absorption in vivo [7].
Synthesis of the receptor takes place in Rough Endoplasmic Reticulum (RER), in the crypt enterocytes, and continues throughout maturation of the cells. Once the receptor reaches the mid-villus region, it is transported by vesicular transport and inserted in microvillus pits where it stays for the remaining maturation, thus not distributing evenly on the brush border surface membrane [8].
It is indicated that the main mechanism regulating regional distribution of the receptor is the process of modulation of cubilin mRNA levels [7].

The cubilin receptor is documented to be extensively present in a variety of tissues other than the intestinal tract, such as the proximal tubules of the kidney [9,10], the lungs [6], and especially the yolk sac in an early embryonic state in rodents [9,10]. It's expression in other tissues than the intestinal tract is, however, of little relevance in this study since orally ingested nutrients is the only source of cobalamin in the canine family, but also because cobalamin needs to bind to the glycoprotein Intrinsic Factor (IF) in order to be recognized by the cubilin receptor [5-7]. At this point, only little or non at all gastric IF is believed to circulate in plasma, further arguing why cubilin's expression in other tissue than the intestinal tract can be excluded from investigation when describing cobalamin absorption [5].
As mentioned, cobalamin needs to bind to IF secreted from gastric mucosa and pancreas before binding to, and absorption via the cubilin receptor can occur. It has been documented, that

cobalamin bound to gastric or pancreatic IF increases the uptake significantly compared to free cobalamin, which were prohibited contact with IF in the gastrointestinal tract [11]. The same study supports the suggestion of cobalamin being absorbed through endocytosis, which were proposed in an earlier in vivo study of orally administered cobalamin [12].
The cubilin receptor is build up with a distinctive set of extracellular protein modules covering 8 epidermal growth factor domains and 27 CUB domains. The CUB domains 5-8 on the cubilin receptor harbours the IF-ligand binding site where IF-cobalamin is bound, and taken up by means of receptor-mediated endocytosis [5,12].

Although cubilin harbours the binding site for IF-bound cobalamin, the receptor is not able to function on it's own and requires the function of another protein, the AMN, in order to facilitate IF-cobalamin uptake.
Not much is known about the AMN protein, but it was recently shown that it is correlated to cubilin's expression in the various tissues, it's role being to chaperone the cubilin receptor from the Golgi apparatus and to the cell surface [13].
It has been proven that functional cubam expression is not possible in the absence of AMN [13].
The AMN-part of the cubam receptor carries two initialisation signals for endocytosis of the rare FXNPXF type, which signals the initiation of internalization of IF-bound cobalamin [5]. Only one of these signals is required for the uptake of IF-bound cobalamin through the cubam receptor [14].

Malabsorption of vitamin B_{12} in dogs expressing homozygote CUBN mutation (pathogenesis)

With the role of CUBN and AMN well established, it has been documented that mutations in genes coding for either of these two components can be the cause of IGS in the dog. However, the mutation is not the same in all breeds were IGS have been documented.

In the Border collie breed, a homozygous single nucleotide deletion (cytosine) in exon 53 on canine chromosome 2, a region harbouring the CUBN locus coding for the protein cubilin, is the cause of IGS. This mutation causes a translation reading frameshift and early termination [15].

Fyfe *et al.*, 2013 [15], used a candidate gene strategy with genes encoding for AMN and CUBN being the strongest candidate genes for the investigation. This was based on the knowledge of the functional components in the cubam complex, in both the dog [13] and in humans [16].
An investigation conducted by He *et al.* 2003 [17], documented that canine IGS is homologue of one form of human IGS. It was demonstrated that the deletion mutation causing IGS in Giant Schnauzers, was located on canine chromosome 8 in a region that is orthologous to human chromosome 14q32.2-ter in an interval that contains the AMN gene [17], which further supports the choice of candidate genes.

The consequences of such frameshift and early termination, is that cubam is expressed 16 and 21 fold less in the ileum, as well as a 24 fold less in the kidney, in dogs being homozygote of the mutated allele, compared to the normal healthy dog, as shown in Figure 1 [15].

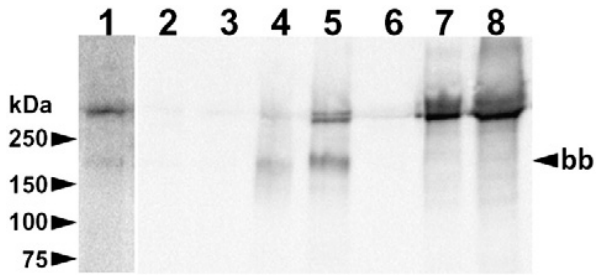

Figure 1, Deficient expression of CUBN in ileum (lane 1-5) and kidney (lane 6-8), in IGS affected dogs (lane 1-3+6) and normal controls (lane 4-5-+7-8). Lane 1 is an overexposure of lane 2 on the same blot. The ileal brush border size of CUBN is marked on the right as bb, ad as it is shown, is only present in normal healthy dogs, indicating a lack of CUBN receptor expression in IGS affected dogs. Fyfe *et al.*, 2013 [15].

When overexposed, it should be noted that a slight band seems to appear in the IGS affected dog in lane 1, indicating that there is not a total lack of CUBN expression in the ileum of IGS affected dogs. Some CUBN receptors may therefore reach the ileum brush-border, but the remaining data with regards to clinical and laboratory findings in the affected dogs suggests, that the CUBN expression is not sufficient to mediate enough cobalamin absorption to maintain metabolic homeostasis [15].
Besides locating the mutation site in the Border Collie breed, the study conducted by Fyfe *et al.*, 2013 [15], established a simple autosomal recessive inheritance of the mutant allele in which the number of affected pups did not differ from the hypothesis, and with both genders represented, which is in accordance with previously conducted studies based on the Giant Schnauzer breed [3,15].

In the Beagle breed, it is also established that a single cytosine base deletion causes a premature termination and is causative of IGS, but it is found to be located in the exon 8 region of the CUBN gene [2,18] and not in exon 53 as described for Border Collies.

By using Sanger sequencing, Drogemüller *et al.*, 2013, were able to illustrate the deletion mutation in Beagles with electropherograms as shown in Figure 2 [18].

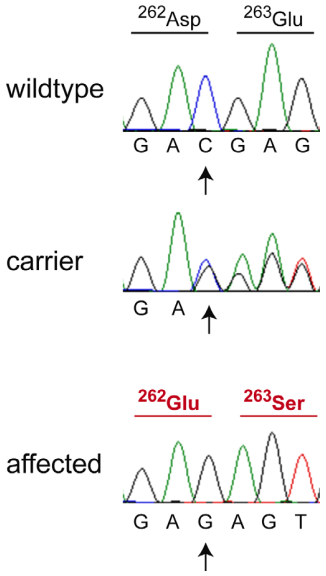

Figure 2, Sanger sequencing of a fragment harbouring the exon 8 and flanking sequences. The arrow indicates mutation site [18]. Drogemüller *et al.*, 2013

Fyfe *et al.*, 2014 [2], used a candidate gene strategy similar to the one used by the same authors in 2013 [15], to document the location of the mutation causative of IGS in Beagles. The candidate gene was chosen based on the knowledge obtained from the previously mentioned studies. The amplicons derived from PCR amplification were compared to the CanFam 3.1 canine reference genome assembly, and AMN and CUBN cDNA sequences (GenBank accession nos. AY368152.1 and AF137068.1) [2]. When comparing PCR amplicons from the cases studied, Fyfe *et al.* found, that the only sequence variation to explain the affected protein coding was a homozygous deletion mutation of a cytosine base in the exon 8 of the CUBN gene. It was therefore concluded that this deletion is the cause of IGS in Beagles. This was confirmed by the fact, that none of the healthy Beagles expressed the exon 8 deletion.

As for Border Collies, the mutation lies in a cytosine base, but the mutation causes a much more severe form of IGS in the Beagles than in the Border Collies, a special feature being the occurrence of a severe form of degenerative liver disease much similar to the one seen in lambs with insufficient cobalt intake [15,18].

Other features of the clinical and laboratory findings will be described in the following section "Clinical presentation".

In the Giant Schnauzer family, as well as in the Australian Shepherd family, the mutation is found to be located in the AMN gene [13]. Firstly, a whole genome scan was carried out, based on DNA material from a large canine family of Giant Schnauzers, in which the IGS figured as a fully penetrant autosomal recessive trait [17]. This was done as a three step process, based on the human

orthologous sequences. The result of this investigation suggested that the genetic locus for canine IGS is located on chromosome 8, where the AMN locus should also be located, but no mutation was observed, even though it was thought highly probable [17].

Later on, He et al., 2005 [13], conducted a new investigation in Giant Schnauzers and Australian Shepherds, to determine the functional domains in both protein and defects caused by CUBN and AMN mutations [13]. This study showed a 33 bp deletion mutation in exon 10 in the transmembrane domain, in the Giant Schnauzer. The mutation seems to occur because of the presence of two repeated 24 bp sequences, which leads to an unequal crossover event after misalignment of these repeats during meiosis I [13]. It was confirmed by the finding of the same 33 bp deletion in the PCR amplicon from gDNA in an affected dog.

In the Australian Shepherd family, the same study revealed a G>A transition at position 3 of the cDNA from an affected dog. This null mutation eliminated the translation initiation codon, and eliminated the restriction enzyme recognition site [13]. This mutation is suggested as being causative of IGS in Australian Shepherds, which was confirmed by the absence of the mutation in 120 chromosomes of unrelated healthy dogs of various breeds [13].

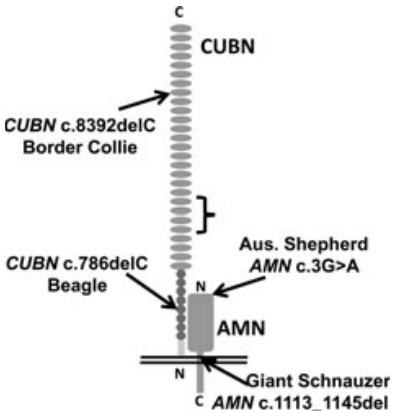

Figure 3. Schematic presentation on the location of the mutations in various breeds. The bracket illustrates the IF-cobalamin binding site, located in domain 5-8 on the cubam complex. Fyfe *et al.*, 2014 [2]

To sum up the documented mutations found in dogs expressing IGS, a schematic presentation is seen in Figure 3 [2]. The figure illustrates the protein domain structure of the cubam receptor complex. The bracket indicates the domain 5-8, which is the binding site for the IF-cobalamin complex. The double line at the bottom illustrates the epithelial cell apical plasma membrane [2].

Clinical presentation

The first clinical presentation occurs when the puppies is about 6-12 weeks old. The main complaint from owners is chronic inappetence, failure to thrive, and failure to gain weight [3]. The clinical investigation will typically reveal poor muscle mass and emaciation of the affected pup [3].

Post mortem examination revealed a variety of pathological changes in various organ systems; kidney, intestinal tracts, lymphoid tissue and bone marrow [3].
The gross findings included emaciation and moderate to severe lymphoid diminution, as well as hypoplasia of lymph nodes and thymus. Diffuse atrophy of the duodenal and jejunal mucosa, oedema, and lymphangiectasia was observed in the entire gastrointestinal tract [3]. Histopathological findings included a hypocellular bone marrow with aggregates of myeloid cells, and megaloblastic erythroid cells. The bone marrow also contains small amounts of hemosiderin, and as does the lymphoid macrophages together with phagocytized erythrocytes [3].

The laboratory findings conducted ante mortem consists of blood – and urine analysis.
The blood analysis shows absolute neutropenia already at 7-16 weeks of age, developing into non-regenerative anaemia in the span of only 4 weeks, but with normal platelet numbers [2,3,19]. In a blood smear the findings will include anisocytosis and poikilocytosis, as wells as few hypersegmented neutrophils, and giant platelets [2,3].
At 8 weeks of age, affected puppies show significantly lower serum cobalamin concentrations than their healthy littermates [3].
The cellular components of the blood analysis in the affected dogs compared to their healthy littermates are summarized in Table 1 below.

	Neutrophils ($x10^9$/L)	Packed Cell Volume	Serum IgG (g/L)	Serum Cbl (pmol/L)	Reference
Healthy dogs	5,2-11,6	0,38-0,45	10-25	203 +/- 11	Fyfe *et al.*,
IGS affected	0,66-3,8	0,21-0,33	1,1-4,2	<75	1991 [3]

Table 1, Summary of values based on blood samples ante mortem in affected vs. non-affected dogs. Fyfe *et al.*, 1991 [3].

The urine analysis shows substantial excretion of various proteins, but it is not considered in larger amount than in the normal healthy dog [2,3,15], and consists mainly of methylmalonic aciduria (MMA) as expected. Even so, the mount of cubam ligands excreted by IGS affected dogs is considered to be greater than the amount excreted by normal healthy dogs. These ligands include proteins such albumin, transferrin, haptoglobulin, apo A1 and vitamin D binding protein [15]. As mentioned earlier, this excretion will prevail lifelong in the affected dog because of the lack of cubam receptor complex for re-absorption of these proteins in the kidney tubules [2,3,5,15].

Diagnosis

In multiple studies, the suspected affected dogs were not chosen randomly but on the basis of clinical symptoms and age of onset [2,15,18,19].

Measurement of total serum cobalamin concentration is an important tool for diagnosis, but cannot stand alone because of the suggestion, that protein bound cobalamin has a very long half life and is therefore active after the total serum cobalamin concentration has dropped after oral intake [19,20].

The diagnosis can be confirmed by measuring total urinary MMA, and total plasma homocysteine (tHcy), which is considered golden standard for diagnosing humans [2,21,22]. Although considered golden standard for diagnosing humans, the two mentioned markers needs further investigation to uncover their full potential as IGS diagnostic tools [19].

The most precise diagnosis is obtained by DNA analysis of genetic material, for example from whole blood or saliva. This method is based on PCR analysis and will be described in more detail in "Materials and Methods", in this report.

This method has been used to detect mutations in either component of the cubam receptor and it is suggested to be useful in any breed [2,13,15,18].

The method should in particular detect mutations in the genes of CUBN, AMN and GIF, the latter yet only described in the human syndrome [16].

This is, however, a complicated and costly method so it is primarily used when all underlying disease has been ruled out, or when presented with breeds proven to carry the mutation, such as Giant Schnauzer, Border collie, Australian Shepherd, Shar pei and Beagle where the mutation is already known [2,13,15,18,19].

Diagnosis of IGS has in several cases been made based on response to treatment with subcutaneous (SC) injections of cyano-cobalamin (CN-Cbl) [15,20]. It is considered that a complete resolution of symptoms is confirmatory for the diagnoses of hereditary selective cobalamin deficiency.

Treatment

Treatment is relatively simple and consists of parenteral injections with CN-Cbl SC. Even given at a critical stage, it has been reported to completely resolve any clinical symptoms in a matter of weeks [2,19].

A summary of routine treatment protocols is seen in Table 2 below.

Breed	Age of onset	Cbl correction	Time until complete resolution	Cbl maintanence	Reference
Giant Schnauzer	6-12 weeks	1 mg IM bolus	ND	1 mg once a month	Fyfe *et al.*, 1991 [3]
Beagle	3 months	50µg/kg SC daily for 3 days	5 days	50µg/kg SC every 2 weeks	Fordyce *et al.*, 2000 [20]
Border collie	Up to 42 months	1 mg SC bolus	4 days	100µg monthly for 14 months	Fyfe *et al.*, 2013 [15]
Border collie	Median age 11,5 months	500µg every 7 days	8 weeks*	ND	Lutz *et al.*, 2013 [19]
Beagle	Up to 4,5 months	50µg/kg SC every 14 days	1 month*	50µg/kg SC 1-2 times a month	Fyfe *et al.*, 2014 [2]

Table 2. Summary of currently used treatment protocols for hereditary cobalamin deficiency [2,15,19]. *Based on time of follow up.

Some variance can be noted as regard to the concentration of maintenance cobalamin dosage, but the primary goal appears to be to keep the dogs free of symptoms and the serum cobalamin concentration within reference frame of 261-1001 ng/L [19,23].

The veterinary literature recommends a dosage of 0,02mg/kg injected SC or IM every 2-4 weeks until the serum cobalamin concentration is normalized and symptoms is resolved [24].

Prognosis

The disease is incurable, but despite of this, the prognosis for affected dogs is quite good if administered the correct form of treatment rapidly after the diagnosis has been determined. Most studies conducted describes a total and rapid recovery from all clinical signs in the affected dogs, except the proteinuria, which will prevail lifelong [2,3,15]

For dogs not receiving the proper treatment in time, the prognosis is more guarded. Examples have been seen on how the disease is rapidly progressing into a severe metabolic crisis characterized by

non-regenerative anaemia with circulating erythroblasts, anisocytosis, schistocytosis, neutropenia, metabolic acidosis, ketonuria, methylmalonic aciduria and hyperammonemia. At this stage, a bone marrow examination will show arrest in the maturation of erythropoeitic cells and only few erythroid precursor cells [15].

Materials and Methods

All procedures were developed and optimized by Department of Veterinary and Clinical Science, section of Animal Genetics, Bioinformatics and Breeding under Copenhagen University, Faculty of Health and Medical Sciences.
The complete laboratory protocol can be seen in Appendix I.

CUBN mutation genotyping

The genotyping was conducted on selected dogs of the Beagle species. The mutation has previously been described in various other breeds such as Giant Schnauzers [3], Australian Shepherds [13], Border Collies [15,19,25], and Beagles [2]. Earlier this year, a similar study was made in Germany, on Beagles, which showed a prevalence of mutation carriers as high as 20% [4]. The data from this study is yet unpublished. Two of the dogs included in this study were of Danish descent, and the Danish Beagle Club, a union under Dansk Kennel Klub (DKK), initiated this investigation to estimate prevalence for the Danish population, in order to discuss future restrictions in breeding dogs carrying the mutant allele.
The results of this investigation will be discussed in the end of this paper, according to importance for the individual dog and the breed as a whole.

Purpose of the study

The purpose of this investigation is to estimate the prevalence of CUBN mutation allele in the Danish Beagle population.
A representative for The Beagle Club has collected samples on a volunteer basis during summer 2015.

Sample Population

A total of 63 assumingly healthy Beagles were studied.
The Danish Beagle Club, a breed specific union under DKK, selected the dogs on a volunteer basis. All dogs are registered in DKK and it is assumed that their results are representative for the DKK registered population of Danish Beagles. Ideally all dogs should have been unrelated, however,

since the dogs have been sampled on the basis of owners volunteering dogs to the project this is not the case as described below.

Sample size

In order to obtain a result that is representative of the entire Danish Beagle population, the needed sample size was calculated according to following equation [26]:

$$n = \frac{Z_{1-a/2}^2 \cdot p \cdot (1-p)}{L^2}$$

n = sample size
$Z_{1-a/2}$= 1,96 with a 95% confidence interval
p = estimated allele frequency
L = maximum allowed error

If the allele frequency is estimated to be between 0,1 and 0,2, based on previously conducted studies [4,18], the required sample size ranges between 139 and 246 dogs, if the maximum allowed error is set to 5%.
The sample size is corrected according to the entire population of Beagles in DK [26]:

$$n_a = \frac{1}{\frac{1}{n} + \frac{1}{N}}$$

n_a = corrected sample size
n = sample size
N = entire population in DK

At present time, the Beagle population registered in DKK is 1001 dogs [27]. This means that the corrected sample size is the same as previously calculated: 123 to 198 dogs, when rounded. This means that at least 123 dogs were needed to be able to get results that can be extrapolated to the entire Danish Beagle population.
The ideal scenario is to only include dogs in the study that is not related on parental site to give the best estimation for the entire population. Since the dogs were selected on volunteer basis, only 25 dogs out of the 63 tested dogs had no relations to any other dogs in the test group.
This means, that this study will not obtain results that are readily representative for the entire Danish Beagle population, and with some uncertainty since the dogs were not randomly selected.

According to protocol acquired from Copenhagen University, Faculty of Health and Medical Sciences, a sterile cotton swap was used to gently wipe the inside of the buccae for 30 seconds. The cotton swap was then used to gently swap the bottom of the oral cavity, preferably under the tongue, to absorb as much saliva as possible. The cotton swap was then smeared repeatedly across

marked areas on FTA-paper, and left to air dry. Each buccae were smeared separately. It is important that the cotton swap is only in contact with the inside of the buccae to ensure the correct DNA is obtained.
The FTA papers were stored refrigerated for a period of 1-3 months until the beginning of the genotyping.
The protocol can be read in Appendix II.

Preparation of the DNA for PCR Analysis

The FTA sample discs was prepared according to QIAamp DNA Investigator Handbook to isolate total DNA from saliva and buccal cells, dried and immobilized on FTA cards.
See Appendix I for details.
After isolation, the DNA was ready for amplification by Polymerase Chain Reaction (PCR).

PCR amplification

Since previous studies have already established the mutation site, and thereby outlined the sequence to be amplified, we used the primers 5'-AGGTTTTCCCAGTCACGACGCTTTCTTGCTCATTTTCGTGAT-3' and 5'-GCATGCTGGAGATTGCACAC-3' to amplify the CUBN mutation site, the same as used in the study by Fyfe *et al.* 2014 [2].
For a single reaction, a mastermix of the following solutions was made:

- 3,0µl Buffer
- 1,2µl Primer F 10 pmol
- 1,2µl Primer R 10 pmol
- 1,2µl MgCl 25mM
- 0,15µl *Taq* polymerase
- 18,75µl Nukleasefree water
- 1,5µl dNTP

Ten samples, a positive control and a negative control were cleansed and prepared at a time.

The conditions for the PCR reaction are shown in Table 3.

	Temperature	**Time**
Step 1	95 °C	15 min
Step 2	95 °C	30 sec
Step 3	60 °C	30 sec
Step 4	72 °C	1 min
Repeat step 2-4, 34 times		
Step 5	72 °C	10 min
Step 6	12 °C	Forever

Table 3. PCR cycles

The end product is a 170 bp amplicon including the sequence where the mutation is located.

Genotyping

When the DNA is processed by PCR, a 170 bp amplicon is the result, and is further processed by the use of the Ms1I restriction enzyme.
Ms1I recognizes the CAYNNNNRTG sequence in both the normal and the mutant allele, and cuts a 36 bp amplicon in both alleles. Additionally, the restriction enzyme cuts the normal allele into two digestion products of 111 bp and 23 bp, because of a sequence change in the normal wild type allele, which was introduced by the reverse primer used in the PCR process. The sequence change is not introduced in the mutant allele, and the digestion product is therefore a 134 bp amplicon.
The fragments were then separated on 4% agarose gels.
The digest products were prepared for gel separation by mixing 10µl of amplification product with 3µl loading buffer and dispensed into the wells in the gel, using a 100 bp markes in the last well.
The amplicons were separated by gel electrophoresis for 30 minutes on 120V.

Statistical analysis

The results were processed manually, and by using the program “R”.
The 95% confidence interval, and the allele frequency was calculated using the function prop.test(c(vectorX,c(vectorY), correct=F) were the vectorX= number of mutant alleles and vectorY= total number of alleles.
The commands used in “R” can be seen in Appendix III.

Results

Genotyping

Of the 63 sampled dogs, it was only possible to obtain enough purified DNA to genotype 42. Not enough purified DNA could be extracted from the last 21 dogs. Since the amplicons from these samples did only show vaguely or not at all, the samples is defined as unreliable and therefore ruled out.

An example of a gel electrophoresis is illustrated in Figure 4.

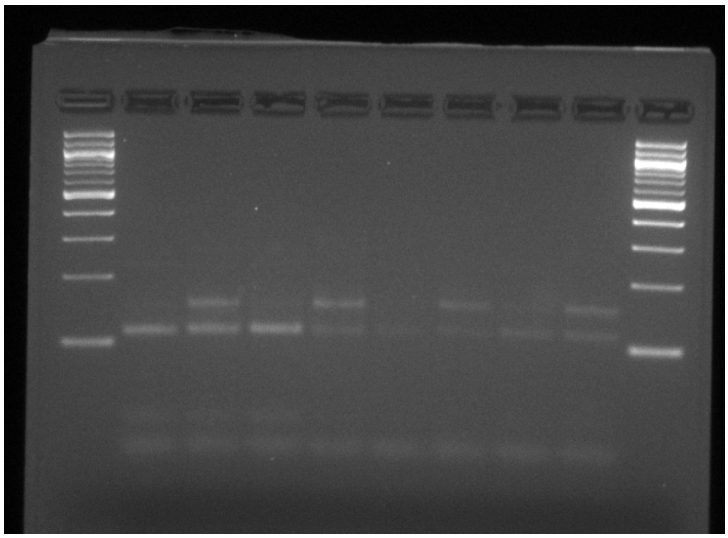

Figure 4, Genotyping of 8 samples. It is illustrated how the wells with 2 bands is dogs being heterozygote for the mutant CUBN allele and wells with 1 band is dogs homozygote for the normal wild type allele. The two outer wells is a 100 bp ladder

As illustrated by Figure 4, dogs exhibiting the homozygote wild type allele demonstrate amplicon size of 111 bp, whereas dogs that are heterozygote for the CUBN mutation exhibits an amplicon size of 111 bp for the normal allele, and of 134 bp for the mutant allele.

Not shown in figure 3 is the use of a positive DNA control obtained from another study design, and a negative control, a blind test. These were used in additional gels to rule out contamination of the samples.

It is important to note that the sample collection was done on a volunteer basis, which means that the study population is not randomized. Therefore only 25 out of the original 63 dogs met the criteria of being non-related. Out of these 25 dogs it was only possible to obtain enough DNA from 14 dogs, but this means, however, that the sample size is still big enough to give an indication of the allele frequency in the Danish population of Beagle dogs.

Therefor it can be concluded that the allele frequency for the 14 unrelated dogs gives a better estimation of the entire population than that of the entire sample population.

Table 2 illustrates the entire result of the genotype analysis.

Sample ID	Country of origin	Genotype result
IGS-101	Denmark	ND
IGS-102	Denmark	CUBN(+/+)
IGS-103	Denmark	CUBN(+/+)
IGS-104	USA	CUBN(+/+)
IGS-105	Denmark	ND
IGS-106	USA	ND
IGS-107	Denmark	CUBN(+/-)
IGS-108	Denmark	CUBN(+/-)
IGS-109	ND	CUBN(+/-)
IGS-110	Denmark	CUBN(+/+)
IGS-111	Denmark	CUBN(+/-)
IGS-112	Denmark	CUBN(+/-)
IGS-113	Denmark	CUBN(+/+)
IGS-114	Denmark	CUBN(+/-)
IGS-115	Denmark	ND
IGS-116	Denmark	ND
IGS-117	Denmark	CUBN(+/+)
IGS-118	Denmark	CUBN(+/+)
IGS-119	Denmark	CUBN(+/+)
IGS-120	Denmark	CUBN(+/+)
IGS-121	Czech Republic	ND
IGS-122	Poland	CUBN(+/+)
IGS-123	Denmark	CUBN(+/+)
IGS-124	Denmark	CUBN(+/+)
IGS-125	Denmark	CUBN(+/+)
IGS-126	Denmark	CUBN(+/+)
IGS-127	Denmark	CUBN(+/+)
IGS-128	Denmark	CUBN(+/+)
IGS-129	Denmark	CUBN(+/+)
IGS-130	Denmark	CUBN(+/+)
IGS-131	Denmark	CUBN(+/+)
IGS-132	Denmark	CUBN(+/-)
IGS-133	Denmark	CUBN(+/+)
IGS-134	Denmark	CUBN(+/-)
IGS-135	Denmark	CUBN(+/+)
IGS-136	Denmark	CUBN(+/-)
IGS-137	Denmark	CUBN(+/+)
IGS-138	Denmark	ND
IGS-139	Spain	CUBN(+/-)
IGS-140	France	ND
IGS-141	USA	ND
IGS-142	Denmark	CUBN(+/+)
IGS-143	Denmark	CUBN(+/+)
IGS-144	Denmark	ND
IGS-145	Denmark	CUBN(+/+)
IGS-146	Denmark	CUBN(+/+)
IGS-147	Denmark	CUBN(+/+)
IGS-148	Denmark	ND
IGS-149	Denmark	CUBN(+/-)
IGS-150	Denmark	CUBN(+/-)
IGS-151	Denmark	ND
IGS-152	Denmark	ND
IGS-153	Denmark	CUBN(+/+)
IGS-154	Denmark	CUBN(+/+)
IGS-155	Denmark	ND
IGS-156	Denmark	CUBN(+/+)
IGS-157	Denmark	ND
IGS-158	Denmark	ND
IGS-159	Denmark	ND
IGS-160	Denmark	ND
IGS-161	Denmark	ND
IGS-162	Denmark	ND
IGS-163	Denmark	ND

Table 2, Genotyping result. CUBN(+/+): homozygote for normal wild type allele, CUBN(+/-): heterozygote for CUBN mutation allele, CUBN(-/-): homozygote for CUBN mutation, ND: Not Defined

From these results, it is possible to calculate the allele frequency in the Danish Beagle population as follows:

$$Allele\ frequency = \frac{12}{84} = 0{,}143$$

Since the ideal situation for the best estimation is, that the dogs is not related on parental side, the frequency is also calculated based only on the number of dogs that are unrelated, 25 in total, and with enough DNA for genotyping, 14 dogs in total. Out of these 14 dogs, 3 is carrying the CUBN mutation allele, giving a total allele frequency of:

$$Allele\ frequency = \frac{3}{28} = 0{,}107$$

There were no observation of dogs being homozygote for the CUBN mutation, CUBN(-/-), which was as expected.

Both results along with genotype frequencies are summarized in table 3.

	Allele frequency	CUBN(+/+)	CUBN(+/-)	CUBN(-/-)	Total
Entire Sample size	0,143	71,4%	28,6%	0%	100%
Non-related	0,107	78,6%	21,4%	0%	100%

Tabel 3, Summary of results

None of the participating dogs proved to be homozygote for the CUBN mutation, which was expected since all of the dogs, except one, were more than 4 months old, this being the primary age of onset for IGS [2,3,20], and in addition all of the dogs presented well and without symptoms at the time of sample collection.

The 95% confidence interval is [0,037; 0,272] for the non-related sample size and [0,084; 0,233] for the entire sample size. This suggests that the results should be interpreted with some caution because of the small available sample size.

It was not possible to find any other study on Beagles to establish prevalence, so it is difficult to compare the allele frequency to that in other countries. One study with the purpose of locating the genetic mutation in Beagles mentioned a carrier frequency of 9% [18], which is close to the established prevalence for the Danish population in this study. Another study established a carrier frequency of 6,3% [15], but this was conducted with Border collies, in which the IGS disease is rarely seen [19].

Several other studies have been conducted on the topic in different breeds with regards to the clinical findings and the inheritance of the genome mutation, but these have been mostly based on known or strongly suspected cases [3,16,19,20,25], which cannot readily be extrapolated for the entire population.
In the yet unpublished German study, a carrier frequency of ~20% was documented, and as mentioned earlier they found that 2 of the carrier dogs were Danish [4].
This is not surprising, as import and export of both dogs and semen for breeding purposes is used vividly between Denmark and Germany in order to expand the current genetic pool and to avoid inbreeding [4].

The dogs included in this investigation will be anonymous, but the owner will be informed on the results of their own dog(s) in the letter figured in Appendix IV. This is done to prevent "bad blood" between the owners and potential future buyers of dogs related to the included dogs.

Discussion

In this chapter there will be a discussion of various problems addressed in this study.

Importance for the individual

For Beagles being homozygote for the wild type allele, the only risk of being cobalamin deficient, is being deprived of food with a high enough dietary concentration of vitamin B_{12}. Mammals do not synthesize this vitamin and is therefore dependent on a nutritional source [6,21,28,29].
In veterinary literature, reference ranges of serum cobalamin is only available for Border collies, and should be interpreted as a suggestion only, since only a single study on the field has been conducted [23]. Even so, it has been shown that the concentration changes when the dietary intake is altered [30], as it also does in humans [31].
Different types of gastrointestinal disease represent another risk of cobalamin deficiency in the homozygote wild type Beagles because of the complex uptake of dietary supplemented cobalamin. In order to be absorbed in the gastrointestinal tract, cobalamin needs to bind to IF secreted by gastric and especially pancreatic mucosa [6,11,32]. This means that any disease affecting the synthesis of pancreatic hormones, such as exocrine pancreas insufficiency, will affect the uptake of cobalamin in otherwise healthy dogs [11].
It is advisable to make sure that dogs of all breeds receive a controlled and balanced feeding with the vitamin concentrations in check.

For Beagles being heterozygote for the CUBN mutation allele, generally the same principles apply as for the homozygote wild type. Cobalamin absorption tests have shown that the absorption of cobalamin in vivo occurs at the same rate and amount as in normal healthy dogs [3]. Extra precaution should be taken when mating these types of animals. According to the law of Mendel [33], CUBN mutant carriers mated with each other gives a risk of 25% that the offspring will be CUBN mutation homozygotes, and 50% risk of them being carriers, as illustrated in Figure 4. This is based on the knowledge that it is a simple autosomal recessive inherited disease [3].

CUBN	-	+
-	-/-	-/+
+	-/+	+/+

Figure 4, the probability for each genotype by mating of CUBN heterozygote dogs.

Dogs that are homozygote for the mutant CUBN allele will express the disease, and need therapeutically management for the rest of their lives in order to thrive.
The main reason for this, is the lack of cubilin expression in the ileum, and despite the fact that the lack is not complete and some cubilin receptors is actually present; it is not enough to uphold the various metabolic processes where cobalamin is an important factor.
If a juvenile/young dog is presented in the clinic on the complaint of failure to gain weight along with poor appetite, low activity level or other signs of not thriving, it is reasonable to suspect cobalamin deficiency early on in the diagnostic work up, especially if the dog belongs to the before mentioned breeds predisposed of inherited cobalamin deficiency. Along with standard blood analysis and blood count, the levels of serum cobalamin should be measured, as serum cobalamin concentration is considered a diagnostic marker for cobalamin deficiency in dogs [34].
It has been shown, that serum cobalamin does not always reflect the cellular cobalamin status, which is important to note, because the most important cobalamin dependent processes happens at intracellular level [35,36]. The liability of serum cobalamin as a deficiency marker therefore needs further investigation.
A better estimate of the cellular cobalamin status in IGS affected dogs, can be obtained by measuring the concentration of MMA in the urine [35]. When cells are deprived of cobalamin, the cobalamin dependent process of converting L-methylmalonyl CoA to succinyl CoA has a reduced activity.

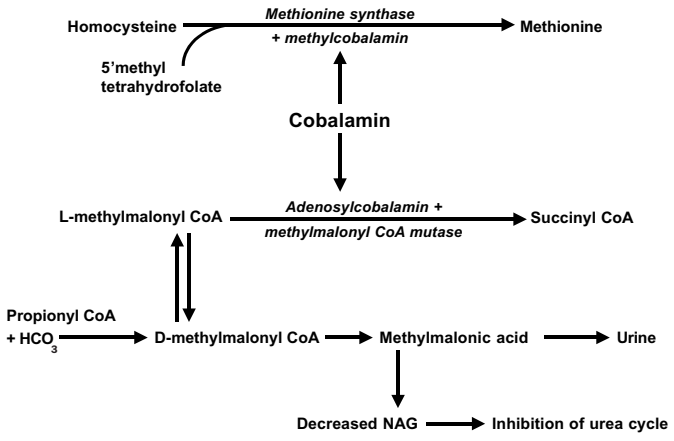

Figure 5. Schematic illustration of the most important cellular metabolic processes dependent on cobalamin. Battersby et al., 2005 [37]. When not enough cellular cobalamin is present to catalyse the processes, an alternative pathway catalysed by D-methylmalonyl

This results in a alternative pathway that is catalysed by D-methylmalonyl CoA hydrolase and

produces MMA, which in turn is excreted in excessive amounts in urine of IGS affected dogs [35,37,38]. The situation is illustrated in Figure 5 [37]. Battersby *et al.*, 2005 further suggests the use of an ammonia tolerance test (ATT) as a tool for diagnosing cobalamin deficiency, based on the case study of a Border collie with hyperammonaemic encephalopathy secondary to inherited selective cobalamin deficiency [37].

Even though IGS should be an important factor to consider when dealing with dogs of the predisposed breeds, various differential diagnoses should still be considered and ruled out in the diagnostic work up.
A DNA test is advised early in the diagnostic work up, especially if no underlying disease that could account for the various symptoms is present.

Importance for the breed

Since the allele frequency is low and the natural way of inheritance is already well established, it would make perfectly good sense to genotype all Beagles that are intended for breeding purposes. This kind of knowledge could be used to set up guidelines for a screening program to ensure that the prevalence of affected, as well as carrier dogs, is reduced. If the screening program was used to make sure that dogs being heterozygote for the mutant allele (CUBN (+/-)) were only mated with dogs being homozygote for the wild type allele (CUBN (+/+)), it would on a long term basis bring down the allele frequency and make it possible to exclude IGS entirely from the Beagle population. This kind of screening program would potentially exclude affected Beagles (CUBN (-/-)) from Danish breeding programs though, but since the genetic pool in Denmark is relatively large and a lot of Beagles from other countries is routinely used, it should not pose to be a problem for the breed.
The Beagle Club already encourages it's members to operate by an ethical code outlined by DKK, which states, that dogs with hereditary defects should not be used for breeding purposes. It is also outlined that mating resulting in litters with significant defects should not be repeated [39]. This indicates a high ethical standard to the benefit of the breed, and therefore good compliance on conducting a screening program, even if some dogs would be ruled out from breeding purposes.
It is, however, important to note, that this code is meant as guidelines only.
According to the standard of the standard for the Beagle breed, outlined by Federation Cynologique Internationale (World Canine Organisation), dogs that show obvious signs of physical or mental

abnormalities should be disqualified [40]. The term "disqualified" could be interpreted as meaning, that the dog is excluded from breeding, and cannot produce any offspring with internationally acknowledged pedigree. This indicates that there is already a great deal of focus on keeping the breed healthy by the use of breeding standards. This, of course, only applies if IGS is seen, as a major physical abnormality, by the FCI, even if there are no symptoms because of correct and adequate treatment.
Of course it is important to keep in mind, that not all Beagles are included in the Danish Beagle Club, and it would be reasonable to suspect a number of matings of Beagles that are not registered, and therefore not with pedigrees, but still predisposed of IGS. It is unlikely that a screening program would affect this population of Beagles, but making the correlation between IGS and it's inheritance common knowledge could hopefully increase the focus of avoiding the disease in this group too.
It would be realistic to focus on controlling IGS in the breed because the allele frequency is relatively low, and because the breed is not concerned about any other hereditary disease that comprises the health of the Beagle more than IGS.

It is advised that mating only takes place between individuals with known genotype. This is also advised when mating with dogs from outside of Denmark, because the IGS has been proven with a relatively high allele frequency in neighbouring countries, and there is a substantial import and export of both dogs and semen between these countries and Denmark.
To this author's knowledge, the Danish Beagle Club is currently not screening for any other type of hereditary disease, and it is therefore thought to be realistic to focus on controlling IGS in the breed as no other hereditary disease is of major concern, and because the allele frequency is relatively low.

Because of the severity of the IGS form expressed in the Beagle, it is most important that this disease is considered a very likely cause of unthriftiness in juvenile patients presented in the clinic. A DNA test should be used to rule out IGS from very early on in the diagnostic work up, especially if the parental IGS status is not known.

Genotyping

In this genetic study of the allele frequency of the mutant CUBN allele, a large sample size was desired in order to get a result representative of the entire Danish Beagle population. The sampling was based on a volunteer basis, which made sure that a large amount of dogs were tested. Unfortunately, the dog's relations were not taken into account, and of the 63 dogs tested, 38 were closely related, which means that the genetic variation is smaller, and the results may not be readily extrapolated to the Danish population. The result was a 3,7% higher allele frequency in the entire sample size, compared to the allele frequency calculated from non-related dogs only. In both cases, a very wide 95% confidence interval was observed, which suggests that the both results should be extrapolated with caution. A larger sample size including only dogs that are not related on parental side would be necessary to give a more precise estimation.
The dogs included in this investigation was chosen on a volunteer basis, and not chosen randomly from the population. This means that the genetic variation is not accounted for in these results, and there is therefore some degree of uncertainty related to the truth of the estimation. There is a risk that some genes may be over- or underrepresented in this study, and it is questionable whether the dogs sampled here represents the entire population, or just a section.

A randomized study design would require a larger sample size, but the result would be precise enough to estimate the likelihood of finding the mutant CUBN allele in a random Beagle. This is because a randomized study would take the genetic variation within the breed into account.
In the study done by Drogemüller *et al.*, 2013, neither a randomized design nor exclusion of related dogs have been used, and the allele frequency is still close to the Danish population. This could be a coincidence as no other study on the allele frequency in Beagles, in any country could be found, except the German study in which the result varies quite a bit from both this study and the one conducted by Drogemüller *et al*., 2013.

It proved to be problematic, that the DNA was obtained from saliva, and clutched to FTA cards. It was difficult to get enough purified DNA for PCR protocol, even though multiple purifying step in the lab was conducted. Because of this complication, only 42 dogs out of the 63 dogs enrolled got a reliable genotyping result. It is possible that this is because DNA from saliva is more readily degraded, even if fixed on FTA paper.

When further investigating IGS in Beagles, it could therefore be beneficial to use blood samples as DNA source rather than saliva. This would be in accordance with other studies, where DNA was obtained from blood or tissue samples [2,3,13,15,18].

Conclusion

The purpose of this study was to estimate an allele frequency of the mutant CUBN gene in the Danish Beagle population, and to discuss the importance for the individual dog, and the breed as a whole.

A single cytosine deletion mutation in the CUBN gene introduces a reading frameshift, and premature termination. This reduces the expression of the cubam receptor complex in the ileum, and the proximal kidney tubules, compromising the uptake of cobalamin dramatically.
The mutant allele is inherited in a autosomal recessive way, which means that only Beagles that are homozygote for the mutant CUBN allele will express IGS, and require lifelong treatment already when 4 months old.
Beagles being heterozygote for the mutant CUBN allele have significant risk of passing on the allele if mated with another mutant CUBN heterozygote Beagle.

Genotyping of 63 Danish Beagles resulted in an allele frequency of 0,107 for non-related dogs included in the sample size, and 0,143 in the entire sample size.
Because this is a not randomized study, these results cannot be readily extrapolated to the entire Danish population, and further investigations should be conducted with a larger and randomized sample size.
If the frequency found in this study is close to a true estimation of the entire population, then it is relatively low, and it would be realistic to focus on bringing the frequency down or completely eliminate IGS from the Danish population, through carefully planning future mating.
Genotyping of dogs intended for breeding purposes, as wells as juvenile Beagles that exhibit failure to thrive makes it possible to influence the allele frequency in the future, and ensure that affected puppies is given the necessary treatment to avoid serious illness and death.

Perspective

Imerslund-Gräsbeck Syndrome happens because of a deletion mutation in a single cytosine base, which lead to a reading frameshift and early termination in the CUBN gene. It has been documented that the mutant CUBN allele is passed on by simple autosomal recessive inheritance.

Because of a relatively low allele frequency, it should be possible to eliminate the disease entirely from the Danish population, if special effort is given. Knowing the dog's genotype when planning future mating should be a major focus point to obtain the goal of total elimination of IGS. This applies for Danish Beagles as well as for foreign Beagles that are used in the Danish population to contribute to the genetic pool and avoid inbreeding. It would therefore be of great interest to determine the allele frequency of the CUBN mutation in other counties as well.

No information exists on the risk of IGS in Beagle crossbreeds or closely related breeds, but this could be interesting to investigate. It would be insight about which breeds, other than the already known, that could be carrying the disease in some degree.

The importance of the lack of CUBN expression in the kidney could be interesting for further research, as it would give more information on the clinical features of the IGS syndrome. Proteinuria is thought of as an acceptable symptom secondary to IGS, but it's influence in the disease calls for further investigation.

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Appendix I

Working protocol Lab

Cleansing of FTA disc

- Make one to three 3.0mm disc from FTA paper containing DNA sample and add to 1.5 ml tube
- Add 280µl of ATL Buffer to each tube
- Add 20µl proteinkinase K and whirlmix
- Incubate in thermomixer at 56 °C for 1 hour with shaking at 900 rpm
- Spin down
- Add 300µl AL Buffer and pulse-whirlmix for 10 sec.
- Thermomix at 70 °C for 10 min with 900 rpm shaking
- Spin down
- Add 150µl ethanol and pulse-whirlmix for 15 sec
- Spin down
- Move entire content to QIAamp MinElute column (750µl solution) without wetting the rim and centrifuge at 6000 x g (8000 rpm) for 1 min
- Discard flow-through and move column to clean tube
- Add 500µl AW1 Buffer without wetting the rim, centrifuge at 6000 x g (8000 rpm) for 1 min
- Discard flow-through and move column to clean tube
- Add 700µl AW2 Buffer without wetting the rim and centrifuge at 6000 x g (8000 rpm) for 1 min (AVOID FLOW-THROUGH CONTACT WITH COLUMN!!)
- Discard flow-through and move to clean tube
- Add 700µl ethanol and centrifuge at 6000 x g (8000 rpm) for 1 min
- Discard flow-through and move to clean tube
- Centrifuge for 3 min (20.000 x g, 14.000 rpm) to dry membrane
- Discard flow-through and move to clean tube
- Add 20-100µl ATE Buffer or water

- Incubate at room temperature for 1 min and centrifuge for 1 min(20.000 x g, 14.000 rpm)
- Product:

PCR amplification preparation

- Whirlmix and spin down all components of PCR Mastermix before mixing
- Mastermix was composed for 10 samples at a time, a positive and a negative control sample as follows:

Component pr. sample	Total Volume for 12 samples
18,75µl denucleated H_2O	225µl
3,0µl Buffer	36µl
1,2µl Primer F	14,4µl
1,2µl Primer R	14,4µl
1,2µl MgCl 25M	14,4µl
1,5µl dnTP	18µl
0,15µl Taq Polymerase	1,8µl
Total of 27µl pr. sample	324/12 = 27µl pr. sample

- Aliquote 27µl into PCR tubes, add cleansed disc into each tube
- Add 3,0µl positive control from BEA31 (known positive for CUBN mutation allele) into positive control tube
- Add 3,0µl of denucleated H_2O into negative sample tube
- Whirlmix and spin down tubes
- Set for PCR with following program:

	Temperature	Time
Step 1	95 C	15 min
Step 2	95 C	30 sec
Step 3	60 C	30 sec
Step 4	72 C	1 min
Repeat step 2-4, 34 times		
Step 5	72 C	10 min
Step 6	12 C	Forever

Restriction cut with restriction enzyme MslI

Procedure is to be conducted ON ICE!

- The PCR amplicon is mixed with CutSmart Buffer and Restriction enzyme MslI as follows:

Component	Volume	
PCR amplicon	30µl	
Denucleated H_2O	14,5µl	
Cutsmart Buffer	5µl	
Restriction Enzyme	0,5µl	
Total volumen	50µl	

- Whirlmix and spin down
- Incubate at 37 C for 15 minutes
- Incubate at 80 C for 20 minutes to inactivate enzyme

Moulding of 4% agarose gel

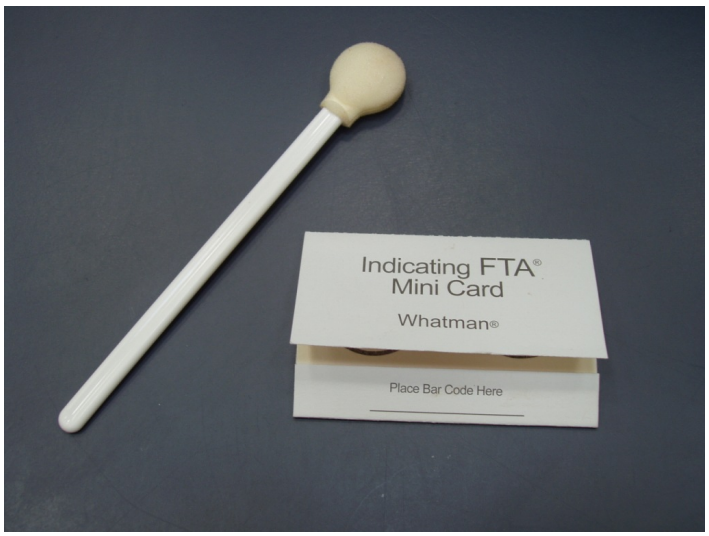
- 2,8g agarose is mixed with 70ml TAE Buffer
- Microwave covered with film for 1 minute at a time until agarose is completely dissolved
- Add 1 drop of ethylenebromide and mix
- Pour gel into mould with 2x20 wells
- Incubate for 20 min

Gel Electrophoresis on 4% agarose gel

- 3µl loading buffer is poured into new PCR tubes
- 10µl PCR amplicon is added to each tube
- Spin down for 8 sec.
- 13µl solution is dispensed into wells on the 4% gel, each row starting and ending with a well containing 10µl 100bp ladder
- Apply 120V for 30 minutes

Appendix II

VEJLEDNING
TIL OPSAMLING AF DNA
FRA MUNDSLIMHINDEN



1. Læs vejledningen inden du går i gang.

2. Tænk på følgende:

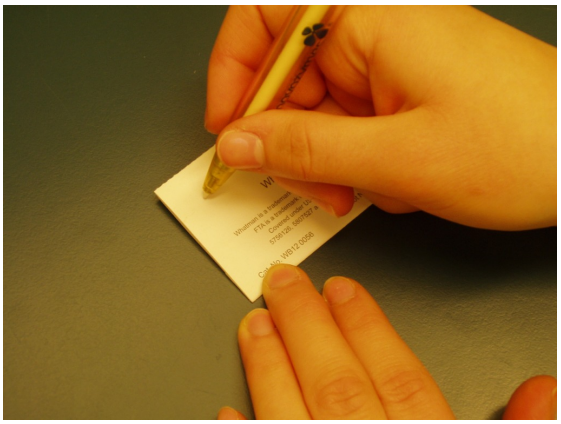
- ✓ Din hund skal ikke have spist eller drukket umiddelbart før prøvetagning.

- ✓ Undgå så vidt muligt at skumgummiet på prøvepinden kommer i kontakt med anden end hundens mundslimhinde og det lyserøde filtrerpapir på FTA-kortet.

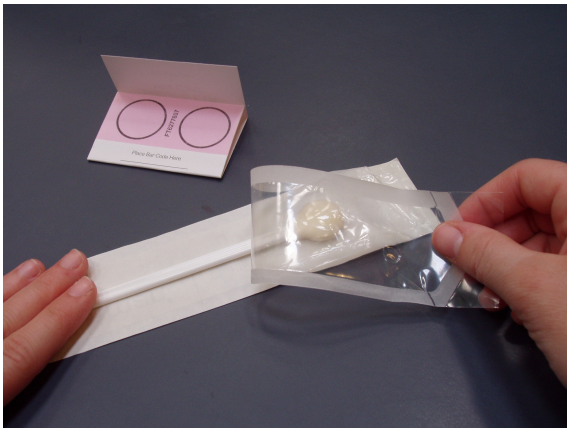
- ✓ Når skumgummipuden har været i kontakt med FTA-kortet skal du ikke bruge den i hundens mund igen. Brug i stedet prøvepind nr. 2, hvis du er i tvivl om der er materiale nok.

3. Placer Jeres FTA-kort på en ren, tør og lige overflade.

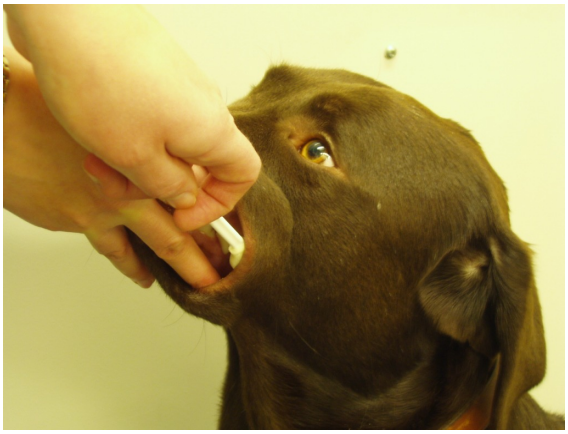
4. Skriv hundens komplette navn (inkl. kennelnavn) og stambogs-nummer på FTA-kortets forside.



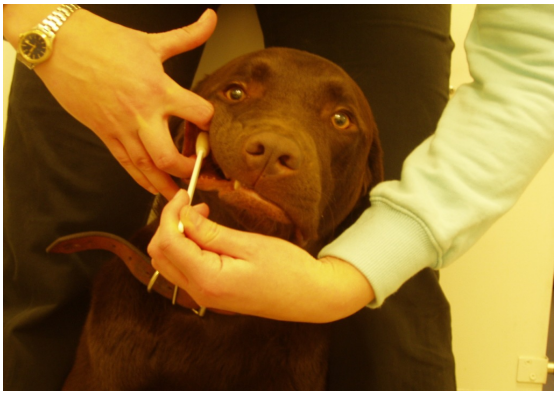
5. Fjern plastikken fra prøvepindens pakning og åbn FTA-kortet så det lyserøde papir med de to sorte cirkler bliver synligt.



6. Adskil hundens over- og underlæbe ved hjælp af dine fingre. Få evt. en hjælper til at holde hunden hvis den er urolig. Hold prøvepinden i den anden hånd og før den ind på indersiden af kinden. Gnub skumgummiet forsigtigt mod indersiden af kinden i ca. 30 sekunder.

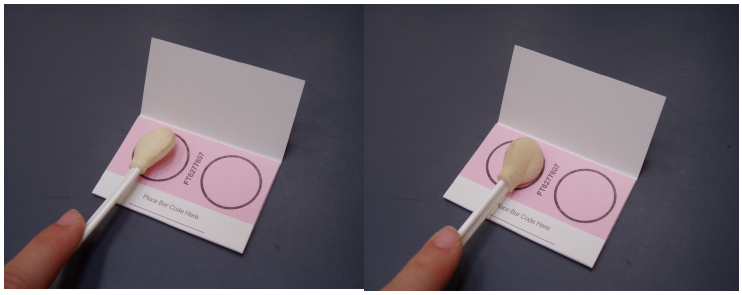


Gentag proceduren med den samme prøvepind i hundens anden kind. Til slut føres skumgummi-puden ned i bunden af mundhulen – evt. ind under tungen – for at opsuge så meget spyt som muligt.



7. Nu skal materialet overføres til filtrerpapiret. Tryk skumgummipuden mod papiret indenfor den ene sorte cirkel

og vip puden fra side til side med en rullende bevægelse. **GNUB IKKE - papiret går nemt i stykker.** Vend skumgummipuden og overfør resten af materialet til den anden cirkel



8. Det lyserøde filtrerpapir skifter farve til hvidt når mundhuleskrabet overføres. Hvis dette ikke sker, er der ikke materiale nok, og du kan gentage proceduren med den vedlagte ekstra prøvepind.

9. Placer FTA-kortet som vist på billedet. Lad det lufttørre ved stuetemperatur i mindst 30 minutter.

10. Luk FTA-kortet, put det i den frankerede kuvert og send det med posten. Husk at vedlægge blanketten med dit navn og din adresse, så vi kan sende svaret til dig.

TUSIND TAK FOR HJÆLPEN!

Tak til Mette Lybeck Ruelokke, Hospital for Mindre Husdyr, for udlåning af labradoren Vitus

Appendix III

```
> mineXværdier <- c(3)
> mineYværdier <- c(28)
> prop.test(c(3),c(28), correct=F)

    1-sample proportions test without continuity correction

data:  c(3) out of c(28), null probability 0.5
X-squared = 17.2857, df = 1, p-value = 3.216e-05
alternative hypothesis: true p is not equal to 0.5
95 percent confidence interval:
 0.03711837 0.27195852
sample estimates:
        p
0.1071429

> mineXværdier <- c(12)
> mineYværdier <- c(84)
> prop.test(c(12),c(84), correct=F)

    1-sample proportions test without continuity correction

data:  c(12) out of c(84), null probability 0.5
X-squared = 42.8571, df = 1, p-value = 5.889e-11
alternative hypothesis: true p is not equal to 0.5
95 percent confidence interval:
 0.08365021 0.23330103
sample estimates:
        p
0.1428571
```

Appendix IV

Kære **(navn på ejer)**

Tak for din deltagelse i Imerslund-Gräsbeck screening i forbindelse med mit speciale:
Malabsorption af vitamin B12 i den danske Beagle Population.
Projektet kunne ikke være blevet gennemført uden denne opbakning.

Som bekendt skyldes sygdommen en mutation i et gen koblet til optagelse af B12 vitamin over tarmen. Sygdommen er reccisivt nedarvet og begge forældre må derfor være anlægsbærere, eller en af dem skal udtrykke sygdommen, for at afkommet kan være anlægsbærer eller udtrykke sygdommen.
For at få klarlagt om hundene i projektet var bærere af den mutante allel blev der foretaget en genotypning af DNA fra spyt på hver enkelt hund.

Resultatet af genotypningen viser at din(e) hund(e) er **positive(e)/negative(e)** for den mutante allel, hvilket betyder at de(n) **er/ikke er** anlægsbærer for IGS.
Det vil være en fordel at medtage denne viden i det videre avlsarbejde således at sygdommens frekvens kan nedsættes med henblik på helt at avle sygdommen ud af den danske population.

Resultatet for alle hunde er anonymt og dermed kun kendt af ejer og projektets fagligt involverede.

Endnu engang TAK for din deltagelse i projektet.

Med venlig hilsen

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