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Master's Thesis

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Genetic testing for cystinuria in the Danish population of English bulldogs

Establishing the frequencies of the mutations c.651A>G and c.2092A>G in *SLC3A1* and c.723G>A in *SLC7A9*

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Topic description:	The purpose of this study was to investigate the allele frequencies of three cystinuria-associated missense mutations in the amino acid transport genes *SLC3A1* (c.651A>G and c.2092A>G) and *SLC7A9* (c.723G>A) in the Danish population of English bulldogs. In this study, 71 English Bulldogs were genotyped for the three mutations using TaqMan assays. The study was conducted at the request of The Bulldog Club with the intent to provide a basis for the Health Committee of Danish Kennel Club (DKK) in their decision-making regarding future breeding recommendations concerning cystinuria in English bulldogs.
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Preface

This study represents the final thesis of the master's degree in veterinary medicine at the Faculty of Health and Medical Sciences, University of Copenhagen, and it was conducted from February to June 2022. The study is conducted at the request of The Bulldog Club with the intention to provide a basis for the Health Committee of the Danish Kennel Club (DKK) in their decision-making regarding future breeding recommendations concerning cystinuria in English bulldogs. Hence, this study targets members of the DKK Health Committee and veterinarians with a special interest in the subject. The study has been limited to investigating the occurrence of the three cystinuria-associated mutations in *SLC3A1* (c.651A>G and c.2092A>G) and *SLC7A9* (c.723G>A) and their association with cystinuria in the Danish population of English bulldogs. The terms "mutation" and "mutated allele" are used despite the continuous uncertainty on whether the observed polymorphisms are the cause of cystinuria. This choice has been made because previous literature has used this nomenclature. Therefore, cystinuria and associated mutations in other dog breeds, and other species, are not presented in detail. Also, the function of the COLA transporter, which is encoded by the genes in question, will only be explained to a degree that concerns the renal transport of cystine. The thesis has been conducted under the supervision of main-supervisor Merete Fredholm, professor in Animal Genetics, University of Copenhagen, and co-supervisor Camilla Vibeke Sichlau Bruun, associate professor in Animal Genetics, University of Copenhagen.

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Abstract

Cystinuria is a genetic disease that can lead to cystine urolith formation, and the English bulldog is the most frequently affected dog breed. In English bulldogs, three mutations have been associated with cystinuria: c.651A>G and c.2092A>G in *SLC3A1*, and c.723G>A in *SLC7A9.* The aim of this study was to determine the allele frequencies of these mutations in the Danish population of English bulldogs to provide a basis for the Health Committee of the Danish Kennel Club in their decision-making regarding possible breeding recommendations concerning cystinuria. Seventy-one English bulldogs were genotyped using TaqMan assays and owners were asked to answer questionnaires concerning the medical history of their dog. High allele frequencies of 0.40, 0.40, and 0.52 were found for the mutated alleles of the mutations c.651A>G, c.2092A>G, and c.723G>A, respectively. For both mutations in *SLC3A1*, a statistically significant correlation was found between cystinuria and homozygosity for the G allele in male, but not in female, English bulldogs. For the mutation in *SLC7A9*, no statistically significant correlation was found between genotype and cystinuria. Due to high allele frequencies, limited genetic diversity, and continued uncertainty of the cause of cystinuria, implementation of genetic screening for cystinuria in the English bulldog breeding program cannot be recommended based on the current available knowledge.

Resumé

Cystinuri er en genetisk sygdom, der kan føre til dannelse af cystin-urolitter, hvor engelsk bulldog er den hyppigst afficerede hunderace. Hos engelske bulldogs er der identificeret tre mutationer, som menes at være associeret med cystinuri; c.651A>G og c.2092A>G i *SLC3A1* og c.723G>A i *SLC7A9*. Formålet med dette studie var at bestemme allelfrekvensen af disse mutationer i den danske population af engelske bulldogs for at danne basis for Dansk Kennel Klubs sundhedsudvalgs beslutningstagen vedrørende mulige avlsanbefalinger i forhold til cystinuri. Enoghalvfjerds engelske bulldogs blev genotypet ved brug af TaqMan assays, og deres ejere blev bedt om at besvare et spørgeskema vedrørende deres hunds sygehistorik. Høje allelfrekvenser for de muterede alleler på 0,40, 0,40 og 0,52 blev fundet for henholdsvis. mutation c.651A>G, c.2092A>G og c.723G>A. For begge mutationer i *SLC3A1* blev der fundet statistisk signifikant sammenhæng mellem cystinuri og genotype G/G hos engelsk bulldog hanhunde, men ikke for engelsk bulldog tæver. For mutationen i *SLC7A9* blev der ikke fundet statistisk signifikant sammenhæng mellem genotype og cystinuri. På grund af de høje

allelfrekvenser, begrænset genetisk diversitet og fortsat usikkerhed vedrørende årsagen til cystinuri kan implementering af genetisk screening for cystinuri i avlsprogrammet for engelske bulldogs ikke anbefales baseret på den nuværende tilgængelige viden.

Abbreviations

A: Arginine
AA: Amino Acid
B^0AT1: Na^+-dependent neutral amino acid transporter 1
$b^{0,+}AT$: Na^+-independent broad specificity neutral and cationic amino acid transporter
BID: Bis in die, twice daily
BOAS: Brachycephalic obstructive airway syndrome
C: Cytosine
cDNA: Complementary deoxyribonucleic acid
CDS: Coding Sequence
CI: Confidence interval
COLA: Cystine, ornithine, lysine, arginine
DKK: Danish Kennel Club
DNA: Deoxyribonucleic acid
FAM: Fluorescent dye, abbreviation for Fluorescein amidites
G: Guanine
GDPR: General Data Protection Regulation
HPLC: High-pressure liquid chromatography
HW: Hardy-Weinberg
LAT2: Na^+-independent L-type amino acid transporter 2
m: Months
MDCK cells: Madin-Darby Canine Kidney cells
OR: Odds ratio
PCR: Polymerase chain reaction
p.o.: Per os, by mouth
rBAT: Related to $b^{0,+}$ amino acid transporter
***SLC3A1*:** Solute Carrier Family 3 Member 1
***SLC7A9*:** Solute Carrier Family 7 Member 9
T: Thymine
UK: United Kingdom
VIC: Fluorescent dye developed after the modifications of Aequorea victoria Green Fluorescent Protein
y: Years
y+LAT1: L-type cationic amino acid transporter 1
4F2hc: 4F2 heavy chain

Introduction

The English bulldog

The English bulldog has a breeding history of approximately 200 years. Originally, the bulldog breed was used for bull baiting, but in the 1820s it gained popularity as a pet dog in the United Kingdom (UK) (Jung & Pörtl, 2019; Pedersen et al., 2016). In 1860, the first bulldogs appeared in show rings in the UK (Pedersen et al., 2016), and in 1873 the breed was officially recognized by the Kennel Club. Since then, English bulldogs have been genetically altered to suit the role as a pet dog while fulfilling human desires for specific physical traits. Unfortunately, the combination of a small founder population, estimated at 68 individuals, and genetic bottlenecks has resulted in several health problems and a greatly diminished genetic diversity in the contemporary English bulldog population (Pedersen et al., 2016). Some of the more known health issues in English bulldogs include brachycephalic obstructive airway syndrome (BOAS), chondrodysplasia, and dermatitis due to excessive folding of the skin and the corkscrew tail. One of the lesser-known health issues is cystinuria. Although cystinuria has been reported to occur in English bulldogs in various studies (Harnevik et al., 2006; Osborne et al., 1999; Ruggerone et al., 2016), the prevalence of cystinuria, and the occurrence of the mutations believed to predispose English bulldogs to cystinuria, are unknown in the Danish population of English bulldogs. With the rising awareness about English bulldog health issues, The Bulldog Club in Denmark wishes to find ways to improve health by selective breeding.

Cystine

Cystine is a dibasic amino acid composed of two molecules of the nonessential amino acid cysteine joined by a disulfide bond (Kovaříková et al., 2021) (Figure 1). In the lumen of the renal tubules, most of the cysteine is oxidized to its dimer form, cystine, whereas in the tubule cells cystine is quickly reduced to cysteine (Harnevik, 2007). The solubility of cystine is pH-dependent which makes it relatively insoluble at physiological urine pH levels 5-7 with an approximate solubility of 250 mg/L at pH 7.0. With alkalization of the urine, the solubility of cystine increases exponentially, hence precipitation of cystine primarily occurs in acidic urine (Harnevik et al., 2006; Kovaříková et al., 2021; Syme, 2012).

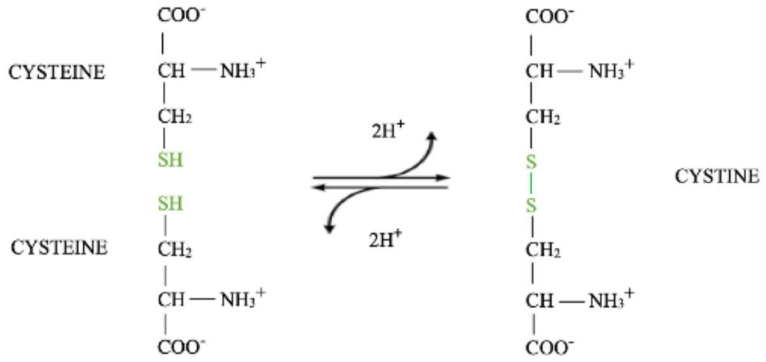

Figure 1 Oxidation of cysteine to cystine and reduction of cystine to cysteine, by signed authors.

Canine cystinuria

Canine cystinuria is an inherited defect characterized by abnormal renal reabsorption of cystine often followed by an abnormal reabsorption of the dibasic amino acids ornithine, lysine, and arginine. These amino acids are collectively referred to as COLA (cystine, ornithine, lysine, arginine). A dog is considered cystinuric at either cystine levels >22.6 mmol/mol creatinine or COLA values >79.2 mmol/mol creatinine in the urine (Kovaříková et al., 2021). Grauer (2014), on the other hand, defines cystinuria as a urine concentration of >35.3 – 58.8 mmol/mol creatinine which is in accordance with a positive cyanide-nitroprusside test used for detecting cystinuria (Bannasch & Henthorn, 2009; Osborne et al., 1999). In a study performed by Hoppe & Denneberg (2001), a normal cystine excretion is defined as < 10 mmol/mol creatinine. In other studies, dogs are considered cystinuric when they have formed one or more cystine uroliths (Bovee et al., 1974; Harnevik et al., 2006; Holtzapple et al., 1971; Hoppe et al., 1993; Hoppe & Denneberg, 2001). However, not all cystinuric dogs form cystine uroliths (Bartges & Callens, 2015; Grauer, 2014; Hoppe et al., 1993; Osborne et al., 1999) or present with cystine crystals in their urine (Grauer, 2014), and neither do all dogs with cystine crystalluria form cystine uroliths (Koehler et al., 2009; Osborne et al., 1999).

Cystinuria and urolith formation

Due to the relative insolubility of cystine in physiological urine pH, dogs with cystinuria are predisposed to cystine crystalluria and cystine urolithiasis (Bannasch & Henthorn, 2009; Kovaříková et al., 2021). In a publication by Hoppe & Denneberg (2001), it was shown that, most frequently, dogs with recurrent cystine urolith formation had a markedly higher urinary cystine excretion compared to dogs with only one episode of cystine urolithiasis. Such results may indicate that the higher the urinary cystine excretion, the more likely it is that cystine uroliths are formed. Other studies have shown cases of cystine urolithiasis where cystine excretion was within the normal range (Harnevik et al., 2006; Holtzapple et al., 1971; Hoppe et al., 1993) and, as earlier mentioned, cases of cystinuria with no urolith formation. In a study performed by Treacher (1964) a dog with a higher urinary cystine excretion than several urolith-forming dogs in the same study did not have a history of urolithiasis at the age of 8 years. Variations as these occur both within and between breeds and suggest that factors other than elevated urinary cystine excretion should be considered as the cause of cystine urolithiasis (Harnevik et al., 2006; Hoppe et al., 1993). For example, Hoppe et al. (1993) reported cystinuric dogs to have lower diuresis compared to normal dogs, and therefore they produce urine with a higher concentration of cystine. Koehler et al. (2009) and Hoppe & Denneberg (2001) argue that normal urinary cystine excretion in urolith forming dogs reflects the daily variations in urinary cystine excretion.

Renal cystine reabsorption

Cystinuria is believed to be caused by a defect in the COLA transporter which under normal circumstances almost completely reabsorbs the COLA amino acids from the renal proximal tubules. The COLA transporter exists in the small intestines as well as in the proximal renal tubules (Kovaříková et al., 2021). In cystinuric dogs, intestinal cystine absorption may be reduced or normal (Holtzapple et al., 1971), but a reduced absorption does not seem to cause deficiency, most likely because cystine is a non-essential amino acid (Bannasch & Henthorn, 2009). An increased excretion of the cystine does not have any other clinical consequences than the risk of developing cystine crystalluria and cystine urolithiasis (Bovee, 1986; Grauer, 2014). Although the variability of isolated cystinuria versus cystinuria with concurrent dibasic aminoaciduria seems irrelevant to the clinical outcome, it might reflect variations in the genetic background of the disease. Variations of specific mutations, their locations, and whether the dog is homozygous or heterozygous might affect the impairment of the COLA transporter and thus result in

phenotypic variabilities in cystinuria with or without dibasic aminoaciduria (Kovaříková et al., 2021).

Clinical signs and epidemiology of canine cystinuria

Conditions as cystine crystalluria and cystine urolithiasis might lead to pollakiuria, stranguria, hematuria, dysuria, and urinary obstruction (Brons et al., 2013; Hoppe et al., 1993; Syme, 2012). Urinary obstructions most often occur in the lower urinary tract (Kovaříková et al., 2021; Osborne et al., 1999), and 98.8% of reported cystine uroliths occur in male dogs (Kovaříková et al., 2021). Canine cystinuria is a disease with a worldwide distribution and it has been reported in more than 70 dog breeds (Kovaříková et al., 2021). A study performed by Kovaříková et al. (2021) shows that the prevalence of cystine uroliths, among analyzed uroliths from dogs, is ranging from 3-26% in Europe (Denmark was not among the studied countries) and approximately 1-3% in North America in the years 1990-2021. Of cystine uroliths submitted and analyzed at the Minnesota Urolith Center in the years 2000-2006, 20% were submitted from English bulldogs, which makes this breed the most frequently affected, followed by a submission of 13% from mixed breeds and 10% from Dachshunds (Koehler et al., 2009).

The genetic aspect of canine cystinuria

The genetic basis of canine cystinuria has been associated with mutations in the two genes *SLC3A1* and *SLC7A9*. These genes encode different parts of the COLA transporter. The underlying mutations and the mode of inheritance have only been determined in some of the affected breeds, as shown in Table 1. Knowledge of prevalence, the underlying mutations, and the mode of inheritance is an important tool to decrease the prevalence with genetic testing and breeding management. So far, the incidence of cystinuria in Newfoundlands and Landseers worldwide has markedly decreased after implementing screening programs using genetic tests to detect carriers of the one mutation found in these breeds (Brons et al., 2013; José et al., 2006).

Table 1 Mutations associated with canine cystinuria in different dog breeds.

Breed	Affected gene	Exon	CDS mutation	Type of mutation	Translational effect	Mode of inheritance	Reference
English bulldog, French bulldog	*SLC3A1*	2	c.651A>G (c.574A>G)	Missense	AA substitution p.Ile192Val	Autosomal recessive	(Harnevik et al., 2006; Ruggerone et al., 2016)
English bulldog, French bulldog	*SLC3A1*	10	c.2092A>G	Missense	AA substitution p.Ser698Gly	Autosomal recessive	(Harnevik et al., 2006; Ruggerone et al., 2016)
English bulldog	*SLC7A9*	6	c.723G>A (c.649G>A)	Missense	AA substitution p.Ala217Thr	Unknown	(Harnevik et al., 2006; Ruggerone et al., 2016)
New-foundland, Landseer	*SLC3A1*	2	c.663C>T	Nonsense	Truncation Ala→ stop codon	Autosomal recessive	(Henthorn et al., 2000; José et al., 2006; Zierath et al., 2017)
Labrador retriever	*SLC3A1*	1	c.350delG	Deletion and Frameshift	Truncation p.Gly117Alafs*41	Autosomal recessive	(Brons et al., 2013)
Australian cattle dog	*SLC3A1*	6	c.1095_1100del	Deletion	Deletion p.Thr366_Thr367del	Autosomal dominant	(Brons et al., 2013)
Miniature pinscher	*SLC7A9*	9	c.964G>A	Missense	AA substitution p.Gly322Arg	Autosomal dominant	(Brons et al., 2013)

As shown in Figure 2, the COLA transporter is a heterodimer consisting of a mainly extracellular heavy chain, rBAT, encoded by *SLC3A1*, and a 12-domain transmembrane light chain, $b^{0,+}$AT, encoded by *SLC7A9.* To form a functional amino acid transporter, the two subunits are joined by a disulfide bridge. This enables the cystine influx in exchange for the efflux of neutral amino acids (Bauch & Verrey, 2002; Harnevik, 2007). To ensure functionality of the COLA transporter, co-expression of the subunits is necessary since neither of the subunits can be expressed on the apical surface in the absence of the other. Moreover, co-expression of the subunits is necessary for rBAT stability and maturation as the protein is terminally glycosylated when it associates with $b^{0,+}$AT (Bauch & Verrey, 2002). Thus, the two subunits are dependent on each other to form a functional COLA transporter, and mutations in *SLC3A1* and/or *SLC7A9* therefore might result in an inhibited function of the collected COLA transporter and thereby restricted renal reabsorption of cystine.

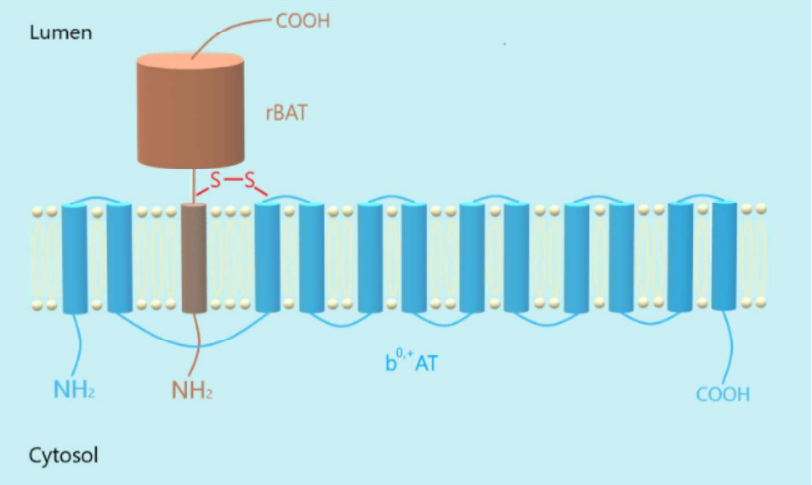

Figure 2 Illustration of the COLA transporter inspired by Andres Otero et al. (2017). The brown element is rBAT. The blue element is $b^{0,+}$AT. S-S is the disulfide bridge joining rBAT and $b^{0,+}$AT.

In 2013, Brons et al. established a classification system for canine cystinuria that describes type I-A, type II-A, type II-B, and type III cystinuria (Table 2). A classification system like this should lead to a classification of phenotypes similar enough to one another that the same medical management and breeding advice apply to all dogs with a certain type of cystinuria. Type III is described as an androgen-dependent type of cystinuria that only occurs in intact adult males, and according to Kovaříková et al. (2021) this is the type of cystinuria seen in English bulldogs. The underlying mechanism of the androgen dependency has not been studied. Thus, it has not been proven that androgens are responsible for the overrepresentation of male cystinuric dogs. The fact that the urinary cystine concentration decreases when cystinuric male Irish terriers are neutered (Giger et al., 2011) supports the idea that androgens are involved in the higher prevalence among male Irish terriers compared to female Irish terriers. However, an alternative explanation could be that the anatomical design of the male urogenital tract increases the risk of urinary obstruction, and thus uroliths are more frequently detected and treated in male dogs. In male dogs the urethra sits in a groove in the penile bone. This causes the urethra to narrow when it enters the penile bone and this site is predisposed to urolith obstruction (Syme, 2012). Regarding female dogs with cystinuria-associated mutations, it is undetermined whether they produce fewer uroliths compared to their male counterparts or if the uroliths are simply not detected since they rarely cause obstruction.

Table 2 Classification system for cystinuria in dogs according to Brons et al. (2013).

Phenotype	Type I-A	Type II-A	Type II-B	Type III
Mode of inheritance	Autosomal recessive	Autosomal dominant	Autosomal dominant	Sex limited
Affected gene	*SLC3A1*	*SLC3A1*	*SLC7A9*	Undetermined
Sex	Males and females	Males and females	Males and females	Intact adult males
Androgen dependent	No	No	No	Yes
Breed	Newfoundland Landseer Labrador	Australian cattle dog	Miniature Pincher	Mastiff and related breeds Scottish deerhound Irish terrier

Mutations associated with cystinuria in English bulldogs

In English bulldogs, two missense mutations associated with cystinuria have been identified in *SLC3A1* (c.651A>G and c.2092A>G), and one missense mutation possibly contributing to cystinuria has been identified in *SLC7A9* (c.723G>A) by Harnevik et al. (2006). Both mutations in *SLC3A1* encode amino acids situated in the extracellular part of the rBAT protein (Harnevik et al., 2006) which, according to Franca et al. (2005), facilitates cystine uptake. All three mutations affect amino acid residues that are not conserved in species used for comparison (human, rat, mouse, and rabbit) (Harnevik et al., 2006). Harnevik et al. (2006) found the three mutations by collecting DNA samples from 13 cystinuric male dogs of different breeds including one English bulldog and one French bulldog. The coding regions of *SLC3A1* and *SLC7A9* were then PCR amplified, sequenced, and analyzed for mutations. Both the examined English bulldog and the French bulldog were homozygous for both mutations in *SLC3A1* (c.651A>G and c.2092A>G), but the English bulldog was the only individual positive for the c.723G>A mutation in *SLC7A9* and it only had one affected allele. In Table 3, the three missense mutations found in English bulldogs are listed together with the resulting amino acid changes.

Table 3 The missense mutations in *SLC3A1* and *SLC7A9* found in English bulldogs and the resulting amino acid changes (Cooper & Hausman, 2004).

Gene	*SLC3A1*	*SLC3A1*	*SLC7A9*
Mutation	c.651A>G	c.2092A>G	c.723G>A
Amino acid exchange	p.I192V Isoleucine → Valine	p.S698G Serine → Glycine	p.A217T Alanine → Threonine
Net. charge at pH 7.4	Neutral → Neutral	Neutral → Neutral	Neutral → Neutral
Polarity	Nonpolar → Nonpolar	Polar → Nonpolar	Nonpolar → Polar

In the studies performed by Harnevik et al. (2006) and Ruggerone et al. (2016), both mutations in *SLC3A1* had an autosomal recessive mode of inheritance and all the studied individuals were either positive for both mutations or neither of them, indicating that these mutations are in linkage disequilibrium. The mutation in *SLC7A9* has been found in both phenotypically affected and unaffected heterozygous individuals, but all affected individuals were also homozygous for the two mutations in *SLC3A1*, leaving the influence of the mutation in *SLC7A9* undetermined. Furthermore, 1 cystinuric English bulldog did not carry the mutation in *SLC7A9* (Ruggerone et al., 2016).

Diagnosing canine cystinuria

Since cystinuria is often discovered due to the presentation of clinical signs associated with urolithiasis, or other urinary abnormalities, the diagnosis is frequently based on a urolith analysis or detection of cystine crystalluria. When uroliths are removed or have naturally passed, a urolith analysis can determine the composition of the urolith, and a treatment and prevention plan can be made. The composition of cystine uroliths can often be "guestimated" from its usually smooth ovoid appearance and light yellow to brown color, but variations in the physical appearance and the occurrence of uroliths with an inner composition different from that on the outer surface make this diagnostic approach unreliable (Bartges & Callens, 2015; Koehler et al., 2009; Osborne et al., 1999). A common way of analyzing uroliths is by using infrared spectroscopy to determine the composition of the uroliths as well as the proportion of the components (Koehler et al., 2009). Detection of cystine crystals in urine raises a strong suspicion of cystinuria since cystine crystals do not occur in healthy dogs. However, cystine crystals are not constantly present in urine from cystinuric dogs (Kovaříková et al., 2021; Osborne et al., 1999). Cystine crystals are colorless, flat, hexagonal crystals that tend to aggregate and therefore appear with a layered appearance (Figure 3) (Kovaříková et al., 2021). When examining urine for cystine crystals, it is important to be aware of diurnal variations in the cystine excretion. In a study performed by Tsan et al. (1972), the varying degree of urinary cystine excretion was argued to be caused by the varying plasma cystine concentration influenced by food intake. In the study, a marked increase in the urinary cystine excretion was observed in cystinuric dogs fed with a protein rich diet, whereas only a small increase was observed in not cystinuric dogs fed the same diet. Due to this variation in urinary cystine excretion, Tsan et al. (1972) stated that "a low cystine value from a single urinary sample does not prove that the dog is not cystinuric." According to Koehler et al. (2009), a postprandial urine sample is preferred when evaluating

crystalluria, whereas Bannasch & Henthorn (2009) argue that a strong correlation between cystine values in a single urine sample and in a 24-hour sample exists if the cystine concentration is adjusted to urinary creatinine concentrations.

Figure 3 Cystine crystals in urine sediment at 100x magnification. Photo by Lance Wheeler, CC BY-SA 4.0 <https://creativecommons.org/licenses/by-sa/4.0>, via Wikimedia Commons.

Qualitative or quantitative methods can be used to estimate the concentration of cystine in the urine. A cyanide-nitroprusside test is a qualitative screening test for cystinuria. When using this test, the sodium cyanide reduces the cystine to cysteine by breaking the disulfide bond. Nitroprusside then reacts with the free sulfhydryl group, and a characteristic purple color is formed (Grauer, 2014; Kovaříková et al., 2021; Osborne et al., 1999). This test, however, is not suitable as an in-house test due to the use of dangerous substances (Kovaříková et al., 2021). High-pressure liquid chromatography (HPLC) is an example of a quantitative urine analysis method where the components of a urine sample are separated and analyzed for their specific chemical properties (Bannasch & Henthorn, 2009; Kovaříková et al., 2021). The method, however, is expensive and not suitable as a routine veterinary test (Bannasch & Henthorn, 2009). Since canine cystinuria is an inherited defect, genetic testing could be a valuable screening tool to discriminate between heterozygous and homozygous individuals and wild type genotypes, and it could possibly have an impact on breeding programs. Today, several laboratories offer genetic testing of cystinuria in English bulldogs among other breeds. PennGen and Laboklin test English bulldogs for the c.2092A>G mutation in *SLC3A1* (P. Werner, Director of the genetic disease discovery laboratory at University of Pennsylvania, personal communication, March 1, 2022; A. Kehl, Diplomat in biology at Laboklin, personal communication, March 2, 2022). VetGen tests for both the c.651A>G and c.2092A>G mutation in *SLC3A1* (Unknown VetGen employee, personal communication, March 30, 2022), and Genimal tests English bulldogs for the c.723G>A mutation in *SLC7A9* (Genimal Biotechnologies, n.d.).

Treatment and prevention of cystine urolithiasis

There is currently no proven method of correcting the genetic defect causing cystinuria, but the disease can be managed in affected dogs by means of urolith dissolution or removal in combination with urolith preventing methods and close monitoring. The current recommendations are that dissolution of cystine uroliths should be attempted before surgical removal (Lulich et al., 2016). Since cystine has a higher solubility in alkaline urine, dissolution of cystine uroliths can sometimes be achieved by alkalizing the urine to a pH of 7-7.5 (Grauer, 2014; Syme, 2012). This can sometimes be obtained with an alkalizing diet alone, but in some patients a potassium citrate supplement is necessary (initial dosage: 75 mg/kg p.o. BID) to achieve the target urine pH (Bartges & Callens, 2015; Osborne et al., 1999). Increased diuresis is an advantage in urolith dissolution and prevention as the urinary cystine concentration is diluted. Feeding canned food or dry food soaked in water can aid in obtaining the target urine specific gravity of less than 1.020. The diet should have a restricted sodium content as natriuresis results in increased cystine excretion (Osborne et al., 1999). Most studies recommend a protein-restricted diet with emphasis on restricting the content of cysteine and its precursor methionine (Bartges & Callens, 2015; Grauer, 2014; Osborne et al., 1999). Commercial canned diets that are low in sulfur-containing amino acids and sodium and that are urine alkalinizing are available (Prescription Diet Canine u/d, Hills' Pet Products and Urinary UC Low Purine, Royal Canin) (Bartges & Callens, 2015). The drug tiopronin (2-mercaptopropionylglycine) can be used to further increase the solubility of cystine in the urine. Tiopronin binds to cysteine in the urine and forms a complex that is 50 times more soluble than cystine (Harnevik, 2007). Tiopronin has been proven effective in dissolving cystine uroliths and preventing urolith recurrence in dogs (Hoppe & Denneberg, 2001). The recommended dose is 15 mg/kg p.o. BID (Grauer, 2014; Hoppe & Denneberg, 2001; Osborne et al., 1999).

If dissolution of the uroliths cannot be achieved, it may be necessary to remove them surgically or mechanically. Minimally invasive techniques should be preferred over traditional open surgical options (Lulich et al., 2016). Minimally invasive techniques include voiding urohydropropulsion, catheter or stone basket retrieval, and lithotripsy. Cystine uroliths can be more difficult to destroy with lithotripsy compared to other mineral types of uroliths, but fragmenting the uroliths will make them easier to dissolve (Osborne et al., 1999).

It has been reported by Giger et al. (2011) that castration of male cystinuric Irish terriers lowers urinary cystine and COLA concentrations and prevents urolith formation. The mutation responsible for cystinuria has not been identified in Irish terriers even though the coding regions

of *SLC3A1* and *SLC7A9* have been sequenced in cystinuric Irish terriers (Giger et al., 2011). It has not been proven that castration has a similar effect in English bulldogs, but Kovaříková et al. (2021) recommend castration in all male dogs with androgen-dependent cystinuria. The American college of Veterinary Internal Medicine recommends neutering cystinuric dogs because neutering, in some cases of cystinuria, is associated with a decreased urine cystine concentration, but also because it prevents unintentional genetic transmission of the disease (Lulich et al., 2016).

After urolith dissolution or removal, the patient should be continuously monitored for recurrence of cystine uroliths. In some patients recurrence can be prevented by continuing to feed the same diet used for urolith dissolution (Grauer, 2014). In dogs with a high recurrence rate, some owners may resort to euthanasia (Kovaříková et al., 2021).

Materials and Methods

Ethical considerations and General Data Protection Regulation (GDPR)

This study was approved by the Animal Ethics Institutional Review Board of the Department of Veterinary and Animal Sciences at the Faculty of Health and Medical Sciences, University of Copenhagen, Denmark (Appendix 1). A written consent to the participation in the study was obtained from all owners (Appendix 2). Owner's consent was obtained by e-mail before contacting the consulted veterinarians.

Dogs

The study was performed on DNA from buccal swabs from a total of 71 English bulldogs, here among 23 males (17 intact, 3 neutered, 3 unknown) and 48 females (38 intact, 6 neutered, 4 unknown) within the age range of 4 months to 10 years and 10 months (average age 3.2 years). Inclusion criteria were dogs of the English bulldog breed registered in the DKK, and the exclusion criteria was full siblings of already participating dogs, in order to give a broad insight into the Danish population of English bulldogs. The dogs were divided into two groups: a study population, and a recruited population. The study population included 62 English bulldogs randomly selected with no prior knowledge of their health status. Dogs in the study population were given study numbers EB_1 to EB_63. One dog (EB_50) was excluded since it had a full sibling already participating in the study. Owners of the dogs were approached by The Bulldog Club at dog shows in Fredericia where the buccal swabs were collected. The bulldog association further contacted members through phone calls and by advertising on their webpage,

encouraging members to let their dogs participate in the study. The recruited population included 9 English bulldogs recruited via posts on social media where English bulldogs with a known genotype and/or phenotype regarding cystinuria were requested. These dogs were given study numbers EB_101 to EB_109. Two of these dogs (EB_102, EB_103) were tested heterozygous for the c.2092A>G mutation in *SLC3A1*, verified by a certificate from Laboklin. One dog (EB_104) was tested heterozygous for the c.651A>G and c.2092A>G mutations in *SLC3A1*, verified by a certificate from VetGen. One dog (EB_109) was tested homozygous for the c.2092A>G in *SLC3A1*, verified by a certificate from Laboklin. Four dogs (EB_105 to EB_108) had formed uroliths consisting of 100% cystine, verified by urolith analysis reports. For the remaining dog (EB_101), documentation of the claimed genotype could not be provided. Buccal swabs from all dogs were obtained by the owners following a written instruction (Appendix 3).

Questionnaires and epicrises

Owners of the dogs were invited by e-mail to complete a questionnaire (Appendix 4). When owners answered “yes” when asked if their dog had a history of uroliths and/or other urinary symptoms, they were asked for permission for the epicrisis to be obtained from the consulted veterinarian.

DNA extraction and genotyping

DNA was extracted from a buccal swab from each dog according to standard techniques (Appendix 5). The concentration of the extracted DNA was measured using spectrophotometry. The cDNA sequences of *SLC3A1* and *SLC7A9* were investigated on www.ensembl.org in a standard boxer genome. The sequences displayed in Figure 2 in the article by Harnevik et al. (2006) were used to confirm the location of the nucleotide substitutions known to occur in English bulldogs. When using ensembl.org, the position of the substitutions slightly differed from the ones stated by Harnevik et al. (2006) and Ruggerone et al. (2016) as shown in Table 4.

Table 4 Mutations associated with cystinuria in English bulldogs.

Affected gene	CDS mutation found on ensembl.org	CDS mutations as stated by Harnevik, Hoppe, and Söderkvist (2006)	Exon	Exon as stated by Ruggerone *et al.* (2016)	Type of mutation	Translational effect
SLC3A1	c.651A>G	c.574A>G	2	2	Missense	I192V
SLC3A1	c.2092A>G	c.2092A>G	10	10	Missense	S698G
SLC7A9	c.723G>A	c.649G>A	6	4+5	Missense	A217T

Extracted DNA was genotyped using TaqMan assays specific for each of the mutations shown in Table 4. Specific primers were designed by supervisors of the study using www.ensembl.org. Probes and primers were manufactured by Life Technologies Europe BV and are shown in Table 5.

Table 5 Taq primers and probes used in this study.

	Forward primer sequence 5'-3'	Reverse primer sequence 5'-3'
SLC3A1 c.651A>G	TGGATTACTTCATTTTACAAATCATCCCTTAAAGA	GCAGATTCTCAAAATCTTTCATTGTTCCA
SLC3A1 c.2092A>G	CGGGCATGCTATTCCAGTGTATTAA	AATGCCAGTGTCTTCATCTCTTTCA
SLC7A9 c.723G>A	CTGGCTTCATTACAGGAAATACAAGGA	CTGATGGCGCCCACAGA
	Reporter 1 sequence (VIC) 5'-3'	**Reporter 2 sequence (FAM) 5'-3'**
SLC3A1 c.651A>G	TCGGAAGTCTTCGATACCA	TCGGAAGTCTTCGACACCA
SLC3A1 c.2092A>G	CATACTGCACAGCTTGT	ATACTGCACGGCTTGT
SLC7A9 c.723G>A	AGCTTCGCGCCCTC	CAGCTTCGTGCCCTC

In each assay, two probes with reporters emitting fluorescent signals of different wavelengths were used. One probe was designed to hybridize with a sequence including the mutation of interest (fluorescent signal FAM), while the other was designed to hybridize with the wild type allele of the same sequence (fluorescent signal VIC). Thus, one fluorescent signal was expected to be registered when the mutation was present and another when the wild type was present. In samples from heterozygous individuals, both fluorescent signals were expected. With TaqMan assay technology, the specific hybridization between the Taq probe and the target sequence, and the 5'-3' exonuclease activity of the Taq DNA polymerase, is necessary to generate a fluorescent signal. Since the Taq probe is covalently attached to a reporter in the 5'-end and a quencher in

the 3'-end, a fluorescent signal will only be detected upon light excitation when the reporter and the quencher are separated from one another (Figure 4). Genotyping using real-time TaqMan PCR was carried out according to standard techniques (Appendix 6 and 7). Based on the lab technicians' good experience using an assay mixture concentration of x20, this was attempted for all three assays. This concentration gave good fluorescent signals (dR Last) in assays testing for the mutations c.2092A>G and c.651A>G in *SLC3A1*. However, in the assay testing for the c.723G>A mutation in *SLC7A9*, fluorescent signals ranging from -3.870 to 363.358 were obtained. When using an assay mixture concentration of x40 in the same assay, as recommended by Life Technologies Europe BV, signals ranging from 800.000 to 45.000.000 were obtained. Therefore, all reported results for the c.2092A>G and c.651A>G mutation in *SLC3A1* have been obtained using an assay mixture concentration of x20 as described in Appendix 6, while the results reported for the c.723G>A mutation in *SLC7A9* have been obtained using an assay mixture concentration of x40 as described in Appendix 7.

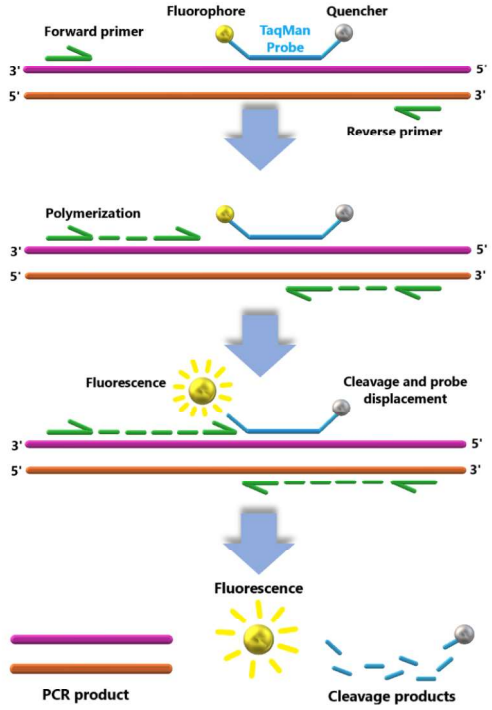

Figure 4 Illustration of the mechanism of the TaqMan PCR method, by signed authors.

DNA sequencing

In order to confirm the validity of the genotyping results obtained from the TaqMan analysis, DNA sequencing of the regions surrounding the three mutations was performed. Primers for PCR amplification and dye-terminator sequencing (Table 6) were designed by supervisors of this study by using the tool: https://bioinfo.ut.ee/primer3-0.4.0/ and manufactured by Sigma-Aldrich.

Table 6 PCR primers used for DNA sequencing.

	Forward primer sequence 5'-3'	**Reverse primer sequence 5'-3'**
SLC3A1 c.651A>G	TTTCAATTTTAGGCATATTGGTTTT	CATCCCTCCGTCACTCAGTT
SLC3A1 c.2092A>G	TCTGCTGACGATCGCAATAC	CATTTGGGAACCTTTGCATT
SLC7A9 c.723G>A	GGTCCTCTGACACCCACAGT	GAGGCTTCTGGCCTAGTCG

One sample representing each genotype, based on results from the TaqMan analysis, was selected for DNA sequencing (Table 7). The DNA samples were PCR amplified, and the expected size of the PCR product was confirmed by gel electrophoresis. The PCR product was purified and then DNA sequenced by the laboratory technician.

Table 7 The samples selected for DNA sequencing and their genotypes according to the TaqMan analyses. Purple shades (light to dark) represent homozygous wild type, heterozygous, and homozygous mutant genotypes.

Study number	*SLC3A1* c.651A>G	*SLC3A1* c.2092A>G	*SLC7A9* c.723G>A
EB_17	A/A	A/A	G/G
EB_46	G/G	G/G	
EB_108	A/G	A/G	
EB_5			A/A
EB_107			G/A

Allele and genotype frequencies and χ^2 test for Hardy-Weinberg equilibrium

The observed allele and genotype frequencies were calculated, using the method described in Hartwell (2011) p. 657. To assess whether the study population was in Hardy-Weinberg (HW) equilibrium, expected genotype frequencies were calculated using the method described in Hartwell (2011) pp. 659-660 and a χ^2 test was performed.

Statistical methods

To determine whether a statistically significant difference in phenotypic outcome exists between different genotypes, a Boschloo's test was performed in the software R using the command:

boschloo(A, (A+B), C, (C+D), alternative = c("two.sided", "less", "greater"),or = NULL, conf.int = FALSE, conf.level = 0.95, midp = FALSE,tsmethod = c("central", "minlike"), control=ucControl())

Where the letters A-D refer to the respective groups in the 2x2 table shown in Figure 5.
This test was chosen because it has a higher statistical power when dealing with small sample sizes compared to Fisher's exact test. p-values from Boschloo's test were compared to corresponding p-values from a $\mathcal{X}^2$ test. All statistical calculations were performed on the study population and recruited population combined. Two possible phenotypic outcomes were defined: "cystinuric" representing dogs with a history of cystine crystalluria or cystine uroliths documented by an epicrisis from the consulted veterinarian, and "not cystinuric" representing dogs with no documented history of cystine crystalluria or cystine uroliths. Dogs for whom a questionnaire was not completed were not included in the statistical analysis.

	Cystinuric	Not cystinuric
Exposed genotype	A	B
Non-exposed genotype	C	D

Figure 5 Standard 2x2 table used for statistical calculations.

In cases where a statistically significant correlation between phenotype and genotype were found, the odds ratio (OR) was calculated. The following command was used in R to calculate the OR:

exact2x2(matrix(c(A,C,B,D),nrow=2), y = NULL, or = 1, alternative = "two.sided",tsmethod = NULL, conf.int = TRUE, conf.level = 0.95,tol = 0.00001, conditional = TRUE, paired=FALSE,plot=FALSE, midp=FALSE)

Where the letters A-D refer to the respective groups in the 2x2 table shown in Figure 5.
The OR calculated using the R command was compared to the corresponding OR calculated with the formula $OR = \frac{\text{odds of cystinuria in the exposed group}}{\text{odds of cystinuria in the non-exposed group}} = \frac{A/B}{C/D}$.

Systematic literature search

The study was supplemented by systematic literature search using primary sources as well as secondary literature. Databases used in the research were chosen due to their content of publications within veterinary medicine and biomedicine from various recognized journals. The following combinations of search items were deployed in PubMed, Medline (ovid), Embase, Web of Science, Scorpus, and Google Scholar:

PubMed, Medline (ovid), Embase, Google Scholar: [dog*] OR [canine*] OR [bulldog*] OR [“English bulldog*”] AND [cystinuria]
Scorpus: [dog] OR [canine] OR [bulldog] OR [English bulldog] AND [cystinuria]
Web of Science: [canine cystinuria]

The articles were systematically sorted, excluding duplicates and articles in languages other than English and Danish. The remaining articles were evaluated on title and abstract, and irrelevant articles were excluded whereas included articles were red in full. In addition to the literature search in the above-mentioned databases, an active search through the reference lists of the included articles was conducted.

Results

Questionnaires and epicrises

In total, 64 out of 71 questionnaires were completed. In the recruited population, 9 out of 9 questionnaires were completed, while 55 out of 62 questionnaires were completed in the study population. Nine out of 64 dogs had either had uroliths or experienced other symptoms from the urinary system, here amongst 4 dogs from the recruited population and 5 dogs from the study population. Symptoms from the urinary system included, but were not limited to: stranguria, dysuria, anuria, oliguria, pollakiuria, urinary incontinence, periuria, hematuria, discolored urine, or change in urine odor. Out of the 5 dogs from the study population, 3 had consulted a veterinarian, and 2 epicrises were obtained (EB_17, EB_46), both in which cystine crystalluria was confirmed by the veterinarian. For the third dog (EB_19), the veterinarian was no longer in possession of the medical record. All 4 dogs from the recruited population (EB_105 to EB_108) had confirmed cystine uroliths. The remaining 55 respondents answered that their dogs had never had uroliths nor had they experienced any other symptoms from the urinary tract. Thus, a

total of 6 dogs were categorized as cystinuric. An overview of information gathered from epicrises and questionnaires can be found in Appendix 8.

DNA extraction

DNA extraction from the buccal swabs gave DNA concentrations from 16,54 ng/µl to 279,27 ng/µl. In 7 cases, a new DNA extraction from the second buccal swab was made, after the first extraction product provided a fluorescence signal in the TaqMan analysis that closely resembled the fluorescence of the control sample of nuclease free H_2O.

Genotyping

Genotypes derived from the results of the TaqMan analyses are shown in Appendix 9 for dogs in the study population and in Appendix 10 for dogs in the recruited population. Figure 6 A, B, and C show representative scatter plots of the fluorescence signals (dR Last) measured in TaqMan assays testing for the mutations c.651A>G and c.2092A>G in *SLC3A1* and the mutation c.723G>A in *SLC7A9*, respectively.

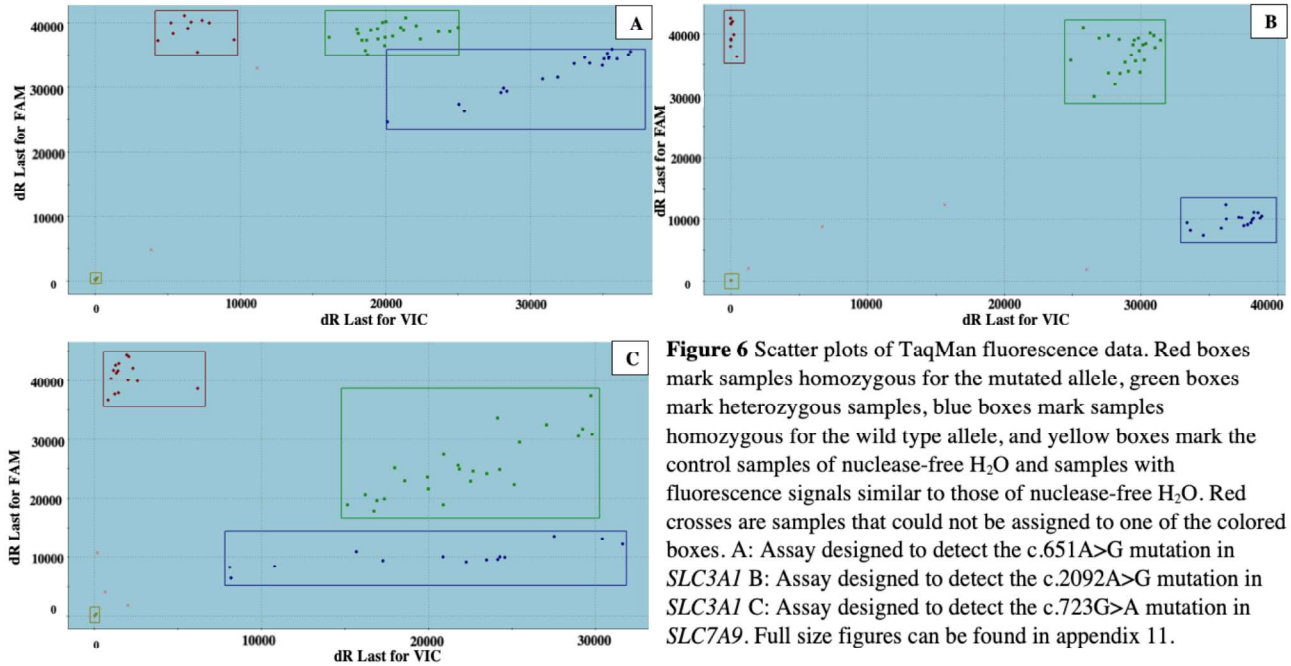

Figure 6 Scatter plots of TaqMan fluorescence data. Red boxes mark samples homozygous for the mutated allele, green boxes mark heterozygous samples, blue boxes mark samples homozygous for the wild type allele, and yellow boxes mark the control samples of nuclease-free H_2O and samples with fluorescence signals similar to those of nuclease-free H_2O. Red crosses are samples that could not be assigned to one of the colored boxes. A: Assay designed to detect the c.651A>G mutation in *SLC3A1* B: Assay designed to detect the c.2092A>G mutation in *SLC3A1* C: Assay designed to detect the c.723G>A mutation in *SLC7A9*. Full size figures can be found in appendix 11.

In all three assays it was possible to distinguish between three separate genotype groups based on the fluorescence signals of each DNA sample. All three TaqMan assays consistently gave a group of samples high in FAM signal and low in VIC signal which was assigned the genotype G/G in the assays designed to detect the c.651A>G and c.2092A>G mutations in *SLC3A1* and the genotype A/A in the c.723G>A in *SLC7A9* assay. When running the TaqMan assay designed to detect the c.2092A>G mutation, a group of samples was consistently high in VIC signal and

low in FAM signal which was assigned with the genotype A/A, and an intermediate group of samples was assigned with the heterozygous genotype. Also the TaqMan assay designed to detect the c.651A>G mutations gave three clearly separated groups in the scatter plot, however, the group of samples assigned with the A/A genotype were consistently high in both VIC and FAM signals. When running the TaqMan assay designed to detect the c.723G>A mutation, samples were not as clearly separated in heterozygous and wild type genotype groups as in the two other assays. In order to distinguish between the two genotypes, each sample was closely inspected for its VIC and FAM signals through the temperature cycles. In this way, two distinguishable groups were found based on the similar progression in registered VIC and FAM signals. Samples that did not clearly follow one of the two groups were rerun and results from the different runs were compared in order to assign the samples with a genotype. Placement of genotype boxes, as shown in Figure 6 A, B, and C, were critically evaluated by comparing assigned genotypes to the genotype of family members through family studies as shown in Appendix 12. The 4 dogs (EB_102-104, and EB_109) that were previously genetically tested by other labs were used as references to assess the normal level of VIC and FAM fluorescence in their respective genotypes. Moreover, selected samples were sequenced with the purpose of validating their assigned genotypes as described in the following.

DNA sequencing

The PCR amplification of the sample from dog number 17 did not produce a product when using the primers designed for sequencing the area surrounding the c.651A>G mutation in *SLC3A1* or the c.723G>A mutation in *SLC7A9.* Hence, it was not possible to sequence the two areas from this sample. All other samples were successfully sequenced. When using the BLAST tool on ensembl.org, the produced sequences almost perfectly matched the expected sequences from a standard dog genome with identity percentages ranging from 96.7% to 100%. The only exception was when the forward primer was used for sequencing the region surrounding the c.651A>G mutation in *SLC3A1.* In this case, the produced sequence was of low quality and the BLAST tool did not find any good matches. For this reason, only the sequence produced when using the reverse primer was considered for the region surrounding the c.651A>G mutation in *SLC3A1*, whereas both produced sequences were considered for the two other analyzed regions. An excerpt of the sequenced regions can be found in Table 8. All the genotypes derived from sequencing were in accordance with the genotypes derived from TaqMan analyses.

Table 8 Excerpts of sequenced regions. Heterozygosity is indicated by (A/G) or (G/A).

Study number	Genotype result from TaqMan analysis	*SLC3A1* sequence from c.646-656	*SLC3A1* sequence from c.2087-2097	*SLC7A9* sequence from c.718-728
EB_ 17	*SLC3A1* c.651A>G: A/A *SLC3A1* c.2092A>G: A/A *SLC7A9* c.723G>A: G/G	PCR amplification not successful	5'GTCACAGCTTG3'	PCR amplification not successful
EB_ 46	*SLC3A1* c.651A>G: G/G *SLC3A1* c.2092A>G: G/G	5'ATGGTGTCGAA3'	5'TGCACGGCTTG3'	
EB_ 108	*SLC3A1* c.651A>G: A/G *SLC3A1* c.2092A>G: A/G	5'ATGGT(A/G)TCGAA3'	5'TGCAC(A/G)GCTTG3'	
EB_ 05	*SLC7A9* c.723G>A: A/A			5'AGGGCACGAAG3'
EB_ 107	*SLC7A9* c.723G>A: G/A			5'AGGGC(G/A)CGAAG3'

Linkage disequilibrium

In accordance with the findings of Harnevik et al. (2006) and Ruggerone et al. (2016), the genotypes found in this study also suggest that the G allele in the c.651 locus and the G allele in the c.2092 locus in *SLC3A1* are in linkage disequilibrium. This study found that all tested individuals either carried the A allele in both loci or the G allele in both loci.

Allele frequencies, genotype frequencies, and test for HW equilibrium in the study population

Observed allele and genotype frequencies in the study population are shown in Table 9 and 10, respectively.

Table 9 Allele frequencies in the study population.

	Frequency of allele A	Frequency of allele G
***SLC3A1* c. 651A>G**	0.60	0.40
***SLC3A1* c.2092A>G**	0.60	0.40
***SLC7A9* c.723G>A**	0.52	0.48

Table 10 Genotype frequencies in the study population.

	Frequency of genotype A/A	Frequency of genotype G/A	Frequency of genotype G/G
***SLC3A1* c. 651A>G**	0.37	0.47	0.16
***SLC3A1* c.2092A>G**	0.37	0.47	0.16
***SLC7A9* c.723G>A**	0.29	0.47	0.24

A $\mathcal{X}^2$ test found the study population to be in HW equilibrium in regard to all three mutations. The $\mathcal{X}^2$ test showed $\mathcal{X}^2 = 0.0287$ for the c.651A>G and c.2092A>G mutations in *SLC3A1* and $\mathcal{X}^2 = 0.2408$ for the c.723G>A mutation in *SLC7A9*. At 1 degree of freedom, ${\mathcal{X}^2}_{0.05} \approx 3.84$. Thus, we cannot reject the null hypothesis of HW equilibrium in the study population at a significance level of 0.05. The calculations are shown in Appendix 13.

Association between genotype and phenotype

In this study, 6 out of 71 English bulldogs were categorized as cystinuric. The genotypes of these 6 dogs are presented in Table 11 from which it appears that there is no univocal association between cystinuria and a specific genotype. In the following, results from statistical analysis investigating the correlation between cystinuria and specific genotypes are presented. All results of statistical calculations can be found in Appendix 14.

Regarding the mutations c.651A>G and c.2092A>G in *SLC3A1*, a statistically significant correlation was found between the cystinuria phenotype and the genotype G/G in English bulldogs ($p = 0.014$). The OR showed that the odds of an English bulldog having the genotype G/G are 10.29 times greater if the dog has cystinuria than if it does not ($OR = 10.29$ with 95% $CI = [1.67; 86.93]$). When investigating male and female English bulldogs separately, a statically significant correlation between cystinuria and the genotype G/G was found in male English bulldogs ($p = 0.003$), but no statistically significant correlation was found for female English bulldogs ($p = 1.000$). The OR showed that the odds of having the genotype G/G are 36.28 times greater if a male English bulldog has cystinuria than if it does not ($OR = 36.28$ with 95% $CI = [2.52; 1438.91]$). When investigating the correlation between cystinuria and the genotype A/A or A/G, no statistically significant correlations were found.

Regarding the c.723G>A mutation in *SLC7A9*, no statistically significant correlations between cystinuria and any genotype were found in neither male nor female English bulldogs.

Table 11 Genotypes of English bulldogs with cystine crystalluria or cystine uroliths. Age indicated by y and m refers to years and months, respectively.

Study number	Sex	Age (registered in April 2022)	Genotype c.574A>G mutation in *SLC3A1*	Genotype c.2092A>G mutation in *SLC3A1*	Genotype c.723G>A mutation in *SLC7A9*	Clinical signs of cystinuria
EB_46	Intact male	10y 11m	G/G	G/G	A/A	Cystine crystalluria
EB_105	Neutered male	6y 6m	G/G	G/G	A/A	Cystine cystoliths
EB_106	Neutered male	2y 3m	G/G	G/G	A/A	Cystine cystoliths
EB_107	Intact male	2y 8m	G/G	G/G	G/A	Cystine urethroliths
EB_108	Intact male	3y 3m	A/G	A/G	G/A	Cystine cystoliths
EB_17	Intact female	4y 10m	A/A	A/A	G/G	Cystine crystalluria

Discussion

Study design

Statistical analysis

The statistical calculations of ORs in this study are attributed with some uncertainty as they are based on a relatively small sample of English bulldogs, especially concerning the population of male English bulldogs consisting of only 20 individuals with completed questionnaires. Although an R command suitable for small sample sizes was used to calculate the ORs, their associated 95% confidence intervals are large, especially for the OR concerning male English bulldogs. Regarding the calculated p-values, Boschloo's test was chosen to compensate for the small sample sizes. The conclusions from the Boschloo's test and $\mathcal{X}^2$ test were in agreement in all tests. The biggest difference in p-values calculated by using Boschloo's test and $\mathcal{X}^2$ test, respectively, was found in the smaller population of male English bulldogs. All results of statistical calculations are shown in Appendix 14.

Selection bias

The aim was for the study population to be representative of the population of English bulldogs registered in the DKK, but some degree of selection bias cannot be excluded. A total of 73% of the dogs in the study population were female. This sex distribution is statistically significantly different from the expected 1:1 male to female ratio at a significance level of 0.05 (calculations are shown in Appendix 15). This uneven sex distribution might be due to the choice of recruiting dogs mainly through dog shows, where more female dogs were attending compared to male dogs. Another selection bias could be that owners of cystinuric dogs might be either more or less likely to let their dogs participate in this study compared to owners of not cystinuric dogs. Also, it is possible that a certain number of cystinuric male dogs are euthanized, thereby making male dogs with the genotype G/G in *SLC3A1* underrepresented in the study population. To investigate whether this was the case, a $\mathcal{X}^2$ test was carried out to test if the genotypes regarding the mutations in *SLC3A1* were in HW equilibrium among male dogs in the study population (calculations are shown in Appendix 16). Only the male dogs were chosen for the test, since cystinuria primarily affects male English bulldogs (Kovaříková et al., 2021), and the mutations in *SLC3A1* were chosen since a statistically significant correlation was found between cystinuria and the genotype G/G. The test showed that HW equilibrium exists, and thus it is unlikely that the selection is biased by this.

With how much certainty are the phenotypes determined?

The phenotypes in this study have been determined by a combination of questionnaires completed by the owners of the dogs and information derived from 6 different veterinarians that have examined dogs with symptoms from the urinary system. Thus, not all phenotypes have been established with the same level of certainty. The certainty of the diagnosis of dogs categorized as cystinuric varies depending on whether the dogs have been diagnosed with cystine urolithiasis or cystine crystalluria. In dogs with cystine urolithiasis, this diagnosis was confirmed by a urolith analysis, which provides a relatively high level of certainty. In dogs with cystine crystalluria, the certainty of the diagnosis is highly dependent on the veterinarian's ability to correctly identify cystine crystals in the urine which may lower the certainty of the diagnosis. Both EB_17 and EB_46 have been categorized as cystinuric based on medical records where the occurrence of cystine crystals in urine have been noted.

In the study population, dogs with a completed questionnaire and no history of cystine crystalluria or cystine uroliths were categorized as not cystinuric. This conclusion, however, is encumbered with great uncertainty. Firstly, this conclusion relies on the assumption that

cystinuria causes symptoms that the owner notices and reports in the questionnaire. However, cystinuria without urolith formation is unlikely to cause any symptoms and even when uroliths are formed, they might go unnoticed, if they do not cause urinary obstruction or give rise to inflammation. This is especially important to keep in mind when discussing female dogs with no clinical signs of cystinuria. Ideally, a measurement of cystine concentration in urine compared with the creatinine concentration in urine should be used to determine whether a dog is cystinuric, but unfortunately this was outside the scope of this study in terms of finance and time.

Association between phenotype and genotype

As previously described, studies by Harnevik et al. (2006) and Ruggerone et al. (2016) have suggested that cystinuria has a recessive mode of inheritance regarding the mutations c.651A>G and c.2092A>G in *SLC3A1*, and that the mutation c.723G>A in *SLC7A9* could possibly be contributing to cystinuria. In the following we will discuss the influence of the two mutations in *SLC3A1* on the phenotype, and the influence of the mutation in *SLC7A9*.

SLC3A1

If cystinuria has a simple mendelian inheritance caused by one of the two mutations in *SLC3A1*, we would expect all dogs with the G/G genotype to be cystinuric, while dogs with the A/G or A/A genotype would be expected not to be cystinuric. In this study, 14 dogs had the G/G genotype and only 4 of them had confirmed cystine crystalluria or cystine uroliths. Among the remaining 10 dogs, 9 were female and 1 was male. As previously mentioned, cystinuria in English bulldogs has been classified as type III cystinuria, described as an androgen-dependent type of cystinuria that only occurs in intact adult males. This could explain why the 9 female dogs with the genotype G/G did not have a history of cystinuria. The one not cystinuric male dog (EB_101) with the genotype G/G was only 23 months old, and since Ruggerone et al. (2016) found the age of onset of cystinuria in English bulldogs to vary from 6 to 36 months, this dog might not have debuted yet. Another explanation could be that the mutations in *SLC3A1* are only associated with an increased risk of cystinuria, and that other risk factors are included in the development of cystinuria.

In this study, 2 of the dogs that were categorized as cystinuric did not have the genotype G/G. One was a female with the genotype A/A (EB_17) and 1 was a male with the genotype A/G (EB_108). As previously stated, EB_17 was categorized as cystinuric based on a medical record where the occurrence of cystine crystals in the urine had been noted. Therefore, this diagnosis depends on the veterinarian's ability to correctly identify cystine crystals. The diagnosis of

EB_108, on the other hand, was confirmed by urolith analysis and is thus less prone to human error. Apart from diagnostic errors, a possible explanation for the genotype and phenotype of EB_17 and EB_108 could be that the mode of inheritance is dominant with incomplete penetrance rather than recessive. However, this would only explain the phenotype of EB_108. In this study, 11 males were heterozygous for the mutations in *SLC3A1*: 1 categorized as cystinuric (EB_108), 8 categorized as not cystinuric, and 2 for whom a questionnaire was not completed, making EB_108 the only cystinuric dog with the genotype A/G in *SLC3A1*. Another explanation could be that neither c.651A>G nor c.2092A>G is the cause of cystinuria but that they are merely markers linked to a cystinuria-causing mutation. This suggestion will be discussed later.

SLC7A9

The influence of the c.723G>A mutation in *SLC7A9* on the cystinuria phenotype is questionable. In the studies by Harnevik et al. (2006) and Ruggerone et al. (2016), it was found that all the cystinuric male English bulldogs were heterozygous for this mutation, except 1 dog that was homozygous for the wild type. But interestingly, all these dogs were also homozygous for the c.651A>G and c.2092A>G mutations in *SLC3A1*, making it impossible to distinguish the effect of the mutations in *SLC3A1* from the mutation in *SLC7A9*. Furthermore Ruggerone et al. (2016) also found an undisclosed number of unaffected English bulldogs that were heterozygous for the mutation in *SLC7A9*. This study found that among the 6 dogs with confirmed cystinuria, 3 had the A/A genotype, 2 had the G/A genotype, and 1 had the G/G genotype regarding *SLC7A9*. However, this study also found 3 not cystinuric male dogs with the A/A genotype and 7 with the G/A genotype. Boschloo's test found no statistically significant correlation between cystinuria and *SLC7A9* genotype. This suggests that the polymorphism observed in *SLC7A9* is not associated with cystinuria after all.

Mutations in *SLC3A1*: Disease-causing or linked markers of canine cystinuria?

As previously discussed, it is possible that neither the c.651A>G nor the c.2092A>G mutation in *SLC3A1* is the cystinuria-causing mutation, but rather markers linked to an actual cystinuria-causing mutation. Since both mutations affect amino acid residues that are not conserved between species (Harnevik et al., 2006), and yet the same essential function of the COLA transporter is maintained in these species, it is more likely that the mutations have little or no effect on the COLA transporter function than if they had affected conserved amino acid residues. On the other hand, if one of the mutations in *SLC3A1* is in fact cystinuria-causing, one would expect the mutation to have a deleterious effect on the function of the COLA transporter. As

previously mentioned, both mutations in *SLC3A1* encode amino acids situated in the extracellular part of rBAT, which is believed to facilitate cystine uptake. Thus, loss of function in this part of the protein would be expected to have a crucial effect on the functionality of the COLA transporter.

As shown in Table 1, the known cystinuria-associated mutations in English bulldogs are all missense mutations whereas in most of the other dog breeds they are nonsense mutations or deletions. Since nonsense mutations and deletions of a single base cause premature termination of translation and frameshift, respectively, such mutations pose a higher risk of the formation of a nonfunctional gene product in comparison to missense mutations. Missense mutations can be either conservative or nonconservative, thus resulting in gene products with very little to deleterious changes in structure and function. The c.2092A>G mutation in *SLC3A1* is nonconservative, leading to changes in amino acid polarity as shown in Table 3. The c.651A>G mutation in *SLC3A1*, on the other hand, is a conservative missense mutation and it is thus unlikely to contribute significantly to the change in protein structure and function. Therefore, it is possible that the c.2092A>G mutation has a negative effect on cystine uptake whereas c.651A>G, due to the conservative nature of the mutation and the fact that it is closely linked to c.2092A>G, could be a marker rather than a cystinuria-causing mutation.

If the cystinuria-causing mutation is not in *SLC3A1* or *SLC7A9*: Where else could it be found?

Since *SLC3A1* and *SLC7A9* encode the proteins rBAT and $b^{0,+}$AT, respectively, which constitute the COLA transporter, one would expect the cause of a dysfunctional COLA transporter to be found within the coding or regulatory regions of these genes. In a study performed by Harnevik et al. (2006), urolith forming dogs with only silent mutations in the coding regions of *SLC3A1* and *SLC7A9* presented with cystine excretion levels ranging from 13 to 650 mmol/mol creatinine. Such findings might suggest the existence of a quantitative rather than qualitative cystinuria-associated mutation, i.e., a mutation in the regulatory region. So far, Harnevik et al. (2006) have made a mutation analysis of the coding regions in cystinuric dogs of ten different breeds, but the regulatory regions have not yet been analyzed. Loss-of-function mutations in the promotor sequence of *SLC3A1* and *SLC7A9* could diminish or prevent transcription, and loss-of-function mutations in an enhancer sequence could diminish or prevent stimulations of the transcription. Also, mutations interfering with post-transcriptional splicing could result in incorrect mRNA and thus an incorrect gene product.

Another possible site for the cystinuria-causing mutation could be in other genes. This could be

genes encoding other apical cystine transporters or proteins otherwise involved in the transcellular transport of cystine. In a study performed by Bauch & Verrey (2002), it was shown that apical cystine uptake in MDCK cells only occurred when these cells were transfected with both rBAT and $b^{0,+}$AT, whereas no significant cystine uptake was measured in MDCK cells transfected with only one of the two proteins. Such findings make it unlikely that other cystine transporting systems than the COLA transporter exist in the apical membrane of the renal tubule cells. However, the COLA transporter is merely responsible for the apical reabsorption of cystine. To reabsorb cystine in exchange for the efflux of neutral amino acids, the COLA transporter is dependent on a sufficient intracellular concentration of neutral amino acids. Both the apical transporter B^0AT1 and the basolateral transporters y^+LAT1-4F2h and LAT2-4F2hc contribute to maintaining the intracellular pool of neutral amino acids necessary for reabsorbing the COLA amino acids over the apical membrane (Bauch et al., 2003). The LAT2-4F2hc transporter is of special interest as it is responsible for the basolateral efflux of cysteine in exchange for an influx of neutral amino acids (Bauch et al., 2003; Harnevik, 2007). As suggested by Meier (2002), a dysfunction in this transporter might be a possible candidate for an increased urinary cystine excretion.

Is it advisable to introduce breeding restrictions related to cystinuria-associated mutations in the Danish population of English bulldogs?

The objective of this study was to gain more information to decide whether it would be advisable to introduce breeding restrictions related to the three cystinuria-associated mutations discussed. It is the opinion of the signed authors and supervisors of this study that it cannot be recommended to introduce such breeding restrictions. Reasons for this are presented in the following.

In this study, a definite cause of cystinuria could not be attributed to one or more of the three mutations otherwise associated with cystinuria in English bulldogs. Regarding the mutation c.723G>A in *SLC7A9,* no statistically significant correlation was found between cystinuria and any of the genotypes, which suggests that this mutation is a random polymorphism. Although the genotype G/G regarding the mutations in *SLC3A1* (c.651A>G and c.2092A>G) show a statistically significant correlation with cystinuria in male English bulldogs, the question of whether one or both mutations is the cause of cystinuria or if they are both linked markers of cystinuria remains unanswered. To further investigate this, it would be interesting to genotype a larger population of both cystinuric and not cystinuric male English bulldogs. Moreover, the

varying levels of cystine excretion in dogs with only silent mutations in *SLC3A1* and *SLC7A9*, as described by Harnevik et al. (2006), point in the direction that the cause of cystinuria might be found in the regulatory regions of *SLC3A1* and *SLC7A9*. Therefore, a mutation analysis of these regions would be interesting to further explore the cause of cystinuria. Finally, of the 8 female English bulldogs in this study with the G/G genotype and completed questionnaires, none of them had a history of uroliths. To find out if the type of cystinuria in English bulldogs does, in fact, only occur in male English bulldogs, it would be interesting to measure the cystine concentration in urine in comparison to the creatinine concentration in female English bulldogs with the genotype G/G regarding the mutations in *SLC3A1*. Performing such studies could greatly increase the understanding of the cause of cystinuria in English bulldogs.

Currently, multiple different laboratories offer genetic testing for cystinuria in English bulldogs, but not all laboratories test for the same markers. For instance, one laboratory test for the c.723G>A in *SLC7A9* which, according to this study, is not associated with cystinuria in English bulldogs.

Evaluating whether breeding restrictions based on genetic testing should be introduced or not also requires careful consideration regarding the high frequency of mutated alleles in the Danish population of English bulldogs. Assuming that the study population represents a wide selection of the English bulldogs registered in the DKK, 63% of the population are heterozygous or homozygous for the c.651A>G and c.2092A>G mutations in *SLC3A1*. To avoid breeding dogs homozygous for the mutated allele, at least one parent must be homozygous for the wild type allele. Considering that individuals with the wild type genotype only constitute 37% of the population, introducing such a breeding restriction would limit the number of English bulldogs used for breeding drastically. Moreover, it must be taken into consideration that the English bulldog population is small and has very limited genetic diversity (Pedersen et al., 2016). In the years 2016-2021, 1134 English bulldogs were registered in the DKK (Dansk Kennel Klub, n.d.), and considering that the median age of death of the British population of English bulldogs is estimated to be 6.29 years (Adams et al., 2010), this could represent the entire Danish population of DKK-registered English bulldogs. Improving health by selective breeding requires either enough genetic diversity within the breed or that diversity is added by outcrossing to other breeds. Pedersen et al. (2016) did an extensive study of the genetic diversity of 102 unrelated English bulldogs from seven countries. They found that 77% of the bulldogs had an adjusted internal relatedness value above 0.25, meaning that they had parents which were more genetically similar to each other than full siblings from a randomly bred population of village dogs would be. The authors concluded that “The loss of genetic diversity and extreme changes in

various regions of the genome will make it very difficult to improve breed health from within the existing gene pool." (Pedersen et al., 2016). If we compare cystinuria to a similar genetic disease in English bulldogs, namely hyperuricosuria where urate uroliths can be formed due to a mutation in the urate transporter gene *SLC2A9* (Ruggerone et al., 2016), this mutation is significantly less frequent in English bulldogs with only 25.5% heterozygotes and 3.1% homozygotes (Pedersen et al., 2016). In regard to the mutation causing hyperuricosuria, Pedersen et al. (2016) concluded that: "Elimination of this recessive mutation from the breed could lead to a significant loss of breed-wide genetic diversity." Considering how much more prevalent the cystinuria-associated mutations are, selecting against them in a breeding program would have detrimental effects on the genetic diversity of the English bulldog breed and thus potentially damage the health and sustainability of the breed. This has to be viewed in the light that the English bulldog breed already faces other serious health issues that could be argued to have an even more urgent need for breeders' attention. A study by Lilja-Maula et al. (2017) found that 100% of the 28 examined English bulldogs had clinical signs of BOAS and that 14% had it to a severe degree. BOAS is a debilitating syndrome caused by anatomical malformations, typical for the English bulldog breed, that interferes with the ability to breathe, thermoregulate, exercise, and sleep. Many more severe heritable health issues threaten the English bulldog breed, and with the limited genetic diversity the opportunity to improve the health of the breed is very restricted. Thus, thoughtful consideration is warranted when introducing health-regulating requirements in the breeding program.

For the above-mentioned reasons, the signed authors and supervisors of this study do not recommend implementing the use of genetic tests in the breeding program of English bulldogs. However, we do recommend that English bulldogs with a clinical diagnosis of cystinuria are not used for breeding. The owners of the dogs included in this study will receive a document with the results of the genetic test of their individual dog and a thorough explanation on how to interpret this information (Appendix 17).

Conclusion

The aim of this study was to investigate the occurrence of three mutations associated with cystinuria in the Danish population of English bulldogs. This study found allele frequencies to be 0.40, 0.40, and 0.52 for the mutated alleles of the c.651A>G and c.2092A>G mutations in *SLC3A1* and the c.723G>A mutation in *SLC7A9,* respectively. A statistically significant

correlation was found between cystinuria and the genotype G/G regarding the mutations c.651A>G and c.2092A>G in *SLC3A1* in male English bulldogs. The odds of having the genotype G/G are 36,28 times greater among cystinuric male English bulldogs compared to male English bulldogs without cystinuria. In female English bulldogs, no statistically significant correlations were found between cystinuria and any of the genotypes regarding the mutations in *SLC3A1*. Regarding the mutation in *SLC7A9,* no statistically significant correlation was found between the cystinuria phenotype and any of the genotypes in neither male nor female English bulldogs. Due to the high frequencies of the mutated alleles, the incomplete understanding of the correlation between cystinuria and genotypes of the three mutations, the limited genetic diversity, and the presence of multiple severe genetic diseases in the breed, the signed authors do not recommend introducing any breeding restrictions related to genetic testing for cystinuria in the Danish population of English bulldogs.

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Appendix 1: Approval from Animal Ethics Institutional Review Board

UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

Animal Ethics Institutional Review Board
Department of Veterinary and Animal Sciences
Faculty of Health and Medical Sciences

Application form

The scope of The Animal Ethics Institutional Review Board (AEIRB)
The Animal Ethics Institutional Review Board issues ethical reviews of projects for which an IVH/Science VIP is responsible. The AEIRB project evaluation and approval can be included in publications and papers requiring a project evaluation number on all animal studies independent of the effects of the study on animal welfare.

Please note that The Animal Ethics Institutional Review Board does not review projects covered under the scope of the Animal Experiments Inspectorate. Animal experiments demanding a permit (acc. BEK nr. 2028 af 14/12/2020 §2) must be evaluated by the Danish Competent Authority; The Animal Experiments Inspectorate (Dyreforsøgstilsynet). Moreover, The Animal Ethics Institutional Review Board does not review projects covered under the scope of The Research Ethics Committee for SCIENCE and SUND. It is the sole responsibility of the submitter/the person responsible for the project to ensure that no other permits are required for the project.

The application procedure
- The application must be submitted prior to the start of the study.
- Be sure to fill out all boxes in Part 2 and relevant boxes of Part 3. Do not fill in Part 1.
- Until November 1st 2021, studies in which the practical work has been initialized, finalized or submitted can also be considered.
- A period of up to 6 weeks must be expected for case handling and administrative procedures.
- Submit the form to Helle Vinberg (hvin@sund.ku.dk) and Dorte Bratbo (brat@sund.ku.dk).
- Write “AEIRB project evaluation” in the subject field of the email.

Appendix 2: Consent form

INSTITUT FOR VETERINÆR- OG HUSDYRVIDENSKAB
DET SUNDHEDSVIDENSKABELIGE FAKULTET – KØBENHAVNS UNIVERSITET

Merete Fredholm
Københavns Universitet
IVH, Husdyrgenetik
Grønnegårdsvej 3
1870 Frederiksberg C

Vedlagte mundhulesvaber er udtaget fra:

Hundens navn: _____

Stambogsnummer: _____

Identifikation: Prøven mærkes med hundens stambogsnummer

Ejers navn: _____

Ejers e-mail: _____

Ejer er indforstået med, at prøven bruges til forskning med henblik på at afklare, om det er hensigtsmæssigt at indføre avlsanbefaling for cystinuri.

Ejers underskrift: _____

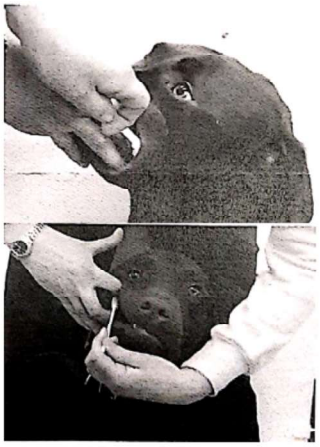
Appendix 3: Buccal swap instruction

INSTITUT FOR VETERINÆR- OG HUSDYRVIDENSKAB
DET SUNDHEDSVIDENSKABELIGE FAKULTET – KØBENHAVNS UNIVERSITET

Københavns Universitet
IVH, Husdyrgenetik
Grønnegårdsvej 3
1870 Frederiksberg C

Vejledning til opsamling af DNA fra mundslimhinden

1. 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 vattet forsigtigt mod indersiden af kinden i ca. 30 sekunder.



2. Før over i den anden kind og slut af med at føre vattet ned i bunden af mundhulen, evt. ind under tungen for at opsamle så meget spyt som muligt.

3. Placer vatpinden i den indpakning de er taget fra og gentag proceduren med den anden vatpind.

4. Når begge vatpinde er tilbage i pakningen, lukkes denne med tape, og hundens navn eller DKK-nummer skrives på pakningen.

5. Prøven sendes sammen med underskrevet informationsark om hund og ejer i den medfølgende returkuvert. Kuverten er frankeret. Har du flere hunde, der indgår i undersøgelsen sendes et sæt af to vatpinde og informationsark for hver hund. Vi har vedlagt ekstra vatpinde, som kan benyttes, såfremt en prøve f.eks. falder på gulvet.

Har du spørgsmål, er du velkommen til at henvende dig på e-mail grj886@alumni.ku.dk

Tak for din deltagelse i projektet!
Med venlig hilsen dyrlægestuderende Julie og Therese

Appendix 4: Questionnaire

Tak fordi du ønsker at deltage i spørgeundersøgelse vedrørende cystinuri hos Engelsk Bulldog. Hvis du har fået taget mundsvaber af flere hunde, er det vigtigt at du udfylder én besvarelse pr hund. Hvis du har spørgsmål til udfyldelsen af spørgeskemaet, er du velkommen til at kontakte os på lgs409@alumni.ku.dk

Hvad er din hunds stambogsnavn?

Hvad er din hunds stambogsnummer? (DKXXXXX/20XX)

_____

Hvad er kønnet på din hund?

- ☐ Intakt han
- ☐ Kastreret han
- ☐ Kemisk kastreret han
- ☐ Intakt tæve (hun)
- ☐ Steriliseret tæve (hun der har fået fjernet æggestokkene og/eller livmoderen)

Har din hund haft urinvejssten? (nyresten/blæresten/...)

- ☐ Ja
- ☐ Nej
- ☐ Ved ikke

Hvilken dyrlæge/-klinik kontaktede du?

Er din hund blevet opereret for at fjerne sten fra urinvejene?

- ☐ Ja
- ☐ Nej
- ☐ Ved ikke

Blev stenen(e) analyseret på et laboratorium?

- ☐ Ja
- ☐ Nej
- ☐ Ved ikke

Hvad bestod stenen(e) af?

- ☐ Cystin
- ☐ Struvit
- ☐ Urat
- ☐ Calcium oxalat
- ☐ Ved ikke
- ☐ Andet_____

Havde dyrlægen et bud på, hvad stenen bestod af?

Hvor gammel var hunden første gang den havde urinvejssten?

Har hunden haft urinvejssten flere gange?

- ☐ Ja
- ☐ Nej
- ☐ Ved ikke

Hvor mange gange har hunden haft urinvejssten og hvor gammel var den og hvilke typer sten har den haft?

Hvad fik dig til at tage hunden til dyrlæge?

- ☐ Urinstenene blev opdaget tilfældigt (fx i forbindelse med røntgenbillede af anden årsag)
- ☐ Hunden viste følgende symptomer:_____

Har du oplevet at din hund har haft tegn på anden sygdom fra urinvejene?

- ☐ Nej
- ☐ Besvær ved urinering
- ☐ Smerter ved urinering
- ☐ Forlænget periode uden urinering
- ☐ Hyppig urinering
- ☐ Ukontrolleret urinering
- ☐ Urenlighed
- ☐ Blod i urinen

- [ ] Urinens lugt var forandret
- [ ] Urinens farve var forandret
- [ ] Andet_____
- [ ] Ved ikke

Søgte du dyrlægehjælp?

- [ ] Ja
- [ ] Nej
- [ ] Ved ikke

Hvilken dyrlæge/-klinik kontaktede du?

Hvad fandt dyrlæge som årsag til symptomerne

Har symptomerne/problemet været tilbagevændende? (forekommet flere gange)

- [ ] Ja, angiv hvor mange gange_____
- [ ] Nej

Tak for din deltagelse!

Studiet om cystinuri forventes afsluttet i sommeren 2022, hvorefter du vil få resultatet på din hunds undersøgelse tilsendt på mail.

Appendix 5 Protocol for DNA extraction

Protocol: Isolation of Total DNA from Surface and Buccal Swabs

This protocol is for isolation of total (genomic and mitochondrial) DNA from surface swabs, sperm swabs, blood swabs, and saliva swabs.

Important points before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- ~~Check whether carrier RNA is required (see pages 10 and 12).~~

Things to do before starting

- Equilibrate Buffer ATE or distilled water for elution to room temperature (15–25°C).
- Set a thermomixer ~~or heated orbital incubator~~ to 56°C for use in step 3 and (optional) step 15, and ~~a second thermomixer or heated orbital incubator~~ to 70°C for use in step 6. ~~If thermomixers or heated orbital incubators are not available, heating blocks or water baths can be used instead.~~
- ~~If processing semen swabs, prepare an aqueous 1 M DTT (dithiothreitol) stock solution. Store aliquots at –20°C. Thaw immediately before use.~~
- If Buffer AL or Buffer ATL contains precipitates, dissolve by heating to 70°C with gentle agitation.
- Ensure that Buffers AW1 and AW2 have been prepared according to the instructions on page 11.
- ~~Optional: To harvest lysate remaining in the swab, QIAshredder spin columns may be required.~~

Procedure

1. **Place the swab in a 2 ml microcentrifuge tube (not provided).**

   ~~If using an Omni Swab, eject the swab by pressing the end of the stem towards the swab.~~

   If using a cotton or Dacron swab, separate the swab from its shaft by hand or by using scissors.

2. **Add 20 µl proteinase K and ~~either 600 µl Buffer ATL (if using an Omni Swab) or~~ 400 µl Buffer ATL (if using a cotton or Dacron swab), close the lid, and mix by pulse-vortexing for 10 s.**

3. **Place the 2 ml tube in a thermomixer ~~or heated orbital incubator~~, and incubate at 56°C with shaking at 900 rpm for at least 1 h.**

   ~~If using a heating block or water bath, vortex the tube for 10 s every 10 min to improve lysis.~~

4. **Briefly centrifuge the 2 ml tube to remove drops from the inside of the lid.**
5. **Add ~~either 600 µl Buffer AL (if using an Omni Swab) or~~ 400 µl Buffer AL (if using a cotton or Dacron swab), close the lid, and mix by pulse-vortexing for 15 s.**

   To ensure efficient lysis, it is essential that the sample and Buffer AL are thoroughly mixed to yield a homogeneous solution.

   A white precipitate may form when Buffer AL is added to Buffer ATL. The precipitate does not interfere with the QIAamp procedure and will dissolve during incubation in step 6.

   ~~**Note:** If carrier RNA is required (see page 10), add 1 µg dissolved carrier RNA to either 600 µl Buffer AL (if using an Omni Swab) or 400 µl Buffer AL (if using a cotton or Dacron swab). Note that carrier RNA does not dissolve in Buffer AL. It must first be dissolved in Buffer ATE and then added to Buffer AL.~~
6. **Place the 2 ml tube in a thermomixer~~ or heated orbital incubator,~~ and incubate at 70°C with shaking at 900 rpm for 10 min.**

   ~~If using a thermoblock or water bath, vortex the tube for 10 s every 3 min to improve lysis.~~
7. **Briefly centrifuge the 2 ml tube to remove drops from the inside of the lid.**
8. **Add ~~either 300 µl ethanol (96–100%) (if using an Omni Swab) or~~ 200 µl ethanol (96–100%) (if using a cotton or Dacron swab), close the lid, and mix by pulse-vortexing for 15 s.**

   To ensure efficient binding in step 10, it is essential that the sample and ethanol are thoroughly mixed to yield a homogeneous solution.
9. **Briefly centrifuge the 2 ml tube to remove drops from the inside of the lid.**
10. **~~If using an Omni Swab, follow step 10a~~. If using a cotton or Dacron swab, follow step 10b.**

~~10a. Carefully transfer 700 µl lysate from step 9 to the QIAamp MinElute column (in a 2 ml collection tube) without wetting the rim, close the lid, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Carefully discard the flow-through from the collection tube and then place the QIAamp MinElute column back into the collection tube. Carefully apply the remaining lysate from step 9 to the QIAamp MinElute column without wetting the rim, close the lid, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.~~

~~If the lysate has not completely passed through the membrane after centrifugation, centrifuge again at a higher speed until the QIAamp MinElute column is empty.~~

~~**Note:** Up to 250 µl lysate remains in the swab. To harvest this remaining lysate, place the swab in a QIAshredder spin column (not supplied) and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 2 min. Transfer the flow-through to the QIAamp MinElute column without wetting the rim, close the lid, and centrifuge at 6000 x *g* (8000 rpm) for 1 min.~~

10b. Carefully transfer the entire lysate from step 9 to the QIAamp MinElute column (in a 2 ml collection tube) without wetting the rim, close the lid, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.

If the lysate has not completely passed through the membrane after centrifugation, centrifuge again at a higher speed until the QIAamp MinElute column is empty.

~~**Note:** Up to 200 µl lysate remains in the swab. To harvest this remaining lysate, place the swab in a QIAshredder spin column (not supplied) and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 2 min. Transfer the flow-through to the QIAamp MinElute column without wetting the rim, close the lid, and centrifuge at 6000 x *g* (8000 rpm) for 1 min.~~

11. **Carefully open the QIAamp MinElute column and add 500 µl Buffer AW1 without wetting the rim. Close the lid and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.**

12. **Carefully open the QIAamp MinElute column and add 700 µl Buffer AW2 without wetting the rim. Close the lid and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.**

Contact between the QIAamp MinElute column and the flow-through should be avoided. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains ethanol, coming into contact with the QIAamp MinElute column. Take care when removing the QIAamp MinElute column and collection tube from the rotor, so that flow-through does not come into contact with the QIAamp MinElute column.

13. **Carefully open the QIAamp MinElute column and add 700 µl of ethanol (96–100%) without wetting the rim. Close the cap and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.**

14. **Centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min to dry the membrane completely.**

    This step is necessary, since ethanol carryover into the eluate may interfere with some downstream applications.

15. **Place the QIAamp MinElute column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the flow-through. Carefully open the lid of the QIAamp MinElute column, and incubate ~~at room temperature (15–25°C) for 10 min or~~ at 56°C for 3 min.**

16. **Apply ~~20–100~~ µl ~~Buffer ATE or~~ distilled water to the center of the membrane.**

    Important: Ensure that Buffer ATE or distilled water is equilibrated to room temperature. Dispense Buffer ATE or distilled water onto the center of the membrane to ensure complete elution of bound DNA.

    QIAamp MinElute columns provide flexibility in the choice of elution volume. Choose a volume according to the requirements of the downstream application. Elution with small volumes increases the final DNA concentration in the eluate significantly, but reduces the overall DNA yield. Remember that the volume of eluate will be up to 5 µl less than the volume of elution solution applied to the column.

17. **Close the lid and incubate at room temperature for 1 min. Centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 1 min.**

    Incubating the QIAamp MinElute column loaded with Buffer ATE or water for 5 min at room temperature before centrifugation generally increases DNA yield.

Appendix 6: Protocol for TaqMan x20

TaqMan Engelsk Bulldog

Husk at tænde apparatet (lampen) i B206 i god tid (Det tager ca. 20 min før lampen er klar)
Mastermix, laves på is **(Husk stinkskab når du arbejder med TaqMan mastermixen!)**:
Der laves Mastermix til hver prøve, 1 blindprøve og 1 ekstra prøve.

Mastermix (20-25ng)	x1	x
TaqMan **(Giftig!)** (x20)	1,0 µl	
TaqMan Universal mix (x2)	10,0 µl	
H_2O	7,0 µl	

18 µl mastermix + 2 µl DNA (20-25ng/µl).

Sæt reaktionerne op i de hvide plader.

	1	2	3	4	5	6	7
A							
B							
C							
D							
E							
F							
G							
H							

Mx3000p opsætning:
Program: MxPro

- Ved opstart vælg "*Allele Discrimination/ SNP's Real-Time*"
- På templaten marker de anvendte brønde, vælg derefter "*Unknown*" under "*well type*"
- Under "*Collect fluorescence data*" (menuen i højre side) marker "*VIC*" (- først) "*FAM*"
- Navngiv brønde: Tryk på  marker én brønd af gangen tryk derefter på "*well information*"(øverst til venstre) , skriv prøvens navn i feltet og tryk "*close*", gentag for alle prøverne på pladen. Tryk derefter på "*Return to setup screen*"
- *Thermal Profile* (øverst venstre side) skal se således ud (vælg eventuelt "*custom*" i menuen til venstre, hvis der ikke kan rettes i programmet):

Segment 1, 1 cycle: 50ºc i 2 min

Segment 2, 1 cycle: 95ºc i 10 min

Segment 3, 45 cycles: 92ºc i 20 sek.; 60ºc i 1 min.

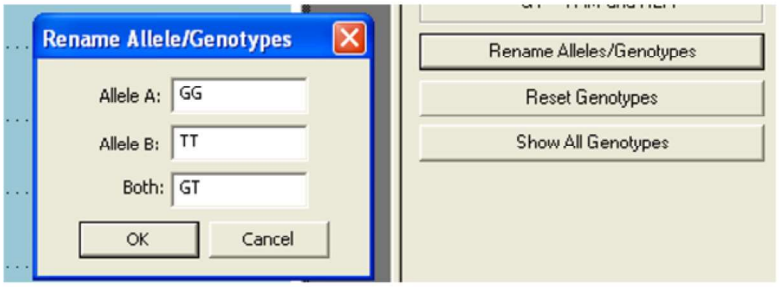
For at tilføje et trin til profilen skal man højre klikke på musen og vælge "*add segment*".

For at redigere en indstilling, skal man klikke på enheden, der skal ændres.

For at slette et trin, skal musen placeres således, at der kommer en dobbeltpil, klikke så stregen bliver rød, 

og derefter trykke "Delete".

- Kørslen startes først når lampen () lyser grøn. Klik på "*Start run*" (nederst i højre hjørne) for at starte kørslen.
- Gem kørslen – vælg evt. "*Lamp off at the end*".
- Efter endt kørsel gå ind på "*Results*" (øverst venstre)
- Vælg "*Dual color scatter plots*" → Under "*Display the values*" vælg "*Fluorescence*"
- Hvis ikke alle 4 bokse kan ses, vælg "*Show all genotypes*"
- Vælg Rename alleles. Ex. (Allel A = GG, Allel B=TT Allel AB =GT) (NB! Tjek at resultatet stemmer overens med kontrollerne, da man kan sætte kasserne forskelligt!!!!!)



- Vælg tekstrapport i menuen i højre side.
- Vælg *well*, *well name* og *Genotype*.
- Tjek kontroller stemmer overens (wellname/genotypes)
- Gem tekstrapport: "*File*" → "*Export text report*" →"*Excel*"

Appendix 7: Protocol for TaqMan x40

TaqMan Engelsk Bulldog

Husk at tænde apparatet (lampen) i B206 i god tid (Det tager ca. 20 min før lampen er klar)
Mastermix, laves på is **(Husk stinkskab når du arbejder med TaqMan mastermixen!)**:
Der laves Mastermix til hver prøve, 1 blindprøve og 1 ekstra prøve.

Mastermix (20-25ng)	x1	x
TaqMan **(Giftig!)** (x40)	0,25 µl	
TaqMan Universal mix (x2)	5,0 µl	
H_2O	3,75 µl	

9 µl mastermix + 1 µl DNA (20-25ng/µl).

Sæt reaktionerne op i de hvide plader.

	1	2	3	4	5	6	7
A							
B							
C							
D							
E							
F							
G							
H							

Mx3000p opsætning:
Program: MxPro

- Ved opstart vælg "*Allele Discrimination/ SNP's Real-Time*"
- På templaten marker de anvendte brønde, vælg derefter "*Unknown*" under "*well type*"
- Under "*Collect fluorescence data*" (menuen i højre side) marker "*VIC*" (- først) "*FAM*"
- Navngiv brønde: Tryk på  marker én brønd af gangen tryk derefter på "*well information*"(øverst til venstre) , skriv prøvens navn i feltet og tryk "*close*", gentag for alle prøverne på pladen. Tryk derefter på "*Return to setup screen*"
- *Thermal Profile* (øverst venstre side) skal se således ud (vælg eventuelt "*custom*" i menuen til venstre, hvis der ikke kan rettes i programmet):

Segment 1, 1 cycle: 50ºc i 2 min

Segment 2, 1 cycle: 95ºc i 10 min

Segment 3, 45 cycles: 92ºc i 20 sek.; 60ºc i 1 min.

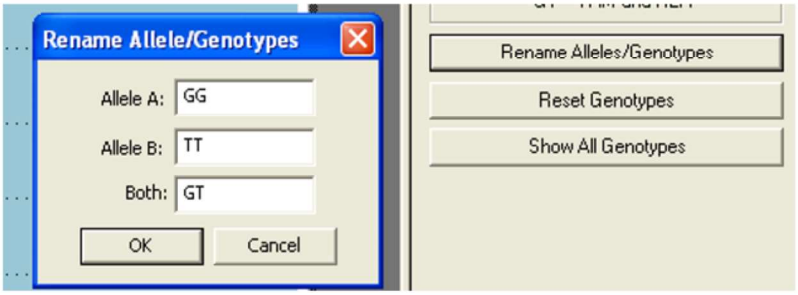
For at tilføje et trin til profilen skal man højre klikke på musen og vælge "*add segment*".

For at redigere en indstilling, skal man klikke på enheden, der skal ændres.

For at slette et trin, skal musen placeres således, at der kommer en dobbeltpil, klikke så stregen bliver rød, 

og derefter trykke "Delete".

- Kørslen startes først når lampen ( ) lyser grøn. Klik på "*Start run*" (nederst i højre hjørne) for at starte kørslen.
- Gem kørslen – vælg evt. "*Lamp off at the end*".
- Efter endt kørsel gå ind på "*Results*" (øverst venstre)
- Vælg "*Dual color scatter plots*" → Under "*Display the values*" vælg "*Fluorescence*"
- Hvis ikke alle 4 bokse kan ses, vælg "*Show all genotypes*"
- Vælg Rename alleles. Ex. (Allel A = GG, Allel B=TT Allel AB =GT) (NB! Tjek at resultatet stemmer overens med kontrollerne, da man kan sætte kasserne forskelligt!!!!!)



- Vælg tekstrapport i menuen i højre side.
- Vælg *well*, *well name* og *Genotype*.
- Tjek kontroller stemmer overens (wellname/genotypes)
- Gem tekstrapport: "*File*" → "*Export text report*" →"*Excel*"

Appendix 8: Overview of questionnaires and epicrises

An overview of information gathered from epicrises and questionnaires.

Study population		
Study number	**Answers to questionnaire**	**Information from epicrises**
EB_02	Urolith formation: No. Urinary symptoms: Periuria. Owner did not contact veterinarian. The symptom was not recurrent.	Owner did not contact a veterinarian.
EB_07	Urolith formation: No. Urinary symptoms: Suspected cystitis based on pollakiuria and "squatting to urinate" for a prolonged time. Owner did not contact veterinarian but has treated the dog with antibiotics. The symptoms were not recurrent.	Owner did not contact a veterinarian.
EB_17	Urolith formation: Diagnosed with uroliths at 4 years of age after recurring cystitis and pseudopregnancies. Uroliths have not been surgically removed and they have not reoccurred. Other urinary symptoms: Pollakiuria, periuria, and change in the urine odor. The veterinarian diagnosed the dog with cystitis due to crystalluria.	Epicrisis confirms cystine crystalluria. Ultrasound of the bladder showed no uroliths.

EB_19	Urolith formation: Diagnosed with uroliths at 3 years of age. Uroliths were found incidentally. Uroliths have not been surgically removed and have not reoccurred. Other urinary symptoms: Change in urine odor. Owner did not contact veterinarian. The symptom did not reoccur.	The veterinarian was no longer in possession of the medical record and an epicrisis has therefore not been obtained.
EB_46	Urolith formation: Diagnosed with uroliths at 5 years of age after presenting with hematuria. Uroliths have not been surgically removed and have not reoccurred. Crystalluria: Owner describes crystalluria that disappeared after feeding the dog with a special diet.	Epicrisis confirms cystine crystalluria, but not urolithiasis.
Recruited population		
Study number	**Answers to questionnaire**	**Information from epicrises**
EB_105	Urolith formation: Diagnosed with uroliths at 3 or 4 years of age after presenting with dysuria, stranguria, pollakiuria, urinary incontinence, and hematuria. The uroliths were surgically removed and analyzed. The owner does not know the result of the urolith analysis. The symptoms have not reoccurred.	Epicrisis confirms cystine cystoliths. Multiple cystine cystoliths were surgically removed. Symptoms were incontinence, hematuria, polakiuria, stranguria, and dolent vesica urinaria.

EB_106	Urolith formation: Diagnosed with uroliths at 11 months of age after anuria for 48 hours. Uroliths were surgically removed and analyzed. The uroliths consisted of cystine. Uroliths have not reoccurred.	Epicrisis confirms cystine cystoliths. Approximately 100 cystine cystoliths, each about 1-5 mm in size, were surgically removed. Symptoms were stranguria and anuria. The authors of this study received a sample of the uroliths from the owner and had them analyzed by Minnesota Urolith Center. The analysis shows an urolith composition of 100% cystine.
EB_107	Urolith formation: Diagnosed with uroliths at 1 year and 8 months of age after dripping urine when urinating. Uroliths were not surgically removed. At 2 years and 2 months of age, the dog presented with anuria.	Epicrisis confirms cystine urethroliths. A large amount of cystine urethroliths were surgically removed. The symptom was dysuria.
EB_108	Urolith formation: Diagnosed with uroliths at 3 years of age after pollakiuria and nocturia. Uroliths were surgically removed. Owner does not know whether uroliths were analyzed. Uroliths have not reoccurred. The dog has also had cystitis with periuria and hematuria and balanitits. The problem has not reoccurred.	Epicrisis confirms cystine cystoliths. Cystine cystoliths were surgically removed. Symptoms were periuria, polakiuria, polyuria, stranguria, and hematuria.

Appendix 9: Genotype results, study population

Genotype results from TaqMan analyses of the study population. Wild type (lightest shade of purple) is A/A in the *SLC3A1* mutations and G/G in the *SLC7A9* mutation. EB_50 has been excluded due to being full siblings to another individual in the study population.

Study number	**Sex**	**Age (registered in April 2022)**	**Genotype c.651A>G mutation in *SLC3A1***	**Genotype c.2092A>G mutation in *SLC3A1***	**Genotype c.723G>A mutation in *SLC7A9***	**Clinical signs of cystinuria**
EB_46	Intact male	10y 11m	G/G	G/G	A/A	**Cystine crystalluria**
EB_07	Intact female	3y 4m	G/G	G/G	A/A	None, but history of cystitis
EB_24	Intact female	4y 6m	G/G	G/G	A/A	None
EB_44	Female	1y 4m	G/G	G/G	A/A	Questionnaire not completed
EB_48	Intact female	0y 9m	G/G	G/G	A/A	None
EB_16	Neutered female	4y 8m	G/G	G/G	G/G	None
EB_23	Intact female	5y 0m	G/G	G/G	G/A	None
EB_37	Intact female	5y 0m	G/G	G/G	G/A	None
EB_53	Intact female	6y 1m	G/G	G/G	G/A	None

EB_57	Intact female	8y 10m	G/G	G/G	G/A	None
EB_05	Neutered male	7y 11m	A/A	A/A	A/A	None
EB_28	Neutered female	10y 11m	A/A	A/A	A/A	None
EB_32	Intact female	0y 8m	A/A	A/A	A/A	None
EB_38	Intact female	2y 3m	A/A	A/A	A/A	None
EB_22	Intact male	0y 11m	A/G	A/G	A/A	None
EB_08	Intact female	5y 5m	A/G	A/G	A/A	None
EB_25	Intact female	1y 0m	A/G	A/G	A/A	None
EB_26	Neutered female	4y 9m	A/G	A/G	A/A	None
EB_39	Intact female	0y 9m	A/G	A/G	A/A	None
EB_03	Intact female	8y 2m	A/G	A/G	A/A	None
EB_43	Intact female	1y 2m	A/G	A/G	A/A	None
EB_45	Intact female	0y 8m	A/G	A/G	A/A	None
EB_49	Intact female	2y 9m	A/G	A/G	A/A	None
EB_14	Intact male	1y 8m	A/G	A/G	G/G	None

EB_18	Intact male	1y 2m	A/G	A/G	G/G	None
EB_62	Intact male	5y 6m	A/G	A/G	G/G	None
EB_61	Male	4y 2m	A/G	A/G	G/G	Questionnaire not completed
EB_11	Female	5y 2m	A/G	A/G	G/G	Questionnaire not completed
EB_12	Intact female	6y 9m	A/G	A/G	G/G	None
EB_34	Intact female	1y 8m	A/G	A/G	G/G	None
EB_02	Intact male	8y 1m	A/A	A/A	G/A	None, but history of periuria
EB_09	Intact male	1y 10m	A/A	A/A	G/A	None
EB_47	Male	8y 5m	A/A	A/A	G/A	Questionnaire not completed
EB_58	Intact male	2y 1m	A/A	A/A	G/A	None
EB_19	Intact female	7y 10m	A/A	A/A	G/A	History of uroliths of unknown type
EB_06	Intact female	4y 8m	A/A	A/A	G/A	None
EB_20	Intact female	3y 1m	A/A	A/A	G/A	None
EB_30	Neutered female	9y 3m	A/A	A/A	G/A	None
EB_33	Intact female	1y 4m	A/A	A/A	G/A	None

EB_36	Intact female	8y 2m	A/A	A/A	G/A	None
EB_51	Intact female	0y 5m	A/A	A/A	G/A	None
EB_60	Intact female	3y 3m	A/A	A/A	G/A	None
~~EB_50~~	~~Intact female~~	~~0y 5m~~	~~A/A~~	~~A/A~~	~~G/A~~	~~None~~
EB_10	Intact male	0y 7m	A/G	A/G	G/A	None
EB_27	Male	2y 6m	A/G	A/G	G/A	Questionnaire not completed
EB_40	Intact male	1y 5m	A/G	A/G	G/A	None
EB_42	Intact male	2y 6m	A/G	A/G	G/A	None
EB_54	Intact male	0y 11m	A/G	A/G	G/A	None
EB_01	Neutered female	5y 0m	A/G	A/G	G/A	None
EB_21	Intact female	3y 9m	A/G	A/G	G/A	None
EB_29	Intact female	0y 7m	A/G	A/G	G/A	None
EB_35	Intact female	3y 11m	A/G	A/G	G/A	None
EB_41	Female	1y 10m	A/G	A/G	G/A	Questionnaire not completed
EB_52	Intact female	3y 11m	A/G	A/G	G/A	None

EB_55	Intact female	2y 5m	A/G	A/G	A/G	None
EB_59	Intact female	4y 10m	A/G	A/G	A/G	None
EB_63	Intact male	2y 6m	A/A	A/A	G/G	None
EB_04	Intact female	1y 11m	A/A	A/A	G/G	None
EB_13	Female	4y 8m	A/A	A/A	G/G	Questionnaire not completed
EB_15	Intact female	1 y 11m	A/A	A/A	G/G	None
EB_17	Intact female	4y 10 m	A/A	A/A	G/G	**Cystine crystalluria**
EB_31	Intact female	2y 5m	A/A	A/A	G/G	None
EB_56	Intact female	0y 8m	A/A	A/A	G/G	None

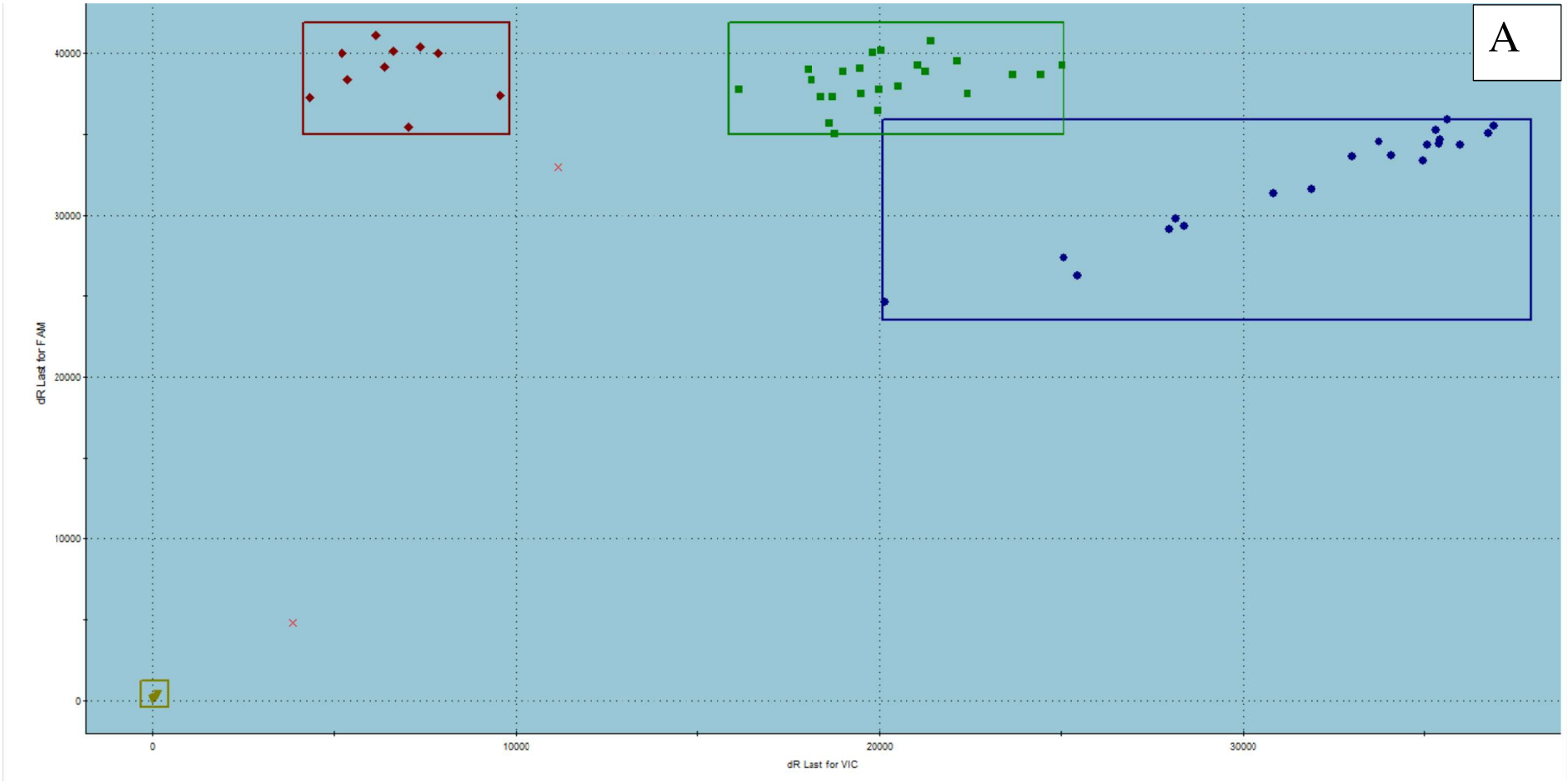
Appendix 10: Genotype results, recruited population

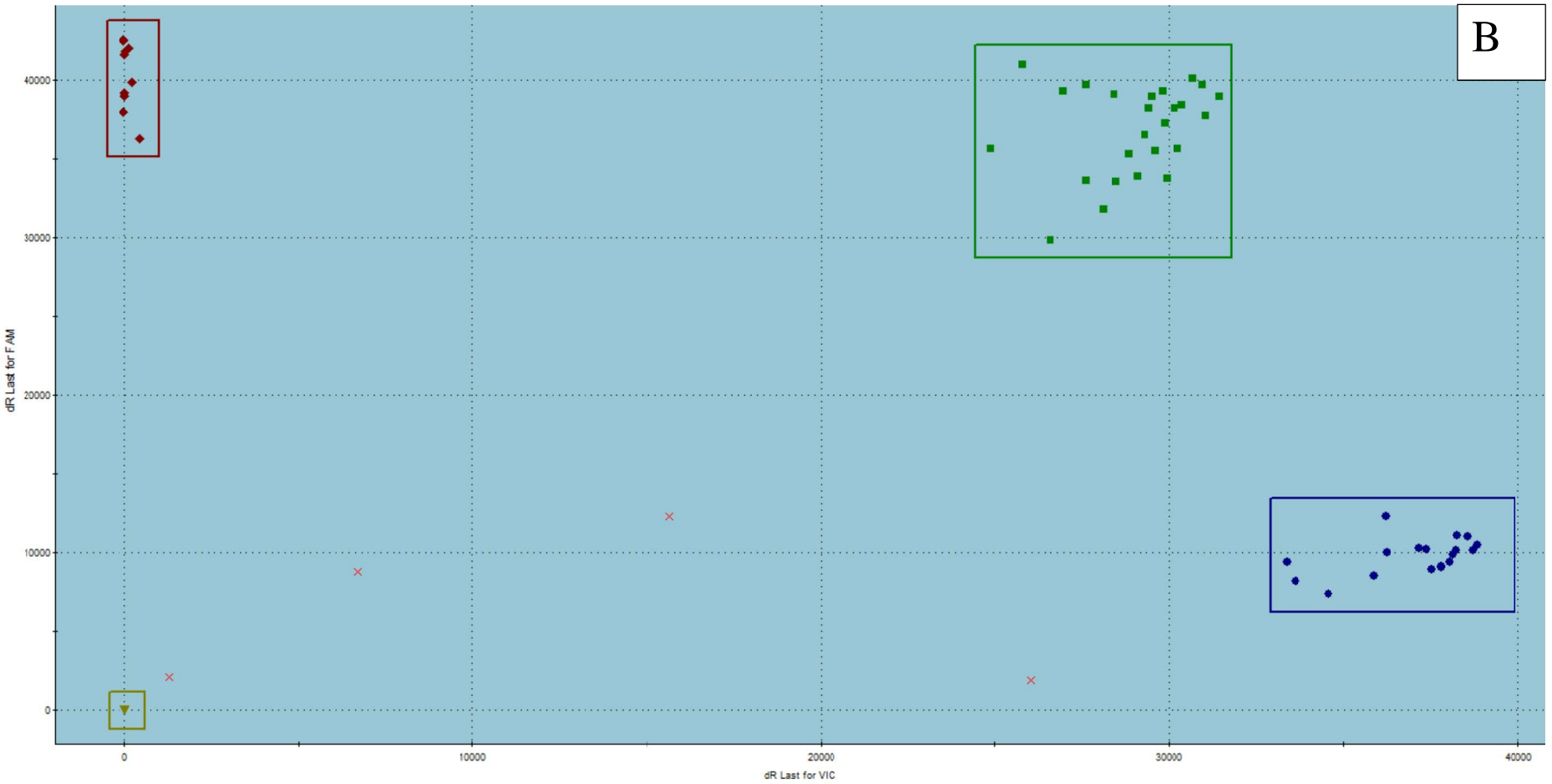
Genotype results from TaqMan analysis of the study population. Wild type (lightest shade of purple) is A/A in the *SLC3A1* mutations and G/G in the *SLC7A9* mutation.

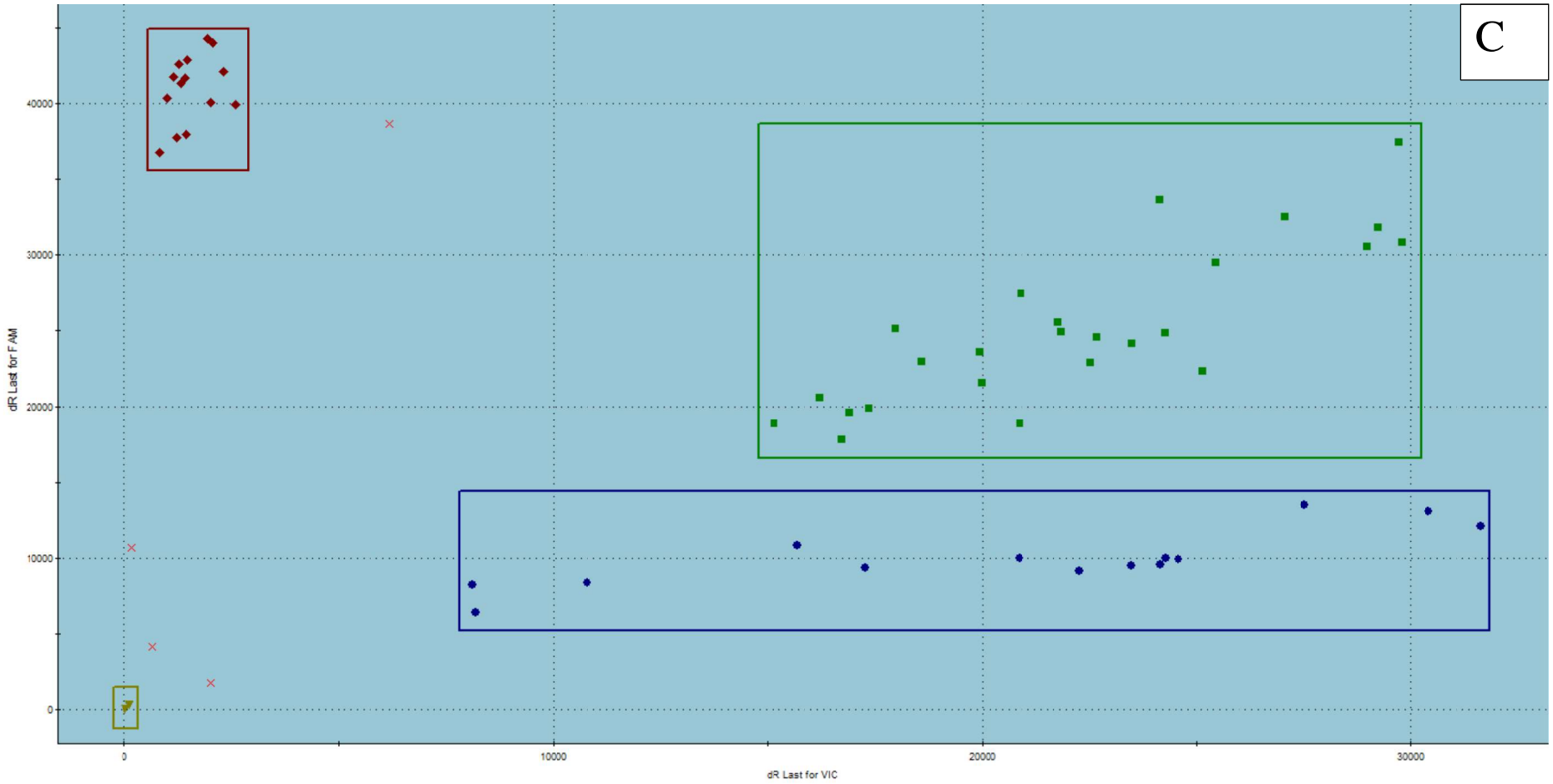
Study number	**Sex**	**Age (registered in April 2022)**	**Genotype c.651A>G mutation in *SLC3A1***	**Genotype c.2092A>G mutation in *SLC3A1***	**Genotype c.723G>A mutation in *SLC7A9***	**Clinical signs of cystinuria**	**Result from previous genetic testing by Laboklin or VetGen**
EB_105	Neutered male	6y 6m	G/G	G/G	A/A	**Cystine cystoliths**	
EB_106	Neutered male	2y 3m	G/G	G/G	A/A	**Cystine cystoliths**	
EB_101	Intact male	1y 11m	G/G	G/G	A/G	None	
EB_107	Intact male	2y 8m	G/G	G/G	A/G	**Cystine urethroliths**	
EB_109	Intact male	3y 10m	A/A	A/A	A/A	None	A/A at locus c.651 in *SLC3A1*
EB_102	Intact female	1y 1m	A/G	A/G	G/G	None	A/G at locus c.651 in *SLC3A1*
EB_104	Neutered female	5y 6m	A/G	A/G	G/G	None	A/G at locus c.651 and locus c.2092 in *SLC3A1*
EB_103	Intact female	1y 1m	A/G	A/G	A/G	None	A/G at locus c.651 in *SLC3A1*
EB_108	Intact male	3y 3m	A/G	A/G	A/G	**Cystine cystoliths**	

Appendix 11: TaqMan scatterplots

Scatter plots of TaqMan fluorescence data from Figure 6, shown in full size.




B
dR Last for FAM
dR Last for VIC
40000
30000
20000
10000
0
0
10000
20000
30000
40000


C
40000
30000
20000
10000
0
dR Last for F AM
0
10000
20000
30000
dR Last for VIC

Appendix 12: Allele frequencies, genotype frequencies and test for HW-equilibrium

Allele frequencies, genotype frequencies, and test for HW-equilibrium in the study population.

***SLC3A1* c.651A>G**

Observed genotypes

	A/A	G/G	A/G	Sum (N)
Dogs	23	10	29	62

Observed allele frequencies

$$f(A)_{\text{observed}} = \frac{23 \cdot 2 + 29}{62 \cdot 2} = 0.60$$

$$f(G)_{\text{observed}} = \frac{10 \cdot 2 + 29}{62 \cdot 2} = 0.40$$

Observed genotype frequencies

$$f(A/A)_{\text{observed}} = \frac{23}{62} = 0.37$$

$$f(G/G)_{\text{observed}} = \frac{10}{62} = 0.16$$

$$f(A/G)_{\text{observed}} = \frac{29}{62} = 0.47$$

Expected genotype frequencies if the population is in HW-equilibrium

$$f(A/A)_{\text{expected}} = (f(A))^2 = 0.60^2 = 0.37$$

$$f(G/G)_{\text{expected}} = (f(G))^2 = 0.40^2 = 0.16$$

$$f(A/G)_{\text{expected}} = 2 \cdot f(A) \cdot f(G) = 2 \cdot 0.60 \cdot 0.40 = 0.48$$

Expected genotypes if the population is in HW-equilibrium

$$A/A_{\text{expected}} = f(A/A)_{\text{expected}} \cdot N = 0.37 \cdot 62 = 22.68$$

$$G/G_{\text{expected}} = f(G/G)_{\text{expected}} \cdot N = 0.16 \cdot 62 = 9.68$$

$$A/G_{\text{expected}} = f(A/G)_{\text{expected}} \cdot N = 0.48 \cdot 62 = 29.64$$

χ^2-test

$$\chi^2 = \sum \frac{(\text{observed-expected})^2}{\text{expected}}$$

$$\chi^2 = \frac{(23-22.68)^2}{22.68} + \frac{(10-9.68)^2}{9.68} + \frac{(29-29.64)^2}{29.64} = 0.03$$

At 1 degree of freedom, $\chi^2{}_{0.05} \approx 3.84$, and because 0.03 < 3.84 the null hypothesis that the genotypes in the study population are in Hardy-Weinberg equilibrium, regarding *SLC3A1* c.651A>G, cannot be rejected at a significance level of 0.05.

***SLC3A1* c.2092A>G**

Since the observed genotypes are exactly the same for c.2092A>G and for c.651A>G, the calculations and conclusion of the χ^2-test are exactly the same.

***SLC7A9* c.723G>A**

Observed genotypes

	G/G	A/A	G/A	Sum (N)
Dogs	15	18	29	62

Observed allele frequencies

$$f(G)_{\text{observed}} = \frac{15 \cdot 2 + 29}{62 \cdot 2} = 0.48$$

$$f(A)_{\text{observed}} = \frac{18 \cdot 2 + 29}{62 \cdot 2} = 0.52$$

Observed genotype frequencies

$$f(G/G)_{\text{observed}} = \frac{15}{62} = 0.24$$

$$f(A/A)_{\text{observed}} = \frac{18}{62} = 0.29$$

$$f(G/A)_{\text{observed}} = \frac{29}{62} = 0.47$$

Expected genotype frequencies if the population is in HW-equilibrium

$$f(A/A)_{\text{expected}} = (f(G))^2 = 0.48^2 = 0.23$$

$$f(G/G)_{\text{expected}} = (f(A))^2 = 0.52^2 = 0.27$$

$$f(A/G)_{\text{expected}} = 2 \cdot f(G) \cdot f(A) = 2 \cdot 0.48 \cdot 0.52 = 0.50$$

Expected genotypes if the population is in HW-equilibrium

$$A/A_{\text{expected}} = f(A/A)_{\text{expected}} \cdot \text{N} = 0.23 \cdot 62 = 14.04$$

$$G/G_{\text{expected}} = f(G/G)_{\text{expected}} \cdot \text{N} = 0.27 \cdot 62 = 17.04$$

$$A/G_{\text{expected}} = f(A/G)_{\text{expected}} \cdot \text{N} = 0.50 \cdot 62 = 30.93$$

$\mathcal{X}^2$-test

$$\mathcal{X}^2 = \sum \frac{(\text{observed} - \text{expected})^2}{\text{expected}}$$

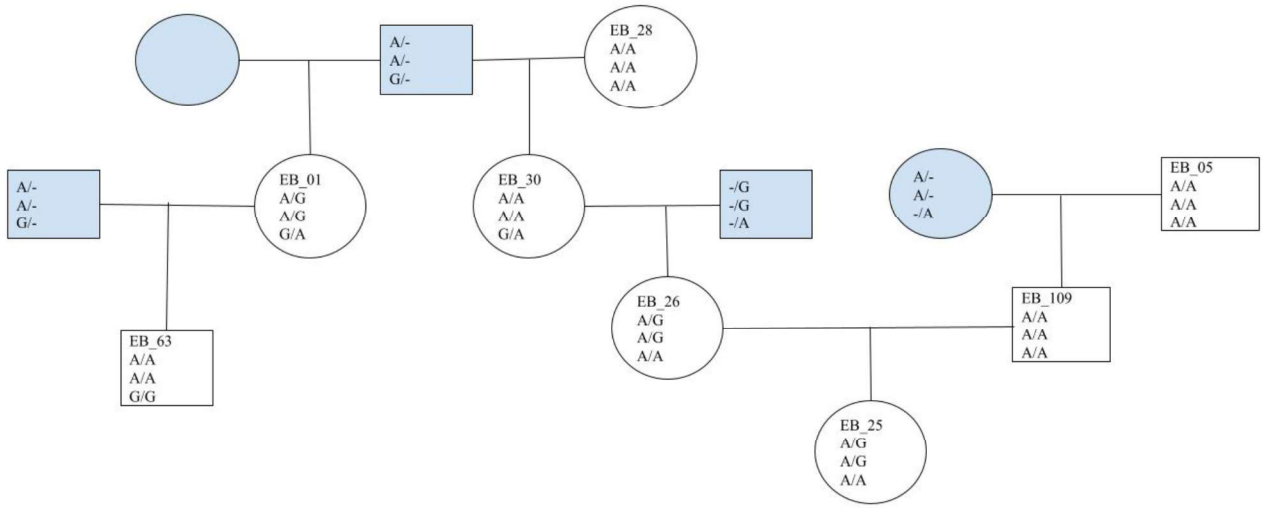
$$\mathcal{X}^2 = \frac{(15-14.04)^2}{14.04} + \frac{(18-17.04)^2}{17.04} + \frac{(29-30.93)^2}{30.93} = 0.24$$

At 1 degree of freedom, $\mathcal{X}^2{}_{0,05} \approx 3.84$, and because 0.24 < 3.84 the null hypothesis that the genotypes in the study population are in Hardy-Weinberg equilibrium, regarding *SLC7A9* c.723G>A, cannot be rejected at a significance level of 0.05.

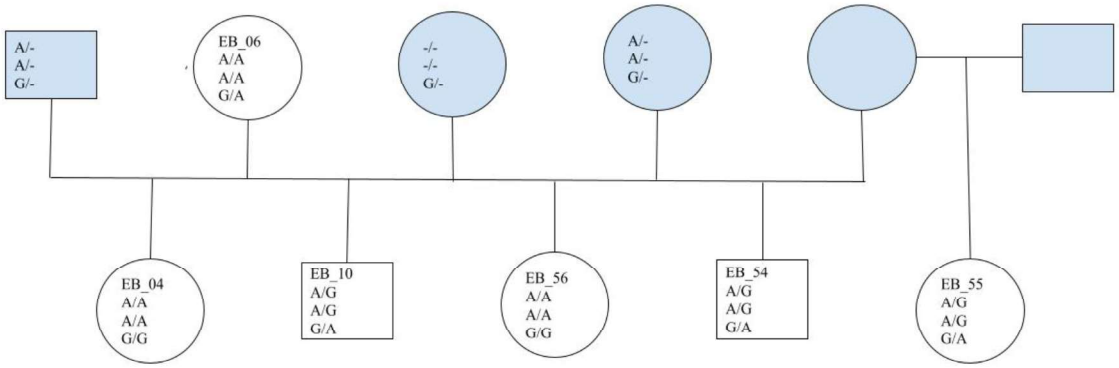
Appendix 13: Genealogical diagrams

Dogs included in this study that are related as parents/offspring or siblings to other dogs in the study are shown in these genealogical diagrams. The blue color marks individuals that are not included in the study. The white color marks dogs with no history of cystinuria, the grey color marks dogs without completed questionnaires, and the red color marks dogs diagnosed with cystinuria. Genotypes are listed in the order: Top: c.651A>G in *SLC3A1,* middle: c.2092A>G in *SLC3A1,* bottom: c.723G>A in *SLC7A9.*

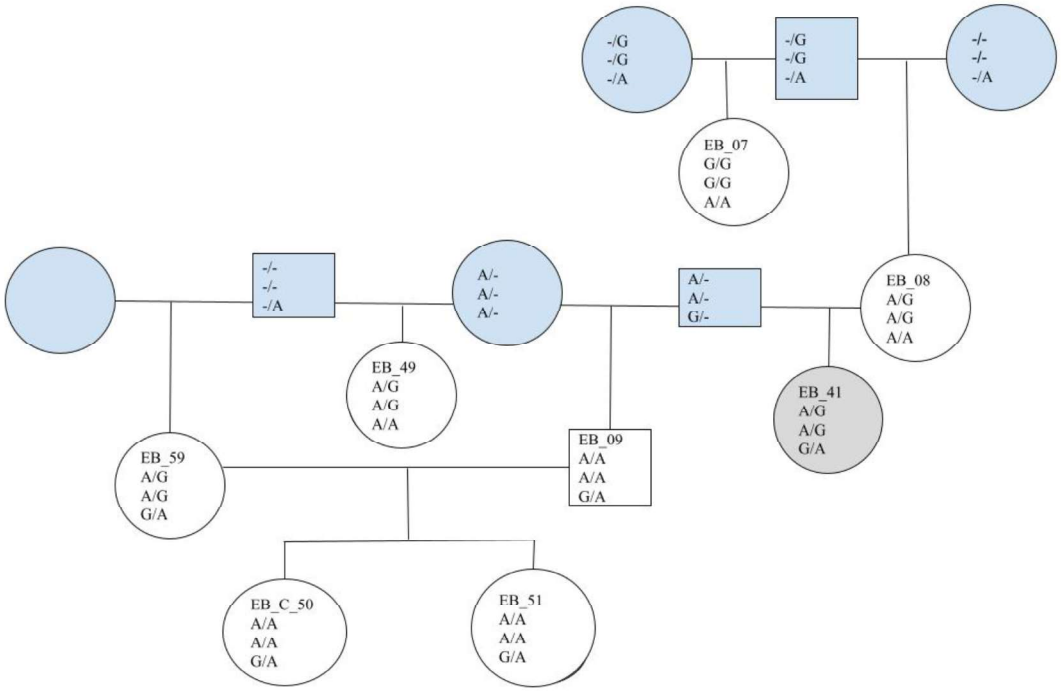
Family 1



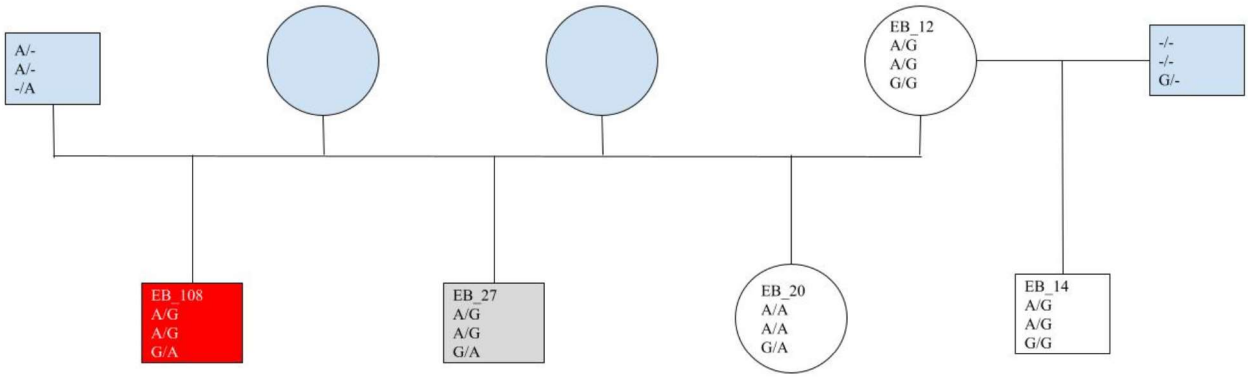
Family 2



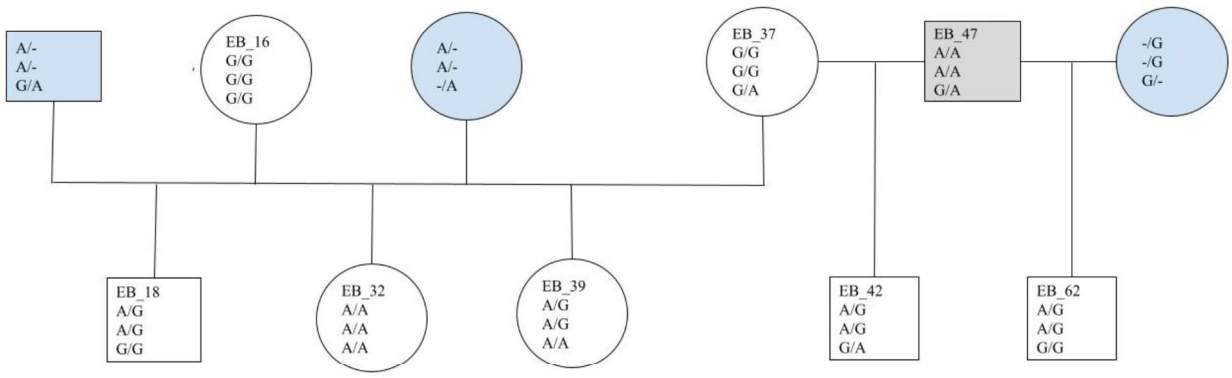
Family 3



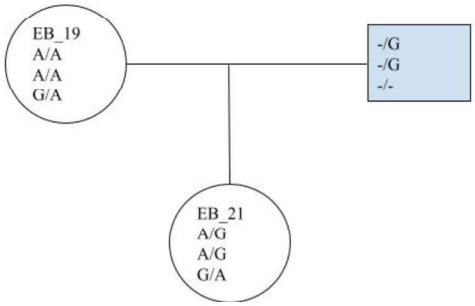
Family 4



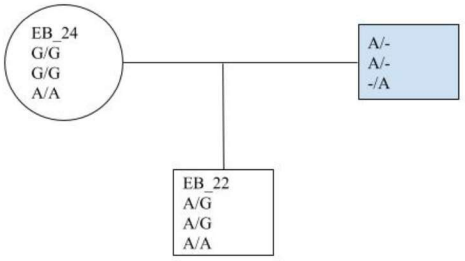
Family 5



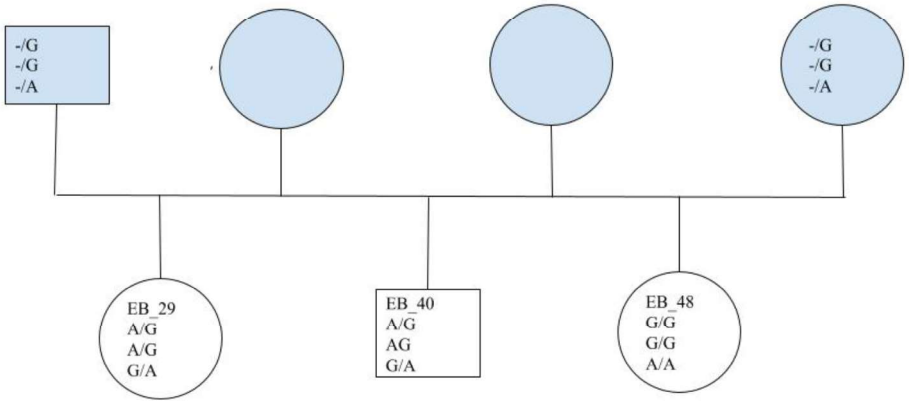
Family 6



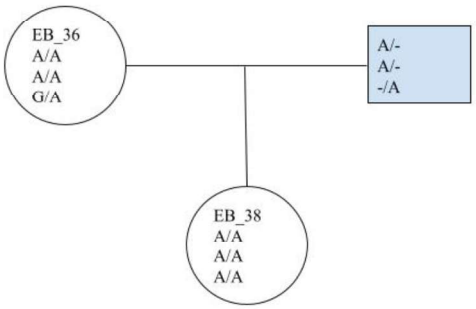
Family 7



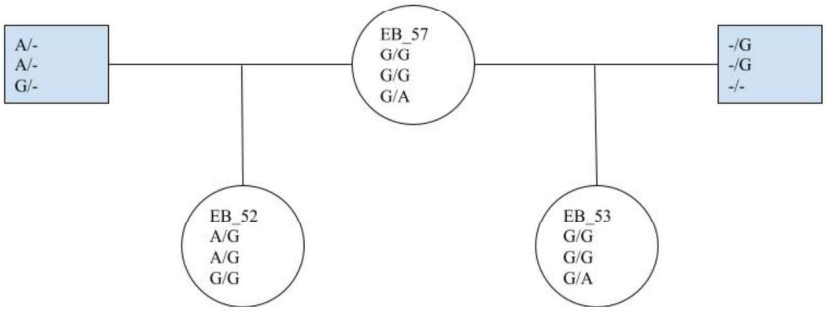
Family 8



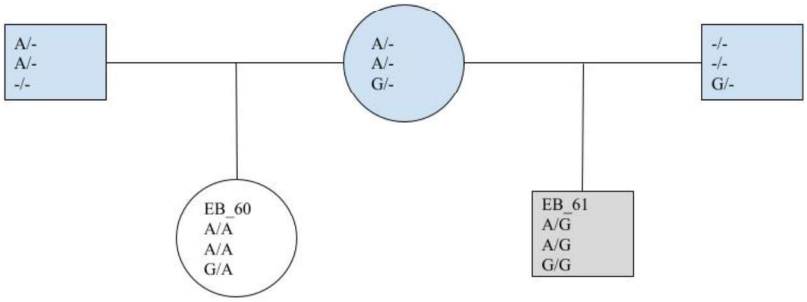
Family 9



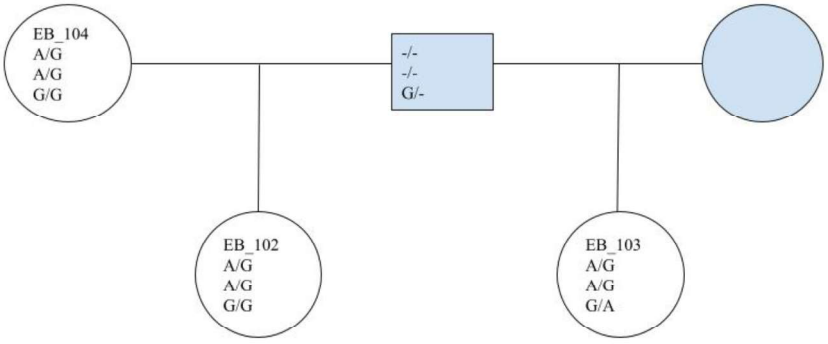
Family 10



Family 11



Family 12



Appendix 14: Results of statistical calculation

<table>
<tr><th>Null hypothesis</th><th>2x2 tables</th><th>p-value (p)</th><th>Odds ratio (OR)</th></tr>
<tr><td>The proportion of cystinuric English bulldogs is the same among dogs with the genotype G/G as among dogs with the genotype A/A or A/G regarding the mutations c.651A>G and c.2092A>G in SLC3A1.</td><td><table><tr><td></td><td>Cystinuric</td><td>Not cystinuric</td></tr><tr><td>G/G</td><td>4</td><td>9</td></tr><tr><td>A/A or A/G</td><td>2</td><td>49</td></tr></table></td><td>Boschloo's test:
$p = 0.014$
$\mathcal{X}^2$ test:
$p = 0.015$
The null hypothesis can be rejected</td><td>R command:
$OR = 10.29$
95% $CI = [1.67; 86.93]$
Formula:
$OR = \frac{(4/2)}{(9/49)} = 10.89$
95% $CI = [1.73; 68.58]$</td></tr>
<tr><td>The proportion of cystinuric English bulldogs is the same among male dogs with the genotype G/G as among male dogs with the genotype A/A or A/G regarding the mutations c.651A>G and c.2092A>G in SLC3A1.</td><td><table><tr><td></td><td>Cystinuric</td><td>Not cystinuric</td></tr><tr><td>G/G</td><td>4</td><td>1</td></tr><tr><td>A/A or A/G</td><td>1</td><td>14</td></tr></table></td><td>Boschloo's test:
$p = 0.003$
$\mathcal{X}^2$ test:
$p = 0.007$
The null hypothesis can be rejected</td><td>R command:
$OR = 36.28$
95% $CI = [2.52; 1438.91]$
Formula:
$OR = \frac{(4/1)}{(1/14)} = 56$
95% $CI = [2.83; 1109.43]$</td></tr>
<tr><td>The proportion of cystinuric English bulldogs is the same among female dogs with the genotype G/G as among female dogs with the genotype A/A or A/G regarding the mutations c.651A>G and c.2092A>G in SLC3A1.</td><td><table><tr><td></td><td>Cystinuric</td><td>Not cystinuric</td></tr><tr><td>G/G</td><td>0</td><td>8</td></tr><tr><td>A/A or A/G</td><td>1</td><td>35</td></tr></table></td><td>Boschloo's test:
1.000
$\mathcal{X}^2$ test:
0.404
The null hypothesis cannot be rejected</td><td>Not calculated since the null hypothesis cannot be rejected.</td></tr>
</table>

The proportion of cystinuric English bulldogs is the same among dogs with the genotype G/G or A/G as among dogs with the genotype A/A regarding the mutations c.651A>G and c.2092A>G in *SLC3A1*.	<table><tr><td></td><td>Cystinuric</td><td>Not cystinuric</td></tr><tr><td>G/G or A/G</td><td>5</td><td>37</td></tr><tr><td>A/A</td><td>1</td><td>21</td></tr></table>	Boschloo's test: 0.469 $\mathcal{X}^2$ test: 0.612 *The null hypothesis cannot be rejected*	Not calculated since the null hypothesis cannot be rejected.
The proportion of cystinuric English bulldogs is the same among male dogs with the genotype G/G or A/G as among male dogs with the genotype A/A regarding the mutations c.651A>G and c.2092A>G in *SLC3A1*.	<table><tr><td></td><td>Cystinuric</td><td>Not cystinuric</td></tr><tr><td>G/G or A/G</td><td>5</td><td>9</td></tr><tr><td>A/A</td><td>0</td><td>6</td></tr></table>	Boschloo's test: 0.126 $\mathcal{X}^2$ test: 0.260 *The null hypothesis cannot be rejected*	Not calculated since the null hypothesis cannot be rejected.
The proportion of cystinuric English bulldogs is the same among female dogs with the genotype G/G or A/G as among female dogs with the genotype A/A regarding the mutations c.651A>G and c.2092A>G in *SLC3A1*.	<table><tr><td></td><td>Cystinuric</td><td>Not cystinuric</td></tr><tr><td>G/G or A/G</td><td>0</td><td>28</td></tr><tr><td>A/A</td><td>1</td><td>15</td></tr></table>	Boschloo's test: 0.519 $\mathcal{X}^2$ test: 0.774 *The null hypothesis cannot be rejected*	Not calculated since the null hypothesis cannot be rejected.
The proportion of cystinuric English bulldogs is the same among dogs with the genotype A/A as among dogs with the genotype G/A or G/G regarding the mutations c.723G>A in *SLC7A9*.	<table><tr><td></td><td>Cystinuric</td><td>Not cystinuric</td></tr><tr><td>A/A</td><td>3</td><td>17</td></tr><tr><td>G/A or G/G</td><td>3</td><td>41</td></tr></table>	Boschloo's test: 0.402 $\mathcal{X}^2$ test: 0.563 *The null hypothesis cannot be rejected*	Not calculated since the null hypothesis cannot be rejected.

<table>
<tr>
<td>The proportion of cystinuric English bulldogs is the same among male dogs with the genotype A/A as among male dogs with the genotype G/A or G/G regarding the mutations c.723G>A in SLC7A9.</td>
<td>
<table>
<tr><td></td><td>Cystinuric</td><td>Not cystinuric</td></tr>
<tr><td>A/A</td><td>3</td><td>3</td></tr>
<tr><td>G/A or G/G</td><td>2</td><td>12</td></tr>
</table>
</td>
<td>Boschloo’s test:
0.128
$\mathcal{X}^2$ test:
0.260
The null hypothesis cannot be rejected</td>
<td>Not calculated since the null hypothesis cannot be rejected.</td>
</tr>
<tr>
<td>The proportion of cystinuric English bulldogs is the same among female dogs with the genotype A/A as among female dogs with the genotype G/A or G/G regarding the mutations c.723G>A in SLC7A9.</td>
<td>
<table>
<tr><td></td><td>Cystinuric</td><td>Not cystinuric</td></tr>
<tr><td>A/A</td><td>0</td><td>14</td></tr>
<tr><td>G/A or G/G</td><td>1</td><td>29</td></tr>
</table>
</td>
<td>Boschloo’s test:
1.000
$\mathcal{X}^2$ test:
0.693
The null hypothesis cannot be rejected</td>
<td>Not calculated since the null hypothesis cannot be rejected.</td>
</tr>
<tr>
<td>The proportion of cystinuric English bulldogs is the same among dogs with the genotype A/A or G/A as among dogs with the genotype G/G regarding the mutations c.723G>A in SLC7A9.</td>
<td>
<table>
<tr><td></td><td>Cystinuric</td><td>Not cystinuric</td></tr>
<tr><td>A/A or G/A</td><td>5</td><td>45</td></tr>
<tr><td>G/G</td><td>1</td><td>13</td></tr>
</table>
</td>
<td>Boschloo’s test:
0.975
$\mathcal{X}^2$ test:
0.846
The null hypothesis cannot be rejected</td>
<td>Not calculated since the null hypothesis cannot be rejected.</td>
</tr>
<tr>
<td>The proportion of cystinuric English bulldogs is the same among male dogs with the genotype A/A or G/A as among male dogs with the genotype G/G regarding the mutations c.723G>A in SLC7A9.</td>
<td>
<table>
<tr><td></td><td>Cystinuric</td><td>Not cystinuric</td></tr>
<tr><td>A/A or G/A</td><td>5</td><td>11</td></tr>
<tr><td>G/G</td><td>0</td><td>4</td></tr>
</table>
</td>
<td>Boschloo’s test:
0.275
$\mathcal{X}^2$ test:
0.519
The null hypothesis cannot be rejected</td>
<td>Not calculated since the null hypothesis cannot be rejected.</td>
</tr>
</table>

The proportion of cystinuric English bulldogs is the same among female dogs with the genotype A/A or G/A as among female dogs with the genotype G/G regarding the mutations c.723G>A in *SLC7A9*.	<table><tr><td></td><td>Cystinuric</td><td>Not cystinuric</td></tr><tr><td>A/A or G/A</td><td>0</td><td>34</td></tr><tr><td>G/G</td><td>1</td><td>9</td></tr></table>	Boschloo's test: 0.289 χ^2 test: 0.510 *The null hypothesis cannot be rejected*	Not calculated since the null hypothesis cannot be rejected.

Appendix 15: Test for HW-equilibrium among male dogs

Test for Hardy-Weinberg equilibrium among male dogs in the study population.

***SLC3A1* c.651A>G**

Observed genotypes

	A/A	G/G	A/G	Sum (N)
Dogs	6	1	10	17

Observed allele frequencies among males in the study population

$$f(A)_{\text{observed}} = \frac{6 \cdot 2 + 10}{17 \cdot 2} = 0.65$$

$$f(G)_{\text{observed}} = \frac{1 \cdot 2 + 10}{17 \cdot 2} = 0.35$$

Expected genotype frequencies if the population is in HW-equilibrium

$$f(A/A)_{\text{expected}} = (f(A))^2 = 0.65^2 = 0.42$$

$$f(G/G)_{\text{expected}} = (f(G))^2 = 0.35^2 = 0.12$$

$$f(A/G)_{\text{expected}} = 2 \cdot f(A) \cdot f(G) = 2 \cdot 0.65 \cdot 0.35 = 0.46$$

Expected genotypes if the population is in HW-equilibrium

$$A/A_{\text{expected}} = f(A/A)_{\text{expected}} \cdot \text{N} = 0.42 \cdot 17 = 7.12$$

$$G/G_{\text{expected}} = f(G/G)_{\text{expected}} \cdot \text{N} = 0.12 \cdot 17 = 2.12$$

$$A/G_{\text{expected}} = f(A/G)_{\text{expected}} \cdot \text{N} = 0.47 \cdot 17 = 7.76$$

$\mathcal{X}^2$-test

$$\mathcal{X}^2 = \sum \frac{(\text{observed-expected})^2}{\text{expected}}$$

$$\mathcal{X}^2 = \frac{(6-7.12)^2}{7.12} + \frac{(1-2.12)^2}{2.12} + \frac{(10-7.76)^2}{7.76} = 1.41$$

At 1 degree of freedom, $\mathcal{X}^2{}_{0,05} \approx 3.84$, and because 1.41 < 3.85 a null-hypothesis about the genotypes among the male dogs in the study population being in Hardy-Weinberg equilibrium in regard to *SLC3A1* c.651A>G cannot be rejected at a significance level of 0.05. Since the observed genotypes are exactly the same for c.2092A>G and for c.651A>G, the calculations and conclusion of the $\mathcal{X}^2$-test are exactly the same.

Appendix 16: $\mathcal{X}^2$-test for sex distribution

Observed sex distribution in the study population

	Male	Female	Sum
Dogs	17	45	62

Expected sex distribution at a 1:1 male to female ratio

	Male	Female	Sum
Dogs	31	31	62

$\mathcal{X}^2$-test

$$\mathcal{X}^2 = \sum \frac{(\text{observed} - \text{expected})^2}{\text{expected}}$$

$$\mathcal{X}^2 = \frac{(17-31)^2}{31} + \frac{(45-31)^2}{31} = 12.64$$

At 1 degree of freedom, $\mathcal{X}^2{}_{0,05} \approx 3.84$, and because 12.64 > 3.84 the null hypothesis that the sex distribution in the study population is not significantly different from a 1:1 ratio can be rejected at a significance level of 0.05.

Appendix 17: Letter to owners of participating English bulldogs

Kære (indsæt navn)

Tusind tak for din deltagelse i specialeprojektet om cystinuri hos engelske bulldogs. Vi vil med dette brev oplyse dig om resultaterne af den gen-undersøgelse, vi har lavet på din hund. For at du kan forstå resultatet, er det vigtigt, at du læser hele brevet. Du er velkommen til at kontakte os, hvis du har spørgsmål til resultatet af undersøgelsen. Vores kontaktoplysninger fremgår nederst i brevet.

Hvad ved vi nu om cystinuri hos engelske bulldogs?
Cystinuri er en arvelig sygdom, der kan føre til udvikling af sten og/eller krystaller i urinvejene pga. en defekt i nyrernes evne til at genoptage aminosyren cystin fra urinen. Nogle hunde mærker intet til sygdommen, mens andre oplever gentagne problemer med stendannelse i urinvejene. Sten i urinvejene kan i værste tilfælde forhindre hunden i at tisse, hvilket kan være livstruende. Sygdommen giver langt hyppigere symptomer hos hanhunde i forhold til hos tæver. Vi har i vores projekt undersøgt engelske bulldogs for 3 mutationer, der ved tidligere forskning har vist sig i engelske bulldogs med cystinuri. To af mutationerne sidder i genet *SLC3A1*, og disse mutationer nedarves altid sammen. Det vil sige, at hvis hunden har den ene mutation, har den også altid den anden. Derfor angiver vi også kun én samlet genotype for dette gen, som gælder for begge mutationer. Den tredje mutation sidder i genet *SLC7A9.*

Hvordan skal du forstå svaret på din hunds genundersøgelse?
Hver hund har to kopier af hvert gen – én er arvet fra hundens mor og én er arvet fra hundens far. En hund kan derfor have arvet udgaven af genet med mutationen fra både mor og far og dermed have to kopier af det muterede gen. Så siger vi, at dens genotype er muteret/muteret. Den kan også have arvet den normale udgave af genet fra den ene forælder og den muterede udgave af genet fra den anden forælder. Så siger vi, at genotypen er normal/muteret. Til sidst kan den have arvet den normale udgave af genet fra begge forældre. Så siger vi, at dens genotype er normal/normal.

Bør engelske bulldogs blive testet for mutationerne?

Der er i vores projekt ikke fundet entydig sammenhæng mellem hundenes genotyper, og hvorvidt de har cystinuri. Ikke alle de hunde, der har cystinuri, har mutationerne, og ikke alle hunde med mutationerne har symptomer på cystinuri. Det gælder dog for hanhunde, at hvis begge deres kopier af genet *SLC3A1* har mutationerne (deres genotype for *SLC3A1* er muteret/muteret), så er de i øget risiko for at udvikle cystinuri. For tæver ses denne sammenhæng ikke. Vi ser ingen sammenhæng mellem mutationen i *SLC7A9* og cystinuri for hverken tæver eller hanhunde, og derfor er der ingen grund til at se på resultatet af denne undersøgelse. Samtidig er alle tre mutationer meget udbredte blandt de engelske bulldogs, vi har testet. 63% af de engelske bulldogs, der blev tilfældigt udvalgt uden forudgående kendskab til, om de havde sygdommen, bærer mindst én kopi af mutationerne i *SLC3A1*.

Derfor vurderer vi, i samarbejde med projektets vejleder Professor Merete Fredholm, at det ikke vil være fornuftigt at tage hensyn til resultatet af gentests for cystinuri i avlen af engelske bulldogs med den nuværende tilgængelige viden.

Dette begrundes med, at usikkerheden omkring mutationernes betydning for udvikling af cystinuri er for stor. Godt nok har vores undersøgelser vist, at størstedelen af hanhunde med cystinuri har genotypen muteret/muteret i *SLC3A1*. Men ingen af tæverne med denne genotype har udvist tegn på sygdom. Anvender man udelukkende hunde uden mutationerne i *SLC3A1* i avlen, vil man udelukke et så stort antal hunde fra avl, at racens samlede sundhed vil forværres. For at mindske forekomsten af cystinuri, er det imidlertid uhyre vigtigt, at hvis din hund har haft blæresten af cystin, så bør den ikke indgå i avlen.

Hvad kan du bruge resultatet af din hunds genundersøgelse til?

Det er vigtigt at understrege, at du ikke skal inddrage resultatet af genundersøgelsen i dine overvejelser omkring, hvorvidt du skal avle på din hund eller ej. Du kan heller ikke bruge resultatet til at udelukke, at din hund kan udvikle cystin-sten i urinvejene, da cystin-krystaller er set hos alle genotyper. Hvis du har en hanhund med genotypen muteret/muteret i *SLC3A1*, så er den i øget risiko for at udvikle cystin-sten i urinvejene. Derfor er det vigtigt, at du holder særligt godt øje med, at din hund tisser ubesværet og regelmæssigt. For alle hundeejere gælder det, at du straks skal kontakte en dyrlæge, hvis din hund ikke tisser, som den plejer.

Testen har vist at (hundens navn) (hundens DKK-nummer) har genotypen (**eksempel**):

SLC3A1

Muteret/Normal

Vi takker igen for din deltagelse i specialeprojektet.
Specialeprojektet forventes afsluttet i slutningen af juni 2022, hvorefter projektet vil være tilgængeligt i dets fulde her.

De bedste hilsener,

Dyrlægestuderende,
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