

Non-Type 1 Cystinuria Marker DNA Test for Mastiff-Type Breeds

The Science and Clinical Implications Behind the Test

There are at least **two types of cystinuria in dogs**. Type-I cystinuria, well documented in Newfoundlands, is simply inherited as an autosomal recessive trait caused by a mutation in a gene that is known to cause cystinuria in people as well. This type of cystinuria is characterized by early age of cystine stone formation in males and females, and very high concentrations of cystine and the dibasic amino acids (together referred to as the COLA amino acids) in the urine of both cystinuric males and females. A DNA test for this type of cystinuria is available for the Newfoundland breed, based on research in our laboratories.

We now have observed a different type of cystinuria in several other dog breeds, which we refer to as **Non-Type I cystinuria**. This type of cystinuria does NOT show a simple, straightforward mode of inheritance, is characterized by more moderately elevated urine concentrations of the COLA amino acids, by cystine stone formation later in life (than in Newfoundland dogs with Type I cystinuria), and is only observed in intact male dogs. The DNA test we have recently developed is for this Non-Type I cystinuria, which we refer to as “Non-Type I Cystinuria Marker DNA Test for Mastiff-Type Breeds”. This test was developed based on studies in the Mastiff breed, and may also be useful for dogs of a few related breeds with similar cystinuria characteristics, such as English and French Bulldogs.

Before explaining our DNA findings, let’s review some **basic genetic terminology**. All mammals (dogs, people, etc) have two copies of every chromosome, except the sex chromosomes, where females have two copies of the X chromosome and males have one X and one Y chromosome. The non-sex chromosomes are referred to as autosomes. So, when looking at an individual’s DNA, there are two copies of each autosome, and therefore two copies of each gene or DNA marker (except in males, for those genes that are on the X and Y chromosomes). The term homozygous refers to the situation when both chromosomes have the same DNA sequence of a gene or marker. Heterozygous refers to when the two chromosomes in an individual differ in the particular DNA sequence being considered, often referred to as mutant (disease-causing) or normal (wild-type) gene or marker.

The DNA sequence differences among individuals, or even between the two different chromosomes in the same individual, are called polymorphisms. If a sequence difference is only in a single base pair, the smallest building block of DNA, this is called a SNP, for **single nucleotide polymorphism**. (For example, there is a SNP at DNA base position 3,286,059 on dog chromosome 1, where either an A (adenine) or a C (cytosine) base has been observed among different dogs, or on the different copies of chromosome 1 in a single dog.) The genetic term for the different versions of a polymorphism is alleles (eg the A allele or the C allele). The “SNP chips” are a technology that can simultaneously determine the alleles at 170,000 different SNPs, located across all of the autosomes and the X chromosome. When SNP chips are used in a genetic trait study involving numerous dogs, it is referred to as a SNP-chip study or GWAS (for **genome wide association study**).

We performed a pilot SNP-chip study in Mastiffs that implicated the region including and surrounding one of the genes associated with cystinuria in humans. We followed this up by examining this region in more than 100 intact male Mastiffs greater than 2 years of age that had been tested for cystinuria in some way (by stone analysis, urinary nitroprusside testing and/or COLA urine amino acid quantitation). We only looked at intact adult males because we fairly

recently recognized that female Mastiffs do not get cystinuria and that male dogs need to be adult and intact to express the disease. We looked at many SNPs across the region, looking for cases where one of the alleles was found in cystinuric dogs more often than expected by chance. So, if we call the SNP/marker allele associated with cystinuria the 2 marker, and the other alleles the 1 marker, each dog could have two copies of marker allele 1 (designated 1-1), one copy of each marker allele(1-2), or two copies of marker allele 2 (2-2). The table below shows the approximate distribution of these marker alleles with respect to cystinuria in intact male Mastiffs.

Before going into the numbers, please keep in mind that this set of dogs is probably NOT representative of the entire Mastiff breed population. (Our sample of dogs is probably biased towards dogs with cystinuric relatives.) All intact male dogs in the study that were homozygous for the 2 marker allele (2-2) had

formed cystine stones at or before 4 years of age. At this time, we do not know of any intact 2-2 dogs over 4 years of age that have not formed a cystine stone. Of all of the stone-forming dogs in our study, about three quarters had the 2-2 result (homozygote).

Cystinuria Status of Intact Adult Male Mastiffs	Marker allele combinations		
	2 - 2	1 - 2	1 - 1
Stone-former	100%	~25%	0%
Nitroprusside positive	n/a	~50%	<15%
Nitroprusside negative	n/a	~25%	~85%

The situation is more complex among the dogs that tested 1-2. About one quarter of the 1-2 dogs have formed stones. Approximately one-half of 1-2 dogs had at least one positive nitroprusside urine test but had not been reported as having formed stones, and the remaining one quarter had only negative nitroprusside test results. The average age of stone formation for the heterozygous 1-2 stone-forming dogs is later than for the homozygous 2-2 stone-formers, with one 1-2 dog forming his first clinical problem due to a cystine stone at 9 years of age, despite being nitroprusside positive for many years.

And lastly, while we have not observed any 1-1 dogs that have formed a cystine stone, more than 10% of 1-1 dogs in the study have had a positive nitroprusside test, with several dogs confirmed to have elevated COLA by urine amino acid quantitation. This is a finding that requires additional research, but may be related to additional genetic causes and/or to diet and dietary supplements.

In summary, while there is still much research to be done, these studies allow us to offer DNA testing to identify Mastiff dogs that are at highest risk for stone formation (intact male dogs of 2-2 marker allele designation), those at lower risk with later stone formation (intact male dogs of 1-2 marker allele designation), and for determining the possibility of producing 2-2 and 1-2 puppies from any particular mating. Combined with the fact that neutering can normalize urinary cystine and COLA levels in male Mastiff dogs (thereby curing them of cystinuria), there are now more options available to identify dogs at risk for stone formation, to avoid having dogs block from cystine stones despite their genetic predisposition, and to help breeders reduce the incidence of cystinuria in the Mastiff breed by allowing informed breeding decisions.

While we recommend DNA testing of Mastiffs and related breeds for cystinuria, be aware that this DNA test alone does not completely predict the cystinuria status of every dog (particularly for 1-2 dogs). Therefore, annual urinary nitroprusside testing is recommended for all adult intact male dogs.

Non-Type 1 Cystinuria Marker DNA Test for Mastiff-Type Breeds

Questions & Answers

1. Blood sample or swab/brush?

We can use blood or cytology brushes (for collecting cells from inside the cheek). Blood samples should be sent in purple/lavender top tubes (1-2 ml EDTA) within a Ziploc bag and in an insulated container (with an ice pack during the summer), by 1-2 day delivery. Cheek/cytology brushes need to be dried completely before placing them in a Ziploc bag and can be sent by regular mail in a standard envelope. We prefer blood, along with a free catch urine sample to test for actual cystinuria. Frozen semen from a semen bank could also be used for DNA testing, but contact Dr. Henthorn to get instructions before sending.

2. At what age can the DNA and nitroprusside tests be done?

This DNA marker test can be done at any age from birth on and independent of neutering status. However, if you want to use cheek brushes (particularly with a puppy that has not been weaned), the dog needs to be fasted for a couple of hours and the cheeks need to be clean from any food so that there is no chance of contaminating the brush with food particles (or the dam's milk which contains her cells and thus her DNA).

In contrast, urinary nitroprusside and COLA tests do not become positive until after puberty and turn negative after castration, and are therefore not as informative for puppies and neutered males. Urine testing for males can be done at any time, but is not generally useful before 2 years.

3. Who takes the sample...me or my veterinarian?

EDTA Blood 1-2 ml – veterinarian or animal nurse/veterinary technician.

Cheek brushes – you or your veterinarian or animal nurse/veterinary technician – rotate brush ten times and let it completely dry before placing in Ziploc bag and shipping.

4. What will the cost be? What is the time line to receive results?

Currently the test costs \$140 per dog, and includes DNA marker and urinary nitroprusside testing if the urine sample is submitted with the blood/cheek brush. The results will typically be reported in 2-4 weeks, although possibly longer now, as the test is first being introduced.

5. Will there be a "litter discount"?

Yes, \$100 each if 3 or more pups from a litter are tested at the same time, with a maximum of \$600 per litter up to 10 puppies.

6. Will the test be made available outside the US?

Yes. It is probably easiest to send airmail cheek brushes from other countries, but we routinely receive FedEx packages from overseas containing urine and blood on frozen ice packs. It might be worthwhile to combine samples to share the cost and paperwork for international shipping of blood and/or urine.

7. I have frozen semen on my deceased dog. Can it be tested?

Yes, but it may take longer to get the results. Contact Dr. Henthorn to discuss specifics.

8. I have provided blood samples for the research. Can I have those tested?

Please send us blood or cheek brushes from dogs you want to have results on, even if you think we already have the DNA. There are a few reasons for this. We may have used the entire previous DNA sample for research or the sample may have become degraded over time, and we may need

additional DNA to run the current test. It is more efficient for us to start with a new sample, which will have arrived with all of the current information that we need. In addition, the exact technology we are using for the current test is not the same as was used in the research, and we want all of the dogs submitted for testing to go through the same workflow pipeline and testing technology. Contact Dr. Henthorn if your dog is deceased, but you sent in blood in the past.

9. Will the genetic test results specify that the dog has one or both copies of the cystinuria-associated marker?

Yes, the results will reflect the possible combinations of the marker alleles as 1-1, 1-2, and 2-2.

- 1-1 indicates two copies of the normal marker allele (no stone-forming males to date).
- 1-2 indicates a dog with one copy of the normal marker alleles and one copy of the cystinuria-associated marker allele (at lower risk with later average age of stone formation in intact males).
- 2-2 indicates both chromosomes carry the cystinuria-associated marker allele (high risk for early stone formation in intact males).

10. Is the test a linked DNA marker test or disease-causing mutation test?

A linked marker test detects DNA differences very close to the actual DNA change responsible, but perhaps not the problem itself. Our ongoing research is aimed at determining the disease-causing mutation. Our preliminary research indicates that one of the markers we are using may be the disease-causing mutation. Until we collect more supporting data, we will continue to refer to our test as a linked marker test. Furthermore our current DNA test addresses the fact that only intact male Mastiffs develop cystinuria and urinary tract cystine stones.

11. At what age should I neuter my cystinuric dog?

Discuss this with your primary veterinarian. If your dog has a 2-2 test result, he is at very high risk for cystine stone formation and urinary tract blockage by age 4 years (range 1.5 to 4, average around 2.5 years). If your dog has a 1-2 DNA test result **and** one or more positive nitroprusside urine test results (NP+), he is also at risk (although at considerably less risk and with clinical signs probably beginning later in life than a 2-2 dog). The average age of stone analysis in 1-2 dogs is about 5.5 years (range 2.5-9 years). We don't yet have a good estimate of what percent of 1-2 NP+ dogs will block from cystine stones during their lifetime.

12. What is the rate of false positives? False negatives?

We are doing our utmost to insure that there are no mistakes in handling the samples, including performing 2 separate marker tests on each dog to serve as an internal control checking for repeatability. False positives or negatives would most likely be caused by misidentification of the dog's sample at some point in the process from your identification of dog and sample at time of collection to our reporting results. The DNA test itself is very accurate. Keep in mind that we have observed Mastiffs with the 1-2 and 1-1 designations that are cystinuric, so it is important to screen the intact males regularly by urinary nitroprusside test. Also, be aware that, as with any genetic disease, other mutations in the same gene or other genes can cause the same disease, and a DNA test only detects one cause. This cystinuria test only detects one of the causes of cystinuria (for example, it does not detect the mutation that causes cystinuria in Newfoundland dogs).

13. If my dog is homozygous for the cystinuria-associated marker allele (2-2), will he/she form cystine stones?

We have not identified any female Mastiffs that have had positive nitroprusside or COLA tests or formed cystine stones. However, all intact male 2-2 Mastiffs in our study have formed stones by 4

years of age. Our data is derived from a relatively small number of stone-forming males, so it is possible that some 2-2 intact males may form stones later than age 4. Environmental factors such as high protein diets and amino acid supplements, low urine pH, dehydration, and anatomical variations in the urinary tract may affect the dog's tendency to form stones and obstruct.

14. If my dog is heterozygous (1-2), will he form a cystine stone?

A heterozygous 1-2 intact male dog may form cystine stones later in life, but we cannot predict with certainty. However, any male dog that has an excess urinary cystine and COLA (determined by nitroprusside testing, urine amino acid quantitation, or presence of cystine crystals in urine) must be considered at some risk for cystine stone formation. An intact male 2-2 dog is clearly at a higher risk than an intact NP+ male 1-2 dog. Again, intact and spayed females with any test result should not develop cystine calculi but females with the 2-2 and 1-2 result can pass on the cystinuria associated marker to their offspring.

15. If my dog does not have a cystinuria-associated marker allele (is 1-1), can he form cystine stones?

We have not yet observed any 1-1 dogs forming cystine stones, but some 1-1 intact male dogs can be cystinuric (meaning that they have increased cystine in their urine), and therefore may still be at risk for stone formation. If your dog is homozygous "normal" (1-1) and has had multiple negative nitroprusside tests since two years of age, he is at the lowest risk that we can currently assess for forming cystine stones.

16. Will the Non-Type I cystinuria DNA Test results specify if the dog is a carrier?

The test result will tell if the dog is homozygous at risk (2-2; two copies of the cystinuria associated marker alleles), heterozygous (1-2; one copy of the cystinuria associated marker allele), or homozygous normal (1-1, no copies of the cystinuria associated marker allele).

17. Please explain the health impact for the individual dog.

Remember that female dogs of several breeds (particularly Mastiffs) apparently do not have any risk for forming cystine stones, no matter which DNA test result. Also castrated males seem to resolve their cystinuria and by two months after neuter have normal urine COLA amino acid concentrations (although their urinary tracts may still contain stones formed before the dog was neutered). Therefore, the interpretations listed below only refer to INTACT MALE dogs:

1-1 An intact male dog with this 1-1 result has no copies of the markers associated with cystine stone formation. While this dog has the lowest risk for forming cystine stones, we recommend annual urine screening for intact male dogs over age 2 years by nitroprusside testing, since we know that some 1-1 dogs can have elevated urine cystine levels.

1-2 An intact male dog with this 1-2 result has one copy of the marker associated with cystine stone formation. This dog is at intermediate risk for developing cystine stones. Urine nitroprusside screening, starting by two years of age, should be performed, since many 1-2 dogs have elevated urine cystine, and some form stones. We have observed intact male 1-2 dogs become nitroprusside positive after age 2 years, so a negative nitroprusside test does not guarantee that the dog will remain nitroprusside negative throughout his life. Castration is an option to prevent the nitroprusside positive dogs from forming cystine stones and to turn nitroprusside positive dogs negative. Both males and females with the 1-2 result can pass on the cystinuria associated marker alleles to their offspring.

2-2 An intact male dog with this 2-2 result has two copies of the DNA markers associated with cystine stone formation. This dog has the highest risk for forming cystine stones, with stones causing clinical signs usually by 4 years of age in intact male dogs. Castration is recommended to reduce both the risk of stone formation and the spread of the disease-causing allele to offspring. While females will not be cystinuric and have no risk for cystine calculi formation, they will pass on the mutant allele to their offspring and thereby can produce cystinuric male offspring if bred to 1-2 or 2-2 males and 1-1 and 1-2 offspring if bred to 1-1 males.

18. Please detail possible breeding combinations.

Please note: these results are based only on testing for the marker allele associated with this non-type I cystinuria found in Mastiffs and related breeds that we have recently identified. At any time, there is a chance (probably extremely small) that male or female dogs could form cystine stones from some other cause, such as a different genetic defect. Please refer to your veterinarian for recommendations for management of puppies (1-1, 1-2, or 2-2) produced by the matings outlined below. As stated above, screen all intact males annually by nitroprusside testing, starting by two years of age.

- a. Both parents are 1-1 (normal/clear for the cystinuria associated marker alleles): All pups produced from this type of mating will be 1-1, like their parents.
- b. Both parents are 1-2 (carriers of one copy of the cystinuria associated markers): Statistically each pup from this type of mating has a 25% risk for being homozygous (2-2) for the cystinuria associated markers, 50% risk for being heterozygous (1-2), and a 25% chance for being homozygous for the normal markers (1-1). Since puppies with all possible DNA test results are possible, DNA test all male offspring by 2 years of age (if left intact) and all females that will be used for breeding.
- c. Both parents are 2-2 (have both copies of the cystinuria associated marker alleles): All pups will be homozygous 2-2 and intact males will therefore be at high risk for cystine stone formation. Female puppies are not expected to have elevated urine cystine concentrations nor to form stones, but like 2-2 males, they will pass on the mutant allele to all of their offspring. This mating must be avoided.
- d. One parent is 1-1, the other is 2-2: All pups will be heterozygous (1-2), with one normal marker and one cystinuria associated marker. These intact male 1-2 pups are at moderate risk for being nitroprusside positive and for forming cystine stones.
- e. One parent is heterozygous 1-2 and the other is homozygous 2-2: Each pup has a 50% chance for being heterozygous 1-2 and a 50% chance for being homozygous 2-2. Intact male 2-2 pups are at very high risk of forming stones, probably before age 4 years. Males with the 1-2 status and positive nitroprusside urine test result (after age two years) will have some risk of forming stones later in life. This mating must be avoided. DNA test all male offspring by 2 years of age (if left intact) and all females that will be used for breeding.
- f. One parent is heterozygous 1-2 and the other is homozygous 1-1: Each pup has a 50% chance for being heterozygous 1-2 and a 50% chance for being homozygous normal 1-1. DNA test all male offspring by 2 years of age (if left intact) and all females that will be used for breeding.

19. What can I do to help the research?

Please continue to test the urine from your intact adult male dogs. Also, let us know if cystinuria in your dog is different from what we have described here. For example, if your female or neutered adult male dog has formed cystine stones or crystals, we need to hear about it. If your dog has formed cystine stones, participate in the research by providing us with blood, urine, pedigree, and the stone analysis report. Contact Dr. Henthorn for instructions for research participation.

20. Who do I contact with questions?

Contact Dr. Giger (PennGen@vet.upenn.edu) with questions about urine testing and medical management of cystinuric dogs.

Contact Dr. Henthorn (CystinuriaDNAtest@gmail.com) with questions about the Non-Type 1 Cystinuria Marker DNA Test for Mastiff-Type Breeds and about research participation.