

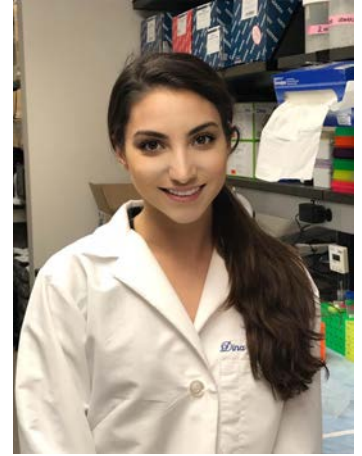
Update from July 2, 2019: Search for the gene and mutation causing inherited cataract(s) in American cocker spaniels



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August 14, 2020

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The working hypothesis has always been that the mode of inheritance is simple autosomal recessive. This has directed the initial phase of the research.

**FAMILIAL CATARACTS
IN THE
AMERICAN COCKER SPANIEL**

J. American Animal Hospital Association, 1971

William L. Yakely, D.V.M., M.S.

G. A. Hegreberg, D.V.M., Ph.D.

G. A. Padgett, D.V.M., M.S.

**A Study of Heritability of Cataracts in the
American Cocker Spaniel**

W. L. Yakely, DVM, MS

3540

JAVMA, Vol 172, No. 7 1978

We now know that is not the case, and have redirected or efforts to examine this new interpretation of heritability of cataracts in the American cocker spaniel.

Cocker Spaniel Inherited Cataract Research Form

INSTRUCTIONS: In addition to collecting 3-5 ml of whole unclotted blood in a purple capped EDTA tube from each dog, please include:

- Completed form by owner
- 5-6 generation pedigree of the dog
- Current and any/all previous eye exams on the dog (*can be sent electronically*)

The blood and paperwork should be sent via US Mail, or a commercial shipper to:

Leonardo Murgiano c/o Lydia Melnyk

School of Veterinary Medicine

University of Pennsylvania

3900 Delancey Street. Ryan #2050

Philadelphia PA 19104-6010.

215-898-9426. lmelnik@vet.upenn.edu

- The blood vial should be protected from breakage during shipping. Place the blood tube inside a sealed plastic bag (or other sealed container).
- Include absorbent material (e.g. paper towel) inside the plastic bag.
- Outside package: Clearly labeled "EXEMPT ANIMAL SPECIMEN"
- Inside package: Paperwork indicating composition of sample (*e.g. non-contagious, non-hazardous canine blood for research*).

OWNER Information

Name: first _____ initial _____ last _____

Address: _____

City: _____ State/Province: _____

Country: _____ Zip/Postal Code: _____

Day Phone: _____ Evening Phone: _____

Fax: _____ Email: _____

DOG IDENTIFICATION (Indicate "N/A" if question not applicable)

Breed : _____ Call Name: _____

Registered Name: _____

Registration #: _____

Birthdate: ____/____/____ (mon/day/yr) Sex: ____ Female ____ Male

Registered Name of Sire: _____

Registered Number of Sire: _____

Registered Name of Dam: _____

Registered Number of Dam: _____

Number of full siblings of dog, including repeat matings of parents: _____

Are there any other cases of inherited cataracts known to have occurred in relatives of this dog? Yes _____
No _____ If yes, please describe relationship to affected dog or identify in pedigree and whether blood samples and clinical examination records are available from any of these dogs:-

Ophthalmologist/Clinician Contact Information **Date of Exam:** _____

Name: first _____ initial _____ last _____

Address: _____

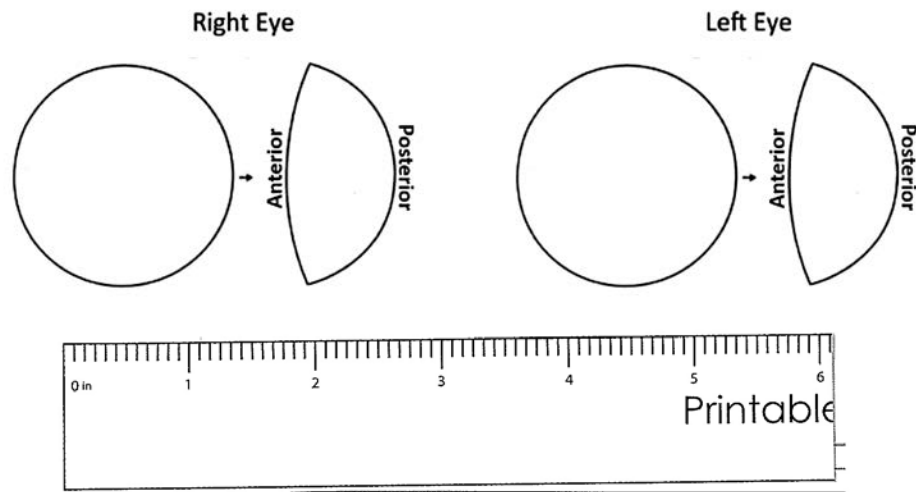
City: _____ State/Province: _____

Country: _____ Zip/Postal Code: _____

Day Phone: _____ Evening Phone: _____

Fax: _____ Email: _____

Clinical examination results (using slit lamp biomicroscope):




Abnormal (describe for each eye). Can you provide digital clinical pictures?) (these should be sent to lmelnik@vet.upenn.edu)

In your opinion, are the cataracts inherited, acquired or of unknown cause?

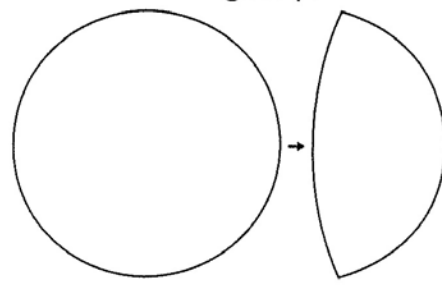
CAER/OFA

CATARACT		LENS						CATARACT		
		Incip.	Incip.	Punc.	Punc.	Incip.	Incip.			
T	N	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	anterior cortex	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	N	T
		<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	posterior cortex	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>		
		<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	equatorial cortex	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>		
		<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	anterior sutures	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>		
A	P	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	posterior sutures	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	A	P
		<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	nucleus	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>		
		<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	capsular	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>		
		<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	generalized/complete	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>		
		<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	resorbing/hypermature	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>		
		suspect not inherited								

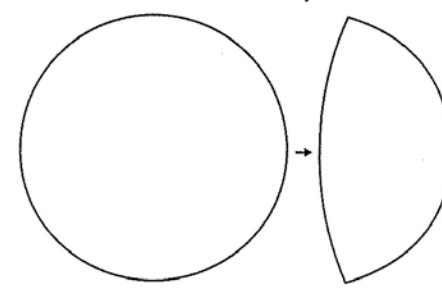


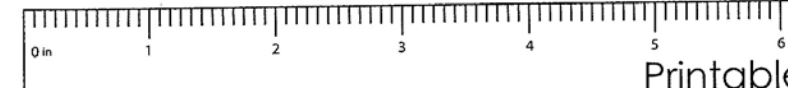
Cocker spaniel research form

Right Eye



Left Eye





Printable

Advantages of the research form:

- lens is anatomically correct.
- larger size of lens schematic to allow for detailed illustration of the clinical findings.
- sufficient space to allow for examiner to write comments or provide interpretation or opinion.
- some ophthalmologists, fortunately a minority, 'know better' and do not use the form and send records that are not useful for the research.

COVID-19 pandemic

- University closed all research labs from March 13-June 8
- Slow-down of activity during the pandemic
- University of Pennsylvania is constantly planning for a progressive restoration of the activities, included lab work, according to all the appropriate safety guidelines
- During the period of general shutdown, we focused on data analysis and into sending samples for high-density genotyping.
- At the moment of this presentation, activities has been restored fully, but we are prepared for any possible upcoming change.



June, 2019 conclusion: *We need samples of dogs diagnosed with bilateral cortical cataracts. We are considering 2 groups of cataract affected dogs: a)-cataracts present between 2-5 yrs of age; b)-cataracts present between >5-8 yrs. To have a good sample set of affected dogs. We need at least 10-25 more "gold standard" cases that fulfill these conditions. We need 10-25 more "gold standard" normal dogs (normal at ≥ 9 years)*

Between July 2017-September 2017

*17 affected
30 normal*

Samples added since previous time period

Between October 2017-March 2018

*29 affected
13 normal*

Between April 2018 and June 2018: 115 samples

*16 affected
10 normal*

Total # samples by June '20 – now: 180 samples

Affected: 62

Normal: 70

Excluded as updates accumulated: 48

State of data as 2020

As of July 2020, we have collected 831 blood samples/records/pedigrees

Total dogs	831
Total of Informative dogs	<u>552</u>
<i>Potential cases</i>	<i>107</i>
Bilateral	79
Unilateral or very Asymmetric	28
<i>Controls</i>	446
Too young to be properly assessed	198
Total of 'Excluded' dogs (as of this stage)	<u>279</u>

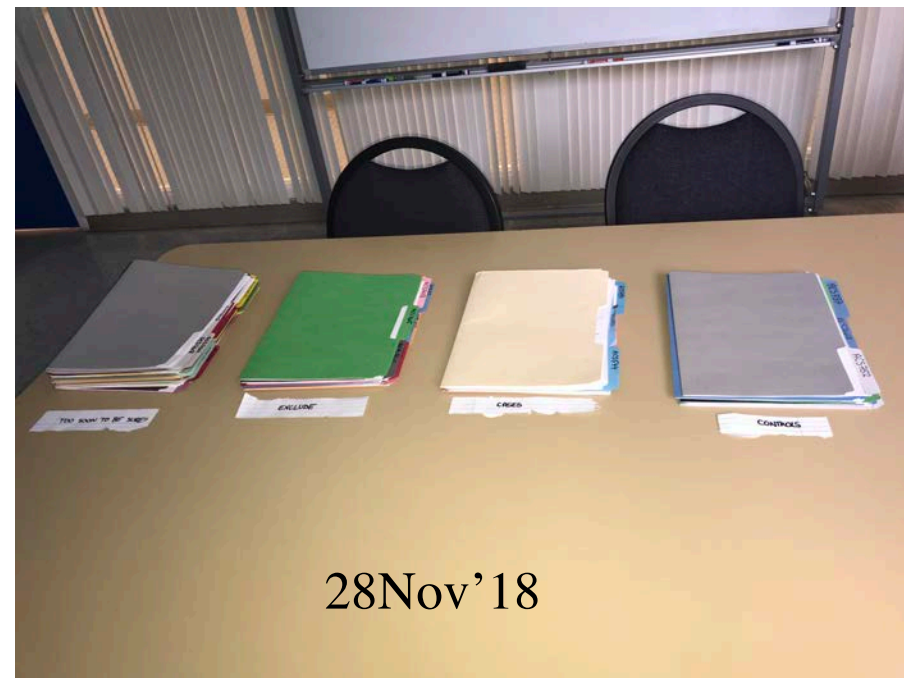
Contacts

Last year:

- Reached out to **~160** people between both email and phone. Of those people, **~100 responded and were very helpful**. But 3-5 people who were responsive, were not very helpful.
- **~150 updates unsolicited, without having to ask. This has been extremely helpful.**

Current year:

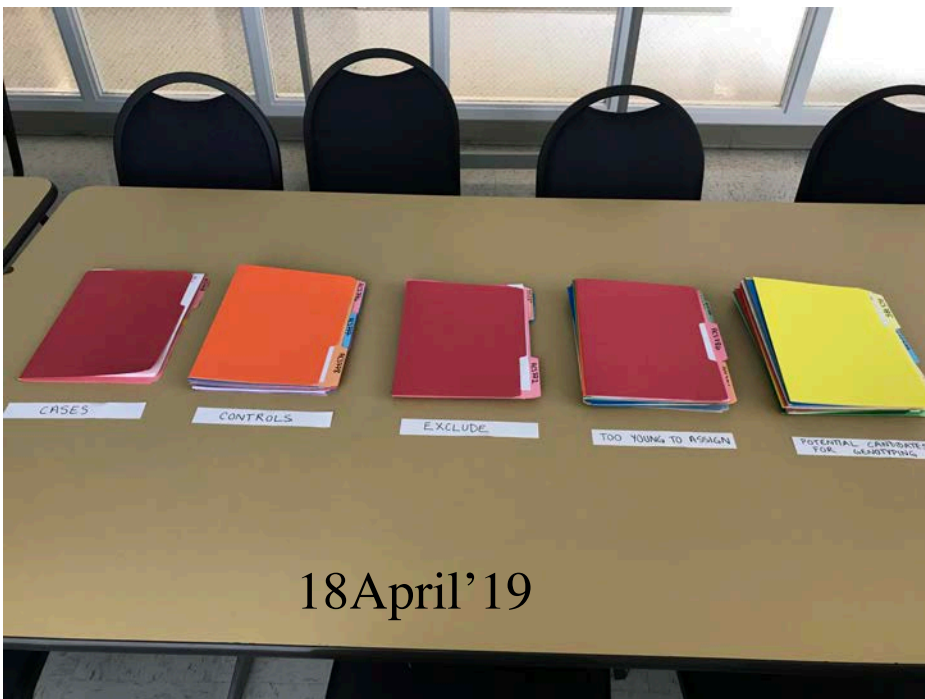
- Spontaneous sending of samples from the breeders.
- Active calling to be restarted



Between 18 April '19 and University closure,
3 more record review sessions carried out

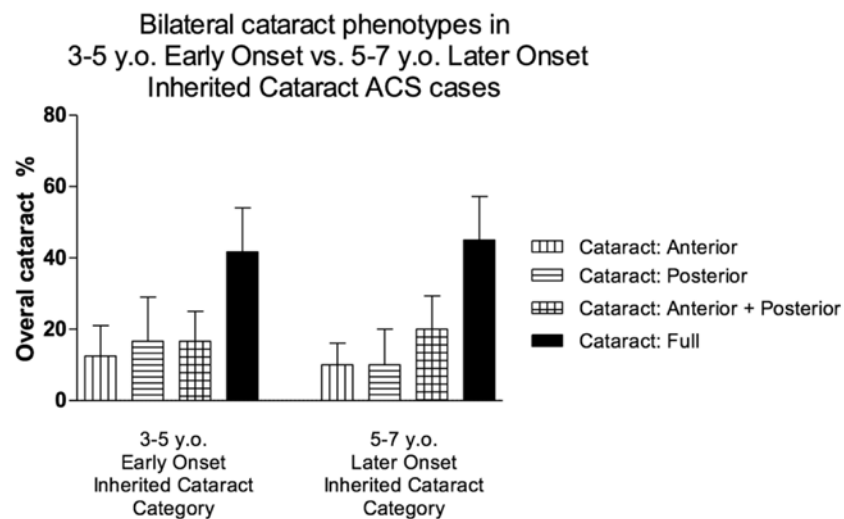
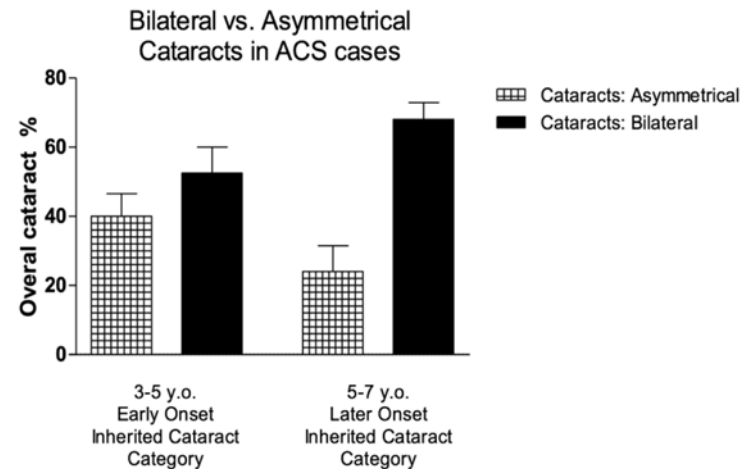
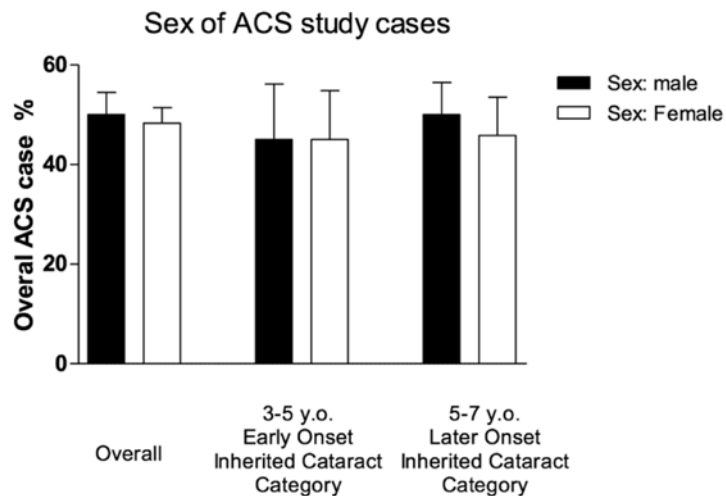
Number of samples received:

-831 as of August, 2020
(~270 examined by GDA)



To be restarted via Zoom

Sample Categorization



State of data as 2020

Reason for exclusion:

- Co-morbidity with another eye condition
- Doubts about diet/medications etc
- Dog prematurely deceased (especially if DNA/blood is missing)
- Lack of feedback on updates (now a **very rare** occurrence)
- Lack of an official diagnosis by a certified veterinary ophthalmologist (or of monitoring post diagnosis)
- Inconsistent records (very rare occurrence)
- Dog too young to tell (will change over time) as dogs are re-examined and enter age range needed for the study.

For selection for the SNP genotyping, we prefer to have multiple records over time for the same dog from both cases and controls for proper phenotype ascertainment.

Our Research Approach

- collect samples from **phenotype ascertained** dogs: normal (controls) and affected with cataracts (cases).
- establish what is the minimum age when dog is considered a control (> 8 yrs of age).
- group cases into specific phenotypes.
- reconsider your groupings as number of cases comes in. Example:
 - initial cases were bilateral ant/post cortical cataracts in 2-5 yr range.
 - after looking at >200 dogs, and receiving close to 800 records, early grouping strategy was revised to:
 - * Cataracts had to be progressive even if they "looked" inherited.
 - * young (2-5 yr) and older (5-8 yrs) cases need to be included.
 - * Unilateral or asymmetric cataract cases need to be included.

Always consider changing approach if data supports such change!!!

Our Research Approach

- New approach evolved not from wishful thinking, but based on data, and we revised our approach depending on the study results.
- With groups, carry out Genome Wide Association Study (GWAS), and also use additional mapping methods.
- Identify chromosomal region of interest and do Whole Genome Sequencing (WGS).
- Although the pandemics slowed down the research pipeline, we managed to carry out our two main aims for this year: high density SNP chip and WGS.

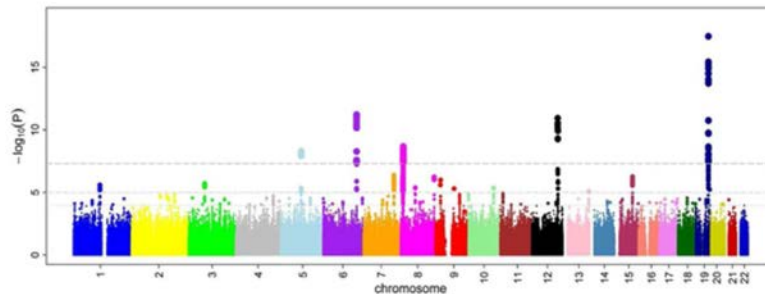
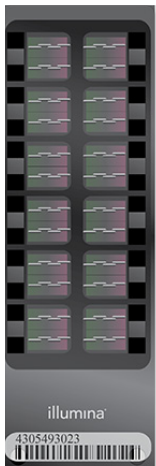
Last year's plans

- Cataracts in the ACS is more complicated than originally suspected.
- It is not a single gene (i.e. monogenic) disorder (otherwise the Manhattan plot would have given a single sharp peak), but cataracts are caused by at least two different genes that result in cataracts that are clinically indistinguishable. There is likely to be a 3rd gene that is a disease modifier.
- Although WGS efforts will focus on dogs in the $>2 - 5<$ yr range (to simplify our effort), GWAS does not distinguish between these dogs and those that are older ($>5 - 8<$) or with those that have symmetrical bilateral cataracts or unilateral cataracts that over the span of several years develop cataracts in the second eye.
- **As planned, WGS has been done in 4 controls and 4 cases that have the haplotypes for the chromosomes being studied.** We also implemented high density mapping.

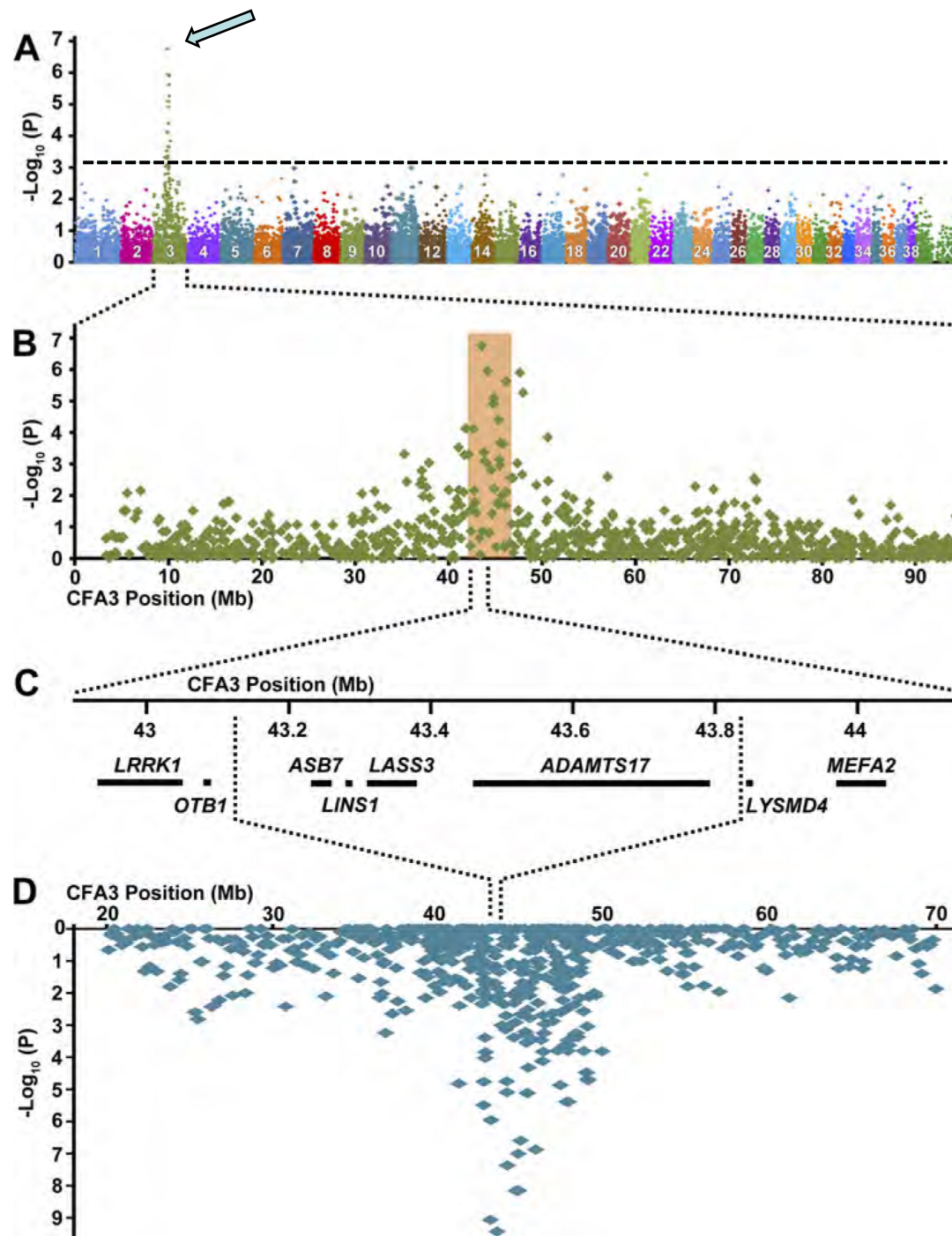
Genome-Wide Association Study (GWAS)

In genetics, GWAS is an observational study of a genome-wide set of genetic variants [aka single nucleotide polymorphisms (SNPs)] in different individuals to see if any variant is associated with a trait. GWAS typically focus on associations between SNPs and inherited traits (e.g. coat color, length of hair, defects-**CATARACTS**).

As long as the trait can be scored accurately in a sufficiently large population of cases and controls, the position of the trait in the genome can be localized. Then the gene/specific defect is identified.



example of a “Manhattan” plot from a human disease study (note: 22 pairs of autosomes + X/Y sex chromosomes)



Samples used for GWAS

- (**'First Batch'**) Dec 2015: 48 initial samples (after analysis of clinical records, 10 cases and 10 controls eliminated)
- (**'2nd Batch'**) Feb 2016: Second batch: 21 samples added (69) - 12 excluded
- (**'3rd Batch'**) Apr 2017: Third batch: 55 samples added (124) - 6 excluded
- (**'4th Batch'**) May 2018: Fourth Batch. 37 samples added (161) - 8 excluded
- (**'5th Batch'**) Sept 2018: Fifth batch: 12 (172) – 6 excluded
- (**'6th Batch'**) Feb 2019 : Sixth batch: 7 (180) – 5 excluded
- *Diagnoses routinely re-analyzed, dogs inserted and excluded from the study as necessary from the updated data*
- Current 220k Illumina dataset of 180 dogs
 - **62 cases (last year 52)**
 - **70 controls (last year 71)**
 - 48 excluded (last year 57)
 - **60+** dogs too young to tell or with incomplete records are potentially going to be integrated in the study

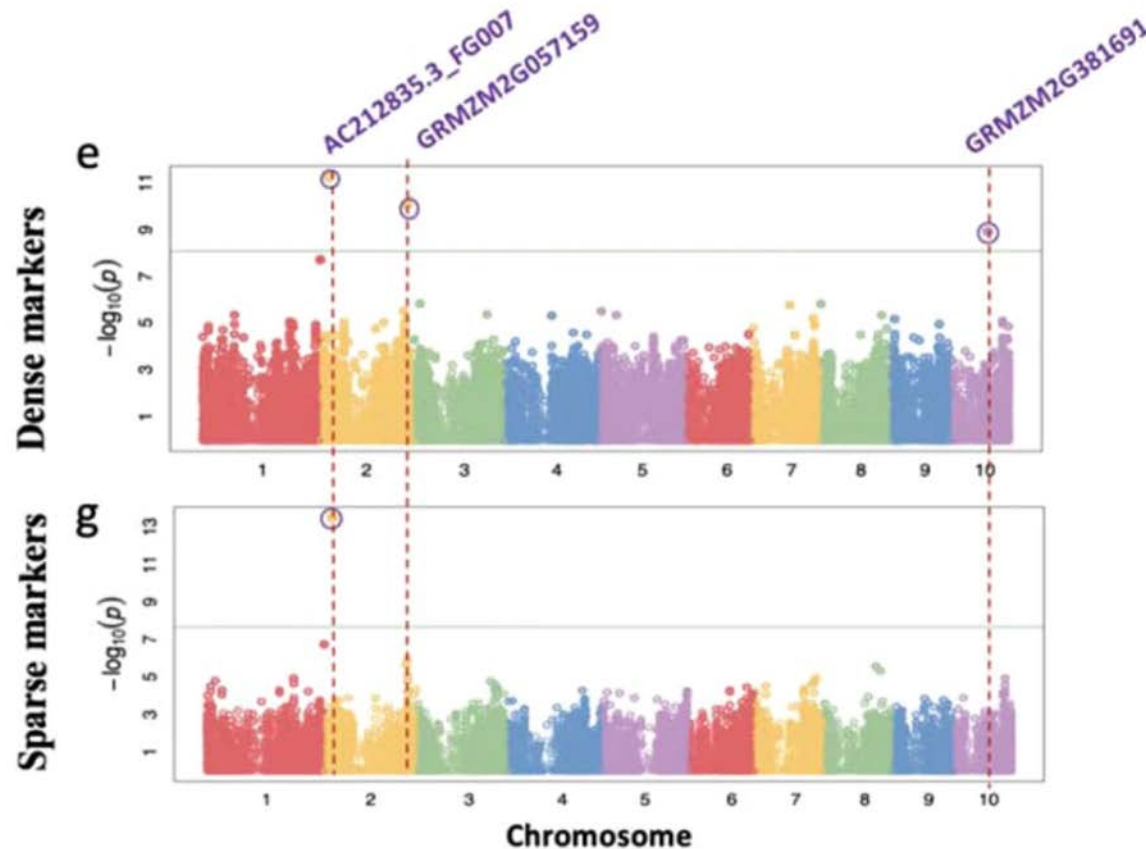
State of data as 2020

Total genotyped	
Cases*	62
First class (>2 – 5< yr)	27
Older age category (>5 – 8< yr)	25
Second class	10
Controls	70
First class	39
Second Class (other lens imperfections-incidental)	17
Third class	14
Excluded	48

*28 bilateral, 17 asymmetrical, 5 unilateral

Second and Third class cases and controls are usually excluded from the analysis

High density markers

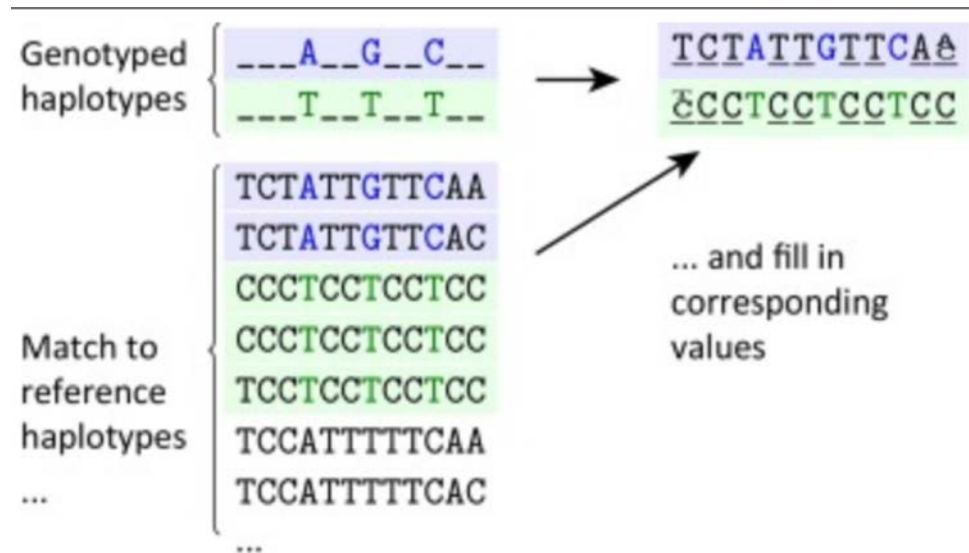


- High density experiments can identify areas of the genome with poor coverage in the older chip versions
- These new areas analyzed might harbor candidate gene(s) that require greater scrutiny

7th “special” batch

Dogs selected from our best samples run in the 220k Illumina platform (60 dogs in total - 26 cases and 34 controls of the highest quality, see above). SNP chip analysis using a new technology of 712k Affymetrix SNP, more than three times the original information!

In addition, the older SNPs are still present and therefore can be used to impute this new information in the rest of the dataset. Additional cycle of GWAS with with the 60 dogs exclusively, and with the imputed dataset as a whole.



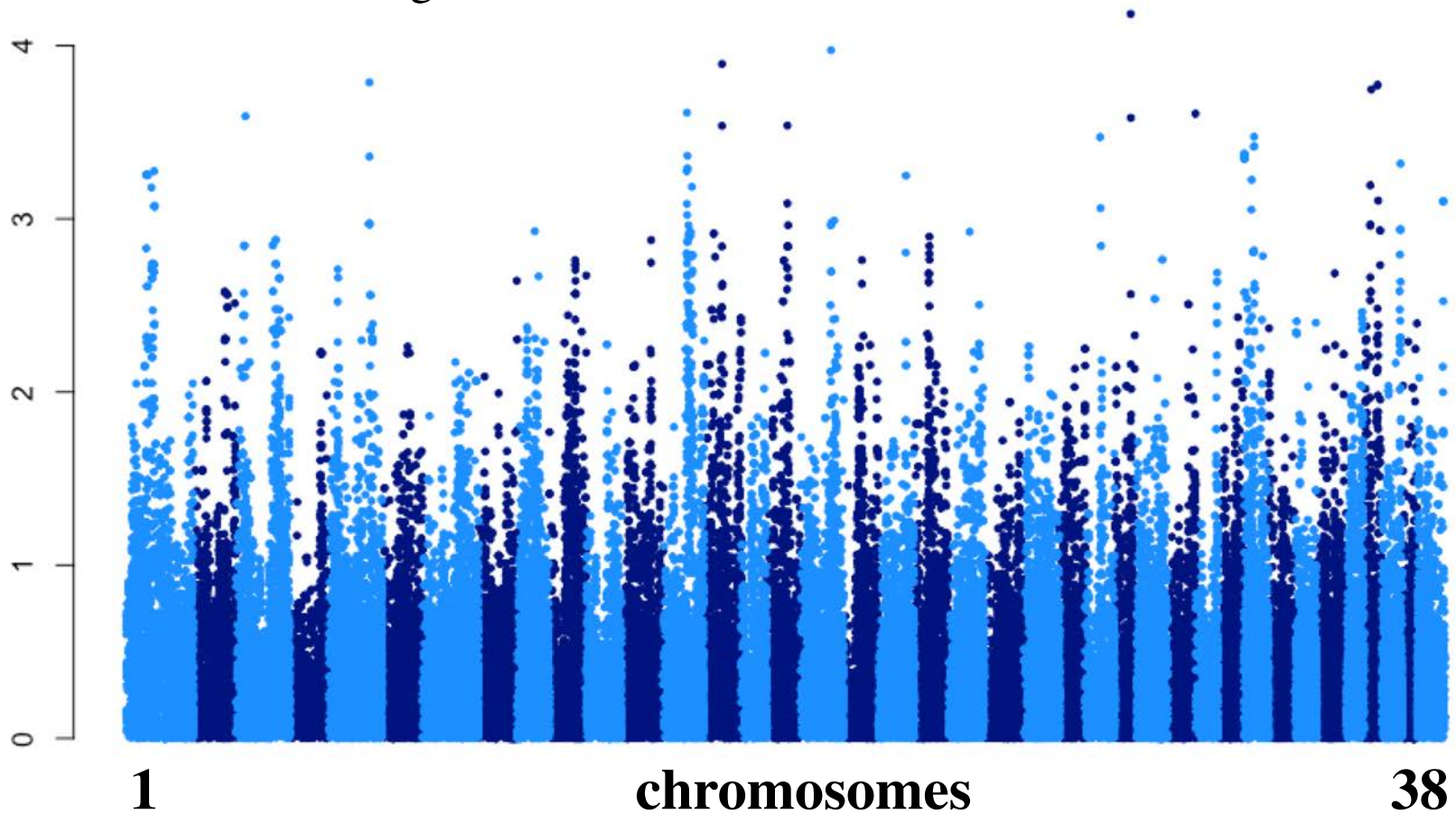
American cocker spaniel-cataract study

Whole population, **first** batch

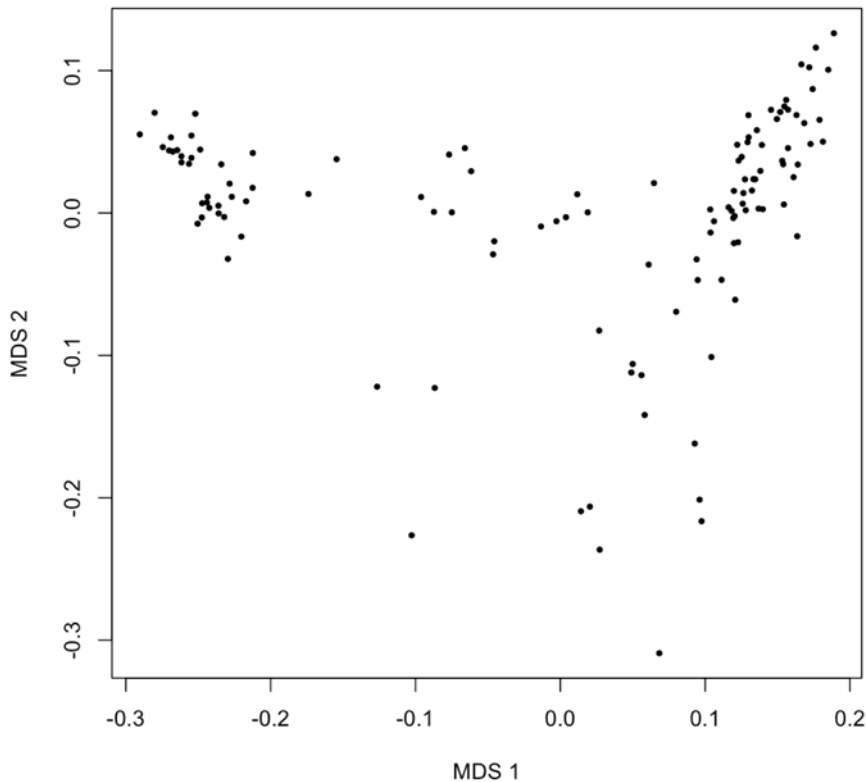
NOT good.

What does it tell you?

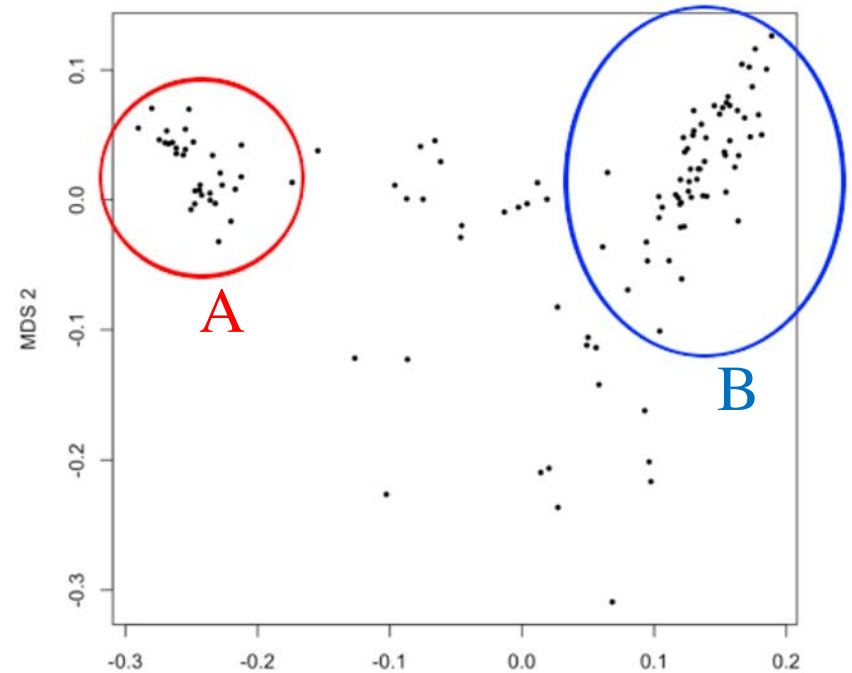
- We have not selected the cases and controls with sufficient rigor.
- Cataracts in cockers not likely to be a single gene defect.
- Back to the drawing board.



MDS plot



Population focused

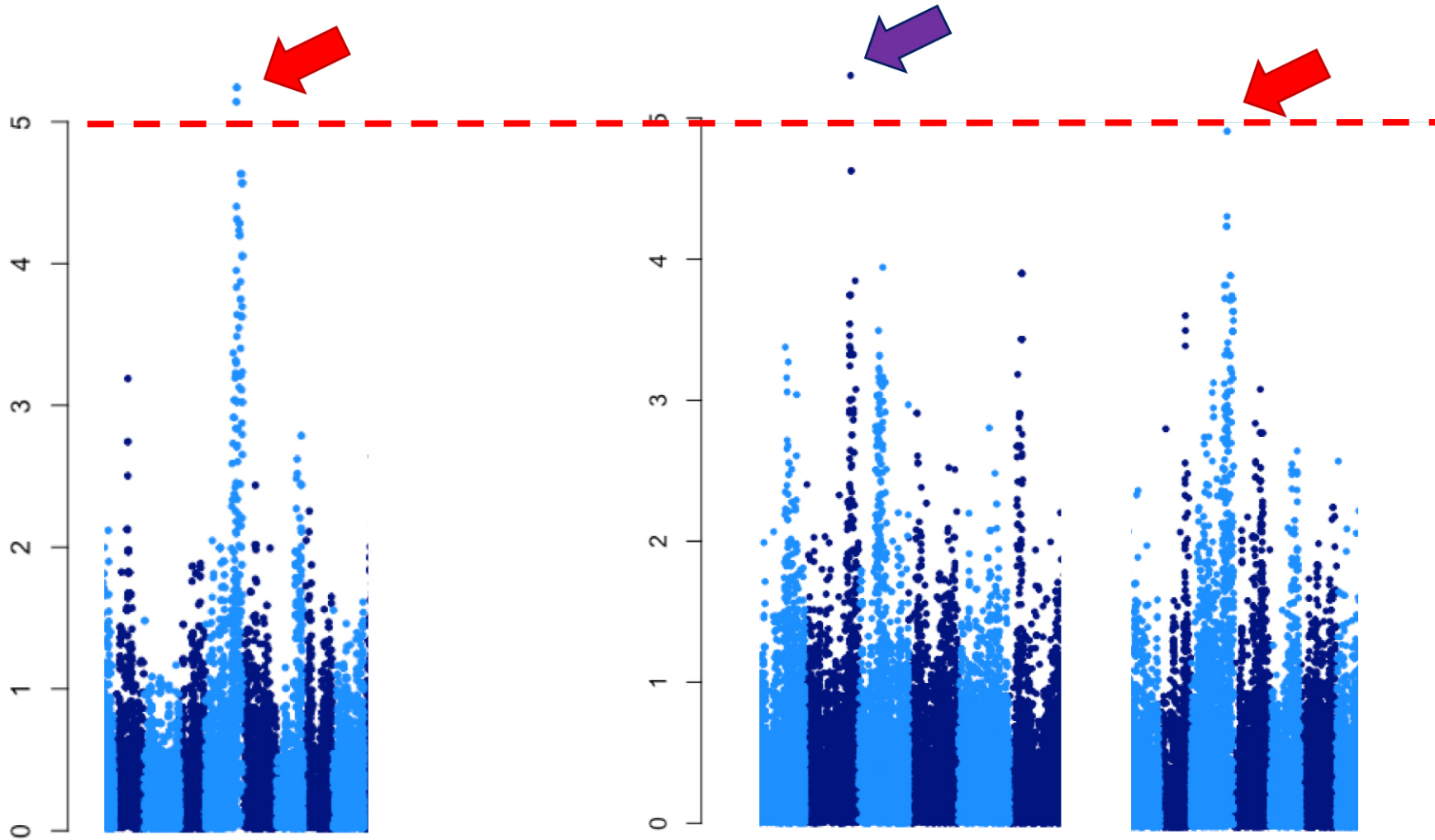


Multidimensional scaling (**MDS**) shows the level of similarity of individual cases of a dataset.

3th, 4th batch... (220k Illumina)

Batch 3

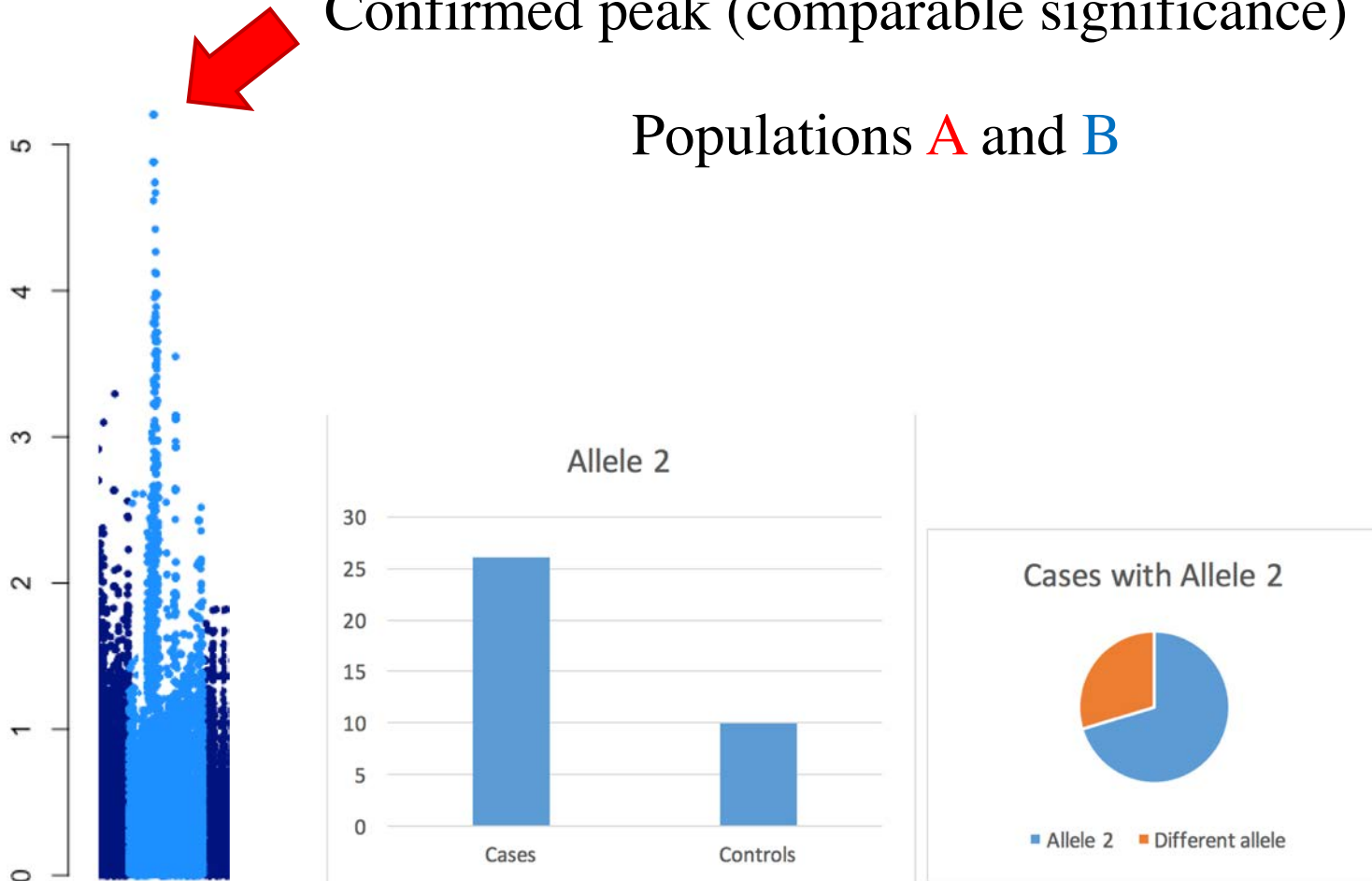
Batch 4



7th batch (712k Affymetrix and 220 Illumina)

Confirmed peak (comparable significance)

Populations **A** and **B**

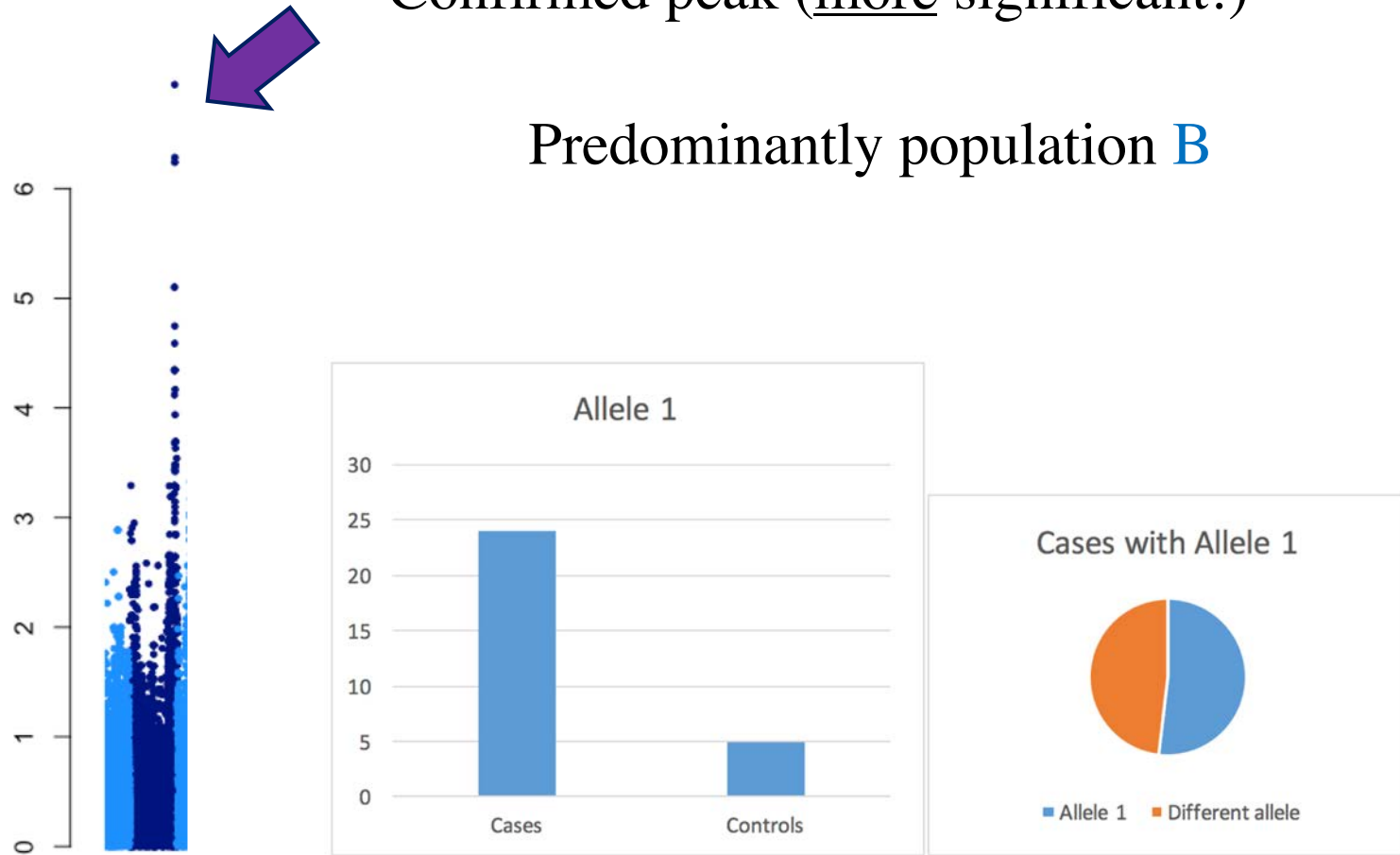


Some peak “disappeared” compared to the previous iteration

7th batch (712k Affymetrix and 220 Illumina)

Confirmed peak (more significant!)

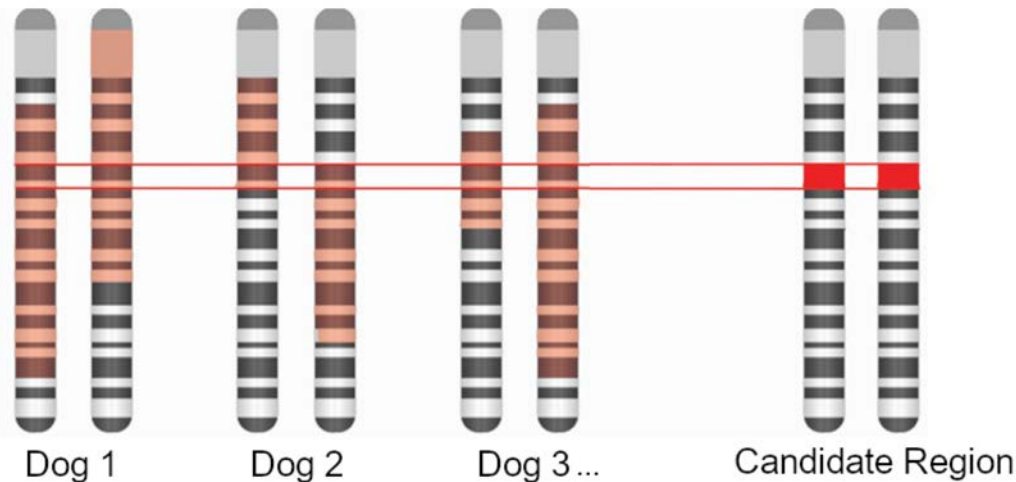
Predominantly population **B**



We will focus on the candidate region ~2-5 Mb

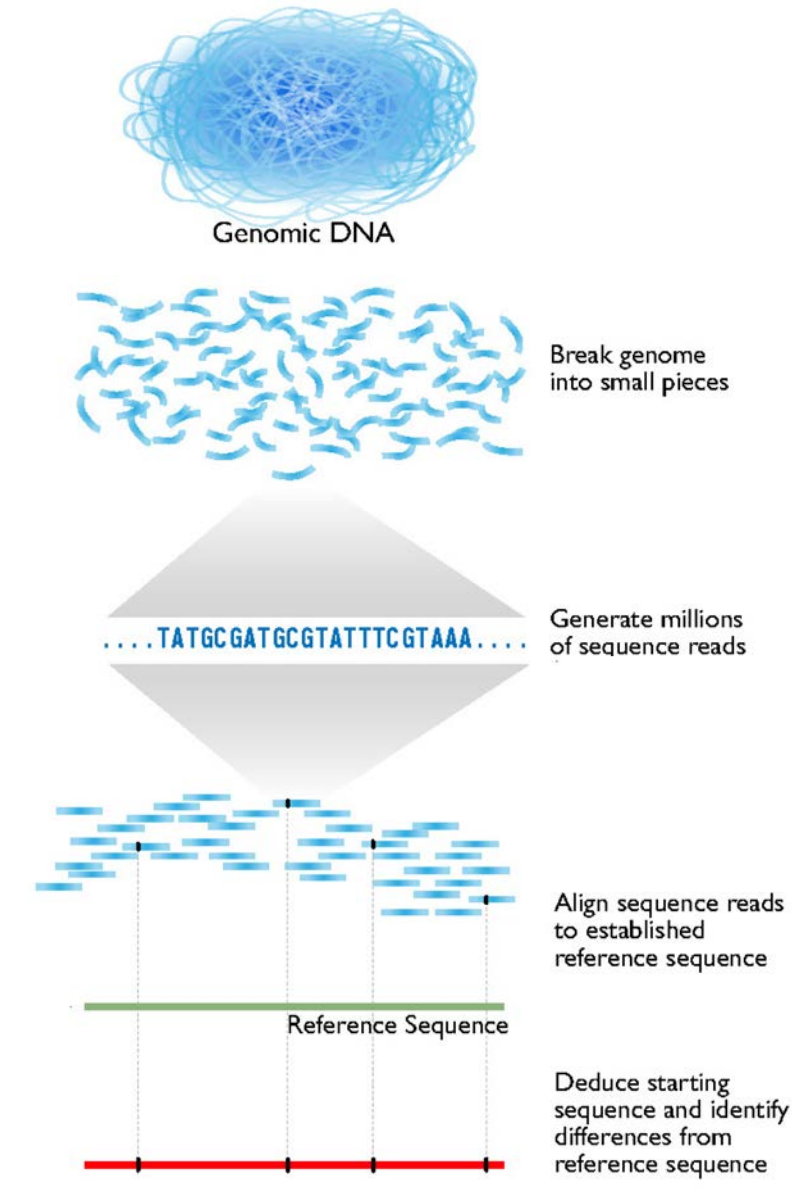
Additional Candidate Regions

- SNP chip technology can be used to find shared regions among the cases.
- Such region could be, as an example, common homozygous intervals (as it happens in recessive diseases).



- Searching in regions of ~ 1 Mb of size, shared by at least 80% of high quality cases (easier/faster case-control analysis compared to the rest of the project, worth checking).

Whole Genome Sequencing



Selected dogs

- 2 “best” cases (2-5 yrs)
- 2 “older” cases (5-8 yrs)
- 4 “top quality” control dogs

Criteria:

- GWAS + other mapping methods (6th batch)
- Sample quality/reliability

Results and ongoing analysis

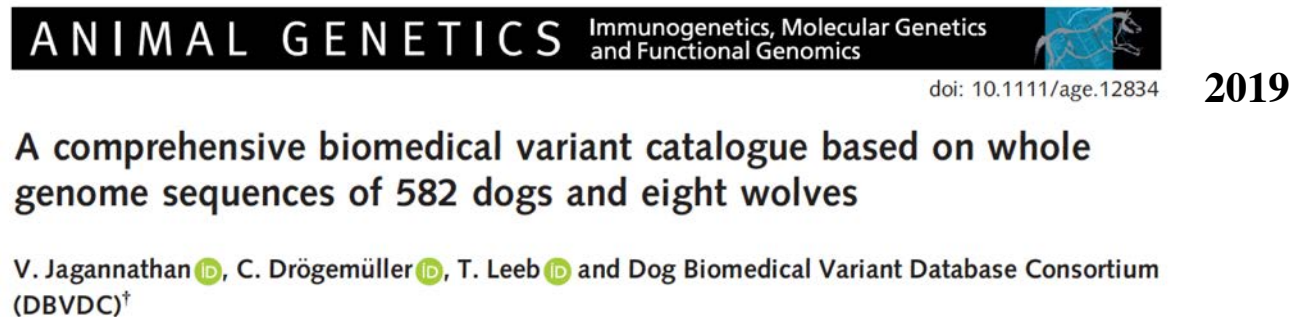
- Thousands of variants and markers. Each one must be assessed for its predicted impact
 - Order of magnitude in the thousands
- Same procedure carried out for small variants will be repeated for (rare) larger variant
 - Order of magnitude in the tens.
- Ultimate aim is to detect a marker suitable for the breeders

Results and ongoing analysis

- Analysis of the region the variant is located to know whether has been linked with cataract in other species
- Analysis of the gene the variant identified in the region to predict any function related to lens function/metabolism
- In preliminary analysis, we found variants in genes associated with cataract, albeit the variants are not of high impact and if causative they must have a regulatory role.
- This is consistent with the phenotype, being not “clear cut”

Results and ongoing analysis

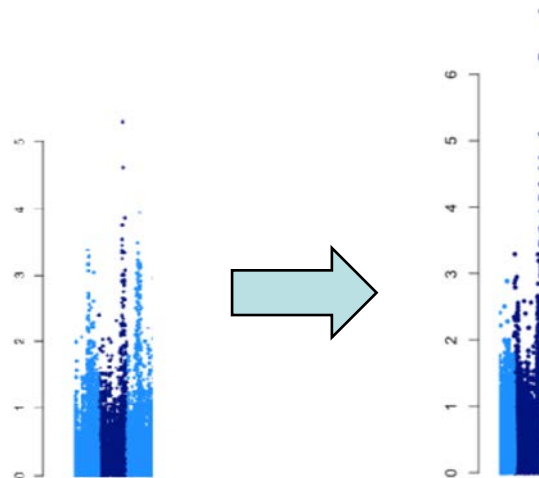
- Filtering through a database of >800 dogs (*not* American Cocker Spaniel) in order to reduce significantly the number of variants we have to look through



- After this first screening, if the results are unclear, will go back and hunt for variant present in other breeds in the (unlikely) case the the same risk variants are present in other breeds
- We will possibly find controls with a candidate marker, but probably not with all of them. This would suggest that modifying factors determine the presence/absence of cataracts

Considerations and further actions

- Using the high-density SNP technology helped us increase the power of the experiment
- The data also helped in the exclusion of regions that were apparently good candidates in the older dataset, reducing “noise”, and giving us more confidence in the regions that were confirmed



Considerations and further actions

- Nonetheless, in order to adjust to the new mapping data, we will sequence additional 1-2 high quality cases with a specific haplotype, and compare the results with the current haplotype information and the other already sequenced dogs
- A new canine reference has been produced and made public, we plan to compare our WGS data on that one, too, in order to detect any possible significant information that the old reference missed
- The marker “hunting” will involve a considerable amount of genotyping

More samples of cases and controls are always welcomed