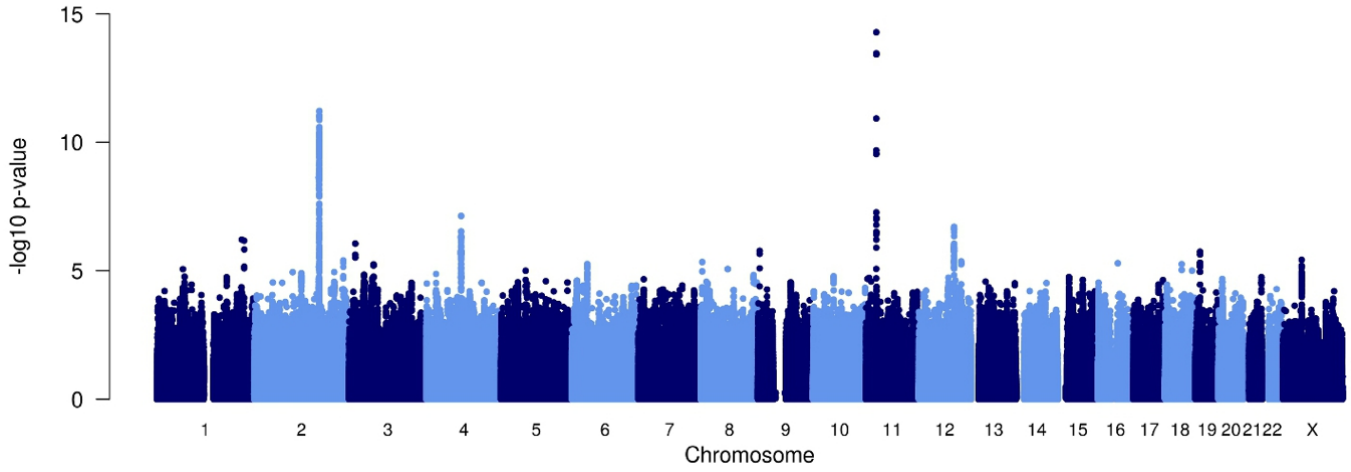
# Association testing and GWAS




Line Skotte, Medical and Population Genetics Course, August 2019

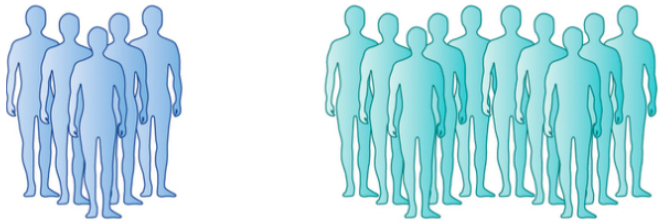
# Outline

# What and why?

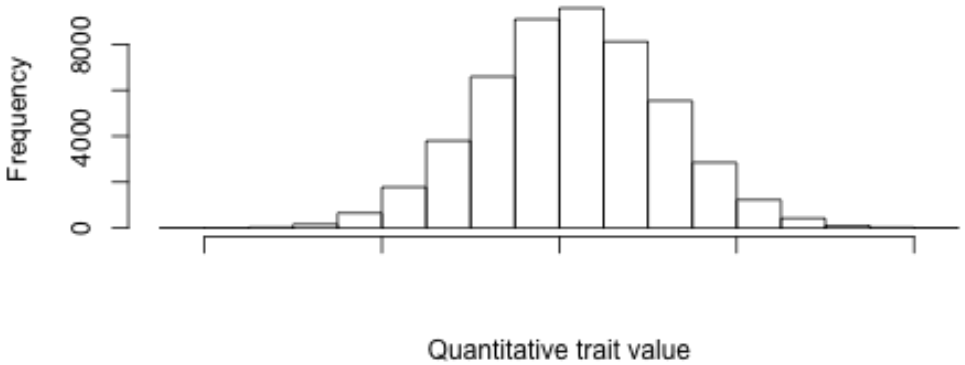
- **Goal: to identify (map) genetic variants that have an effect on a trait**
- Typically **disease related traits**, e.g. febrile seizures
- Motivation: reaching this goal can help
  - reveal the underlying genetic architecture
  - hopefully lead to better understanding of what **causes** the disease
  - in turn ideally lead to better treatment and/or prevention
- Note, **can also be used in e.g. evolutionary studies!**

# Plan for today (to teach you how)

- **This afternoon:**
  - How to test if a genetic variant potentially affects a trait (single SNP tests)
  - How to search the genome for variants that affect a given trait (GWAS)
  - We will assume we have genotyping data (e.g. from SNP chip)
  - We will assume there is no population structure
  - We will look at disease status traits:




  - And quantitative traits:

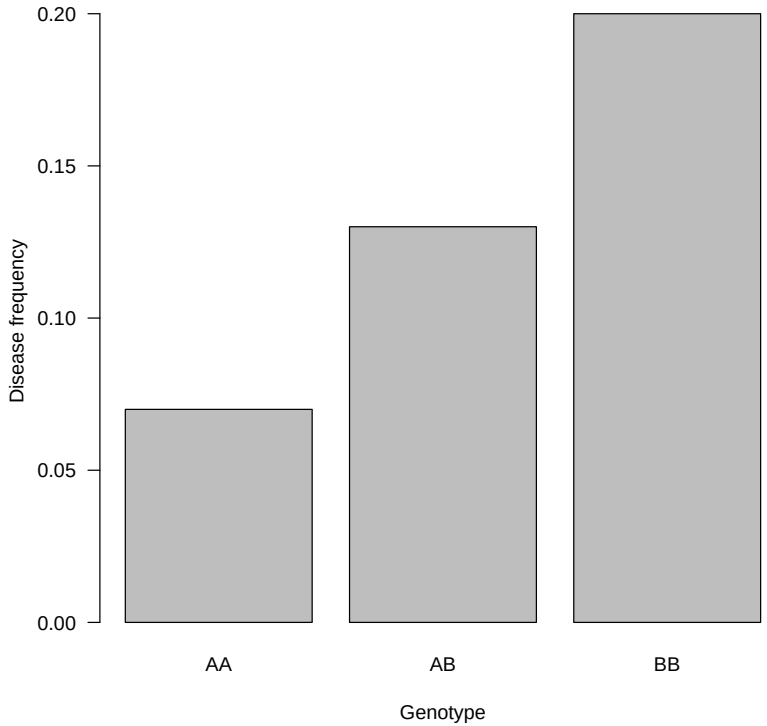


# Outline

# Overall idea in association testing

**How do we test if a genetic variant potentially has an effect on a disease?**

- Idea: test for **association** between the variant and disease status (case/control)




- Rationale: this is what we expect if the variant affects the trait
- Approach: test null hypothesis, $H_0$, of no association (independence)

# Probability of disease given genotype

**What is the probability of disease for the different genotypes?**

- In the previous figure, $P(D|AA) = 0.07$, $P(D|AB) = 0.13$ and $P(D|BB) = 0.20$.
- More formally we model the probability of disease for an individual

$$p = P(D|g)$$

- We can use a logistic regression model

$$\log\left(\frac{p}{1-p}\right) = \beta_0 + \beta_{AB}x_{AB} + \beta_{BB}x_{BB}$$

  where the $\beta$s are *regression coefficients* (effect sizes).
- The $x$s are determined by the genotype of the individual considered:

| Genotypes | $x_{AB}$ | $x_{BB}$ |
|---|---|---|
| AA | 0 | 0 |
| AB | 1 | 0 |
| BB | 0 | 1 |

# Exercise

**What is the probability of disease for the different genotypes?**

- An individual has genotype AA. Can you express $p = p(D|AA)$ in terms of the effect sizes?
- How do you find the probability of disease when the individual has genotype AB?

# Solution

**What is the probability of disease for the different genotypes?**

- We can rephrase the logistic regression model

$$\log\left(\frac{p}{1-p}\right) = \beta_0 + \beta_{AB}x_{AB} + \beta_{BB}x_{BB}$$

into

$$p = \frac{\exp\left(\beta_0 + \beta_{AB}x_{AB} + \beta_{BB}x_{BB}\right)}{1 + \exp\left(\beta_0 + \beta_{AB}x_{AB} + \beta_{BB}x_{BB}\right)}$$

- For individual with genotype AA, we see that $x_{AB} = x_{BB} = 0$ and thus

$$p(D|AA) = \frac{\exp\left(\beta_0\right)}{1 + \exp\left(\beta_0\right)}$$

- For individual with genotype AB, we see that $x_{AB} = 1$ and thus

$$p(D|AB) = \frac{\exp\left(\beta_0 + \beta_{AB}\right)}{1 + \exp\left(\beta_0 + \beta_{AB}\right)}$$

# Testing for association between disease and genotype

**How do we test for association between disease and genotype?**

- The logistic regression model allows the probability of disease to depend on genotype:

$$\log\left(\frac{p}{1-p}\right) = \beta_0 + \beta_{AB}x_{AB} + \beta_{BB}x_{BB}.$$

- If there is **no association** then the probability of disease is the same regardless of genotype, i.e.: $P(D|AA) = P(D|AB) = P(D|BB)$.
- The corresponding logistic regression **null model** is

$$\log\left(\frac{p}{1-p}\right) = \beta_0$$

  and this null model assumes $\beta_{AB} = \beta_{BB} = 0$.
- The **likelihood ratio test** compares the likelihood of the data under these two models and here it has two degrees of freedom.

# Exercise

**How do we test for association between disease and genotype?**

- Load a test dataset into R with one line per person and three columns. First colums is the disease status of each individual and the other two columns contain counts of allele B for two different variants, SNP1 and SNP2.

```
# Read data
dat<-read.table(file = "/home/line/popgen19/case_control_snp1_snp2.
    txt", header = TRUE, as.is = TRUE)

# Check out the data
str(dat)
table(dat$status,dat$snp1)
table(dat$status,dat$snp2)
```

- Which variant looks most associates with disease status?

# Exercise

## How do we test for association between disease and genotype?

- Do the association test for SNP1:

```
# Fit null model
null <- glm(status ~ 1, family = binomial(link = "logit"), data =
    dat)
summary(null)

# Fit full genotype model for snp1
full_snp1 <- glm(status ~ as.factor(snp1), family = binomial(link =
    "logit"), data = dat)
summary(full_snp1)

# Do the LRT test
anova(null, full_snp1, test = "LRT")
```

- Is the variant associated to disease status?

# Exercise

## How do we test for association between disease and genotype?

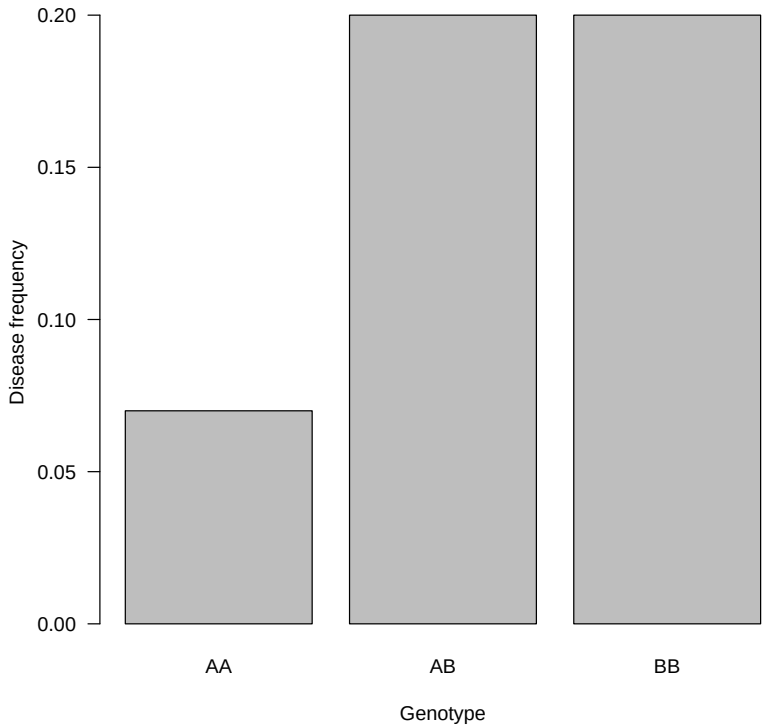
- Do the association test for SNP2:

```
# Fit null model
null <- glm(status ~ 1, family = binomial(link = "logit"), data =
    dat)
summary(null)

# Fit full genotype model for snp2
full_snp2 <- glm(status ~ as.factor(snp2), family = binomial(link =
    "logit"), data = dat)
summary(full_snp2)

# Do the LRT test
anova(null, full_snp2, test = "LRT")
```

- Is the variant associated to disease status?

# Assuming recessive, dominant or "additive" effects

- The full genotype model just described allows different disease probabilities for each genotype.
- Genetic effects can be **dominant**:



# Assuming recessive, dominant or "additive" effects

- Genetic effects can be **dominant**, meaning that we assume

$$P(D|AB) = P(D|BB).$$

- We can then use a simpler logistic regression model

$$\log\left(\frac{p}{1-p}\right) = \beta_0 + \beta_D x_D$$

- The $x$s are determined by the genotype of the individual considered:

| Genotypes | $x_D$ |
|---|---|
| AA | 0 |
| AB | 1 |
| BB | 1 |

- The corresponding **likelihood ratio test** has 1 degree of freedom.

# Assuming recessive, dominant or "additive" effects

- Genetic effects can be **recessive**, meaning that we assume

$$P(D|AA) = P(D|AB).$$

- We can again use a simpler logistic regression model

$$\log\left(\frac{p}{1-p}\right) = \beta_0 + \beta_R x_R$$

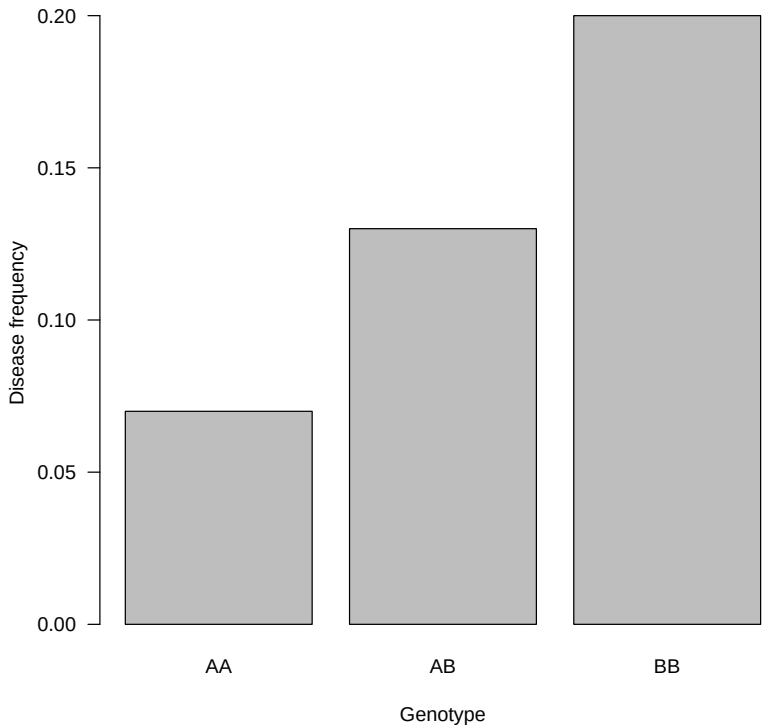
- The $x$s are determined by the genotype of the individual considered:

| Genotypes | $x_R$ |
|---|---|
| AA | 0 |
| AB | 0 |
| BB | 1 |

- The corresponding **likelihood ratio test** has 1 degree of freedom.

# Assuming recessive, dominant or "additive" effects

- In some cases genetic effects are expected to be near-**additive**.
- The the risk of disease for heterozygous carriers $P(D|AB)$ is midway between the two risks for homozygous carriers $P(D|AA)$ and $P(D|BB)$.



# Assuming recessive, dominant or "additive" effects

**Is there a 1 degree of freedom test for the near-additive effects?**

- We can again use a simpler logistic regression model

$$\log\left(\frac{p}{1-p}\right) = \beta_0 + \beta_A x_A$$

- The $x_A$s are determined by the genotype of the individual considered:

| Genotypes | $x_A$ |
|---|---|
| AA | 0 |
| AB | 1 |
| BB | 2 |

- The corresponding **likelihood ratio test** has 1 degree of freedom.

# Exercise

**How do we assume recessive, dominant or "additive" effects?**

- Fit dominant and recessive models for SNP2 in R:

```
# Fit and test dominant model
dat$snp2_x_dom <- 1*(dat$snp2 > 0)
dom_snp2 <- glm(status ~ snp2_x_dom, family = binomial(link = "logit
    "), data = dat)
summary(dom_snp2)
anova(null, dom_snp2, test = "LRT")

# Fit and test recessive model
dat$snp2_x_rec <- 1*(dat$snp2 == 2)
rec_snp2 <- glm(status ~ snp2_x_rec, family = binomial(link = "logit
    "), data = dat)
summary(rec_snp2)
anova(null, rec_snp2, test = "LRT")
```

- Why does the association seem less significant when se assume that the effect is recessive?

# Exercise

**How do we assume recessive, dominant or "additive" effects?**

- Fit the "additive" model for SNP2 in R:

```
# Fit and test "additive" model
add_snp2 <- glm(status ~ snp2, family = binomial(link = "logit"),
    data = dat)
summary(add_snp2)
anova(null, add_snp2, test = "LRT")
```

- Is the p-value lower than for the full genotype model?
- Compare the different nested models:

```
# Comparing nested models
anova(dom_snp2, full_snp2, test = "LRT")
anova(rec_snp2, full_snp2, test = "LRT")
anova(add_snp2, full_snp2, test = "LRT")
```

- What model describes "best" the genetic effect?

# Model assumptions, degrees of freedom and power

**What single-SNP test is the correct one to use?**

- If a sub-model is correctly specified, the test with fewer degrees of freedom have better power than the full genotype test.
- It the model is strongly misspecified, the test may loose power.
- A slightly misspecified model with fewer degrees of freedom can have better power than a completely correct test with more degrees of freedom.

We will discuss statistical power again a bit later.

# Logistic regression in general

- Based on the following general model

$$\log\left(\frac{p}{1-p}\right) = \beta_0 + \beta_1 x_1 + \ldots + \beta_n x_n$$

  $\beta$s are *regression coefficients* (effect sizes) and $x$s are covariates.

- They can also contain additional covariates and allow correcting the model for sex, population structure or batch effects.
- The **design matrix** is a matrix made of the $x$s as columns.
- Single-SNP design matrix calculation:

| Genotypes | Additive | Dominant | Recessive | Full | |
|---|---|---|---|---|---|
| | $x_A$ | $x_D$ | $x_R$ | $x_{AB}$ | $x_{BB}$ |
| AA | 0 | 0 | 0 | 0 | 0 |
| AB | 1 | 1 | 0 | 1 | 0 |
| BB | 2 | 1 | 1 | 0 | 1 |

# Why is logistic regression a good framework to use?

Logistic regression is very convenient due to its flexibility:

- Most inheritance models can be tested (by recoding $x$).
- Can incorporate other factors in the model
  - **discrete** factors such as gender
  - **continuous** factors such as age

  Can be used to correct for possible confounding factors
  Can be used for metaanalysis by incl a factor for the different studies
- Possible to compare nested models using ANOVA

# Effect sizes for case-control data - relative risk

## Relative risk - definition

$$RR = \frac{P(Case|Exposed)}{P(Case|Not\ exposed)}$$

where exposed depends on model, e.g. exposed=aa under recessive model

I.e. how many times higher the *risk* of disease is for exposed

## Relative risk - example with recessive model

| | Cases | Controls | Total |
|---|---|---|---|
| Exposed (g=aa) | 100 | 100 | 200 |
| Not exposed (g=AA or Aa) | 400 | 3600 | 4000 |

- $P(Case|Exposed) = \frac{100}{200} = \frac{1}{2}$
- $P(Case|Not\ exposed) = \frac{400}{4000} = \frac{1}{10}$
- $RR = \frac{1/2}{1/10} = 5$

# Effect sizes for case-control data - odds ratio

## Odds ratio - definition

$$OR = \frac{ODD_{Exposed}}{ODD_{Not\ \ Exposed}} = \frac{\frac{P(Case|Exposed)}{P(Control|Exposed)}}{\frac{P(Case|Not\ \ exposed)}{P(Control|Not\ \ exposed)}}$$

where exposed depends on model, e.g. exposed=aa under recessive model

I.e. how many times higher the *odds* of disease is for exposed

## Odds ratio - example with recessive model

| | Cases | Controls | Total |
|---|---|---|---|
| Exposed (g=aa) | 100 | 100 | 200 |
| Not exposed (g=AA or Aa) | 400 | 3600 | 4000 |

- $\frac{P(Case|Exposed)}{P(Control|Exposed)} = \frac{100/200}{100/200} = \frac{100}{100} = 1$
- $\frac{P(Case|Not\ \ exposed)}{P(Control|Not\ \ exposed)} = \frac{400/4000}{3600/4000} = \frac{400}{3600} = 1/9$
- $OR = \frac{1}{1/9} = 9$ (very high for an association study!)

# Effect size estimates from logistic regression

- In logistic regression the ORs are estimated directly.
- Example: In the recessive model we estimate the effect size $\beta_R$

$$\log\left(\frac{p}{1-p}\right) = \beta_0 + \beta_R x_R$$

- The OR from the recessive model

$$\frac{\text{ODD}_{BB}}{\text{ODD}_{AA/AB}} = \frac{\frac{p_{BB}}{1-p_{BB}}}{\frac{p_{AA/AB}}{1-p_{AA/AB}}} = \frac{\exp(\beta_0+\beta_R)}{\exp(\beta_0)} = \exp(\beta_R)$$

- So we can get OR by taking the exp() of $\beta_R$

# Exercise

**How do we extract effect size estimates from logistic regression models?**

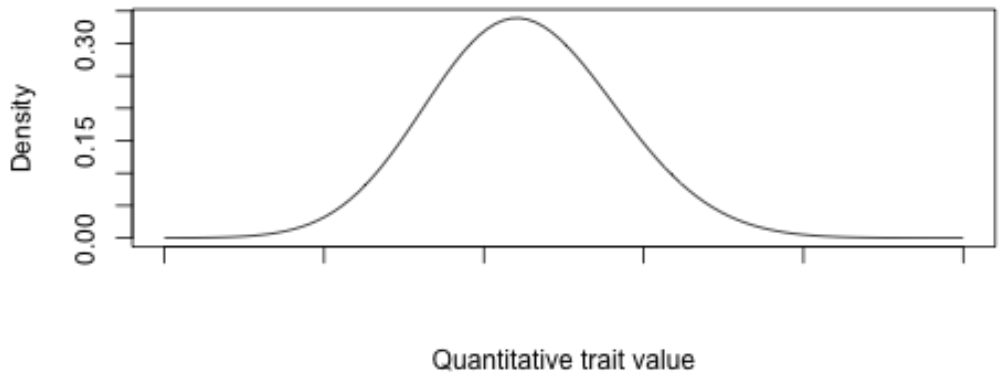
- Calculate ORs from the logistic regression in R:

```
# Inspect coefficients and find ORs in the full genotype model
full_snp2$coefficients
# OR for AB versus AA
exp(full_snp2$coefficients[2])
# OR for BB versus AA
exp(full_snp2$coefficients[3])

# OR from "additive" model
add_snp2$coefficients
exp(add_snp2$coefficients[2])
```

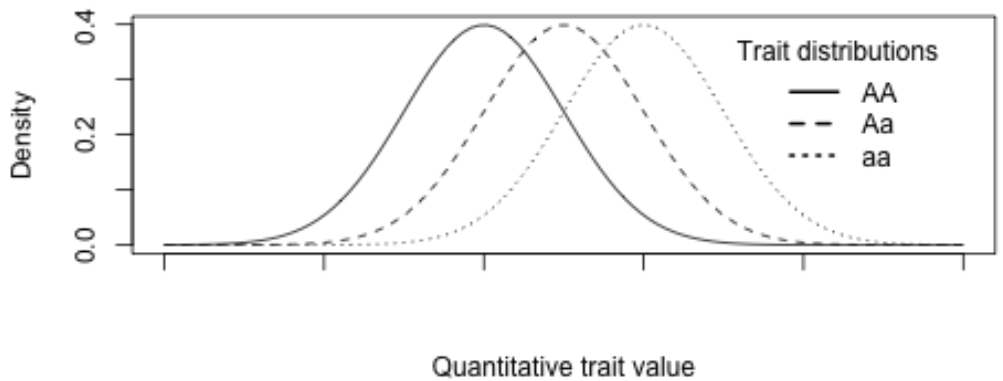
- How can we interpret the OR calculated in the "additive" model?

# Quantitative trait

- Distribution of the trait in the population (assume normal distribution)




- If a variant influence the trait value, we expect:



# Linear regression

- Based on the following general model

$$\mathrm{E}(y) = \beta_0 + \beta_1 x_1 + \ldots + \beta_n x_n$$

  where the $\beta$s are *regression coefficients* (effect sizes).
- The $x$s are determined by the genotype of individual $i$ and the inheritance model
- E.g. for a simple additive inheritance model we have

$$\mathrm{E}(y) = \beta_0 + \beta_A x_A$$

  where $x_A$ is the number number of copies of the variant so 0, 1 or 2
- Test if $\beta_A$ is zero (no association between the variant and the trait)

# Outline

# Causality?

- No, not necessarily!
- We expect to see some loci highly correlated w. causal variant, e.g:

| Causal | Other locus |
|---|---|
| A | G |
| A | G |
| A | G |
| A | G |
| A | G |
| C | T |
| C | T |
| C | T |

- This means that we see association in loci that are in high LD with the causal SNP
  So you have to be careful what you conclude from an association signal!

# Other important limitations

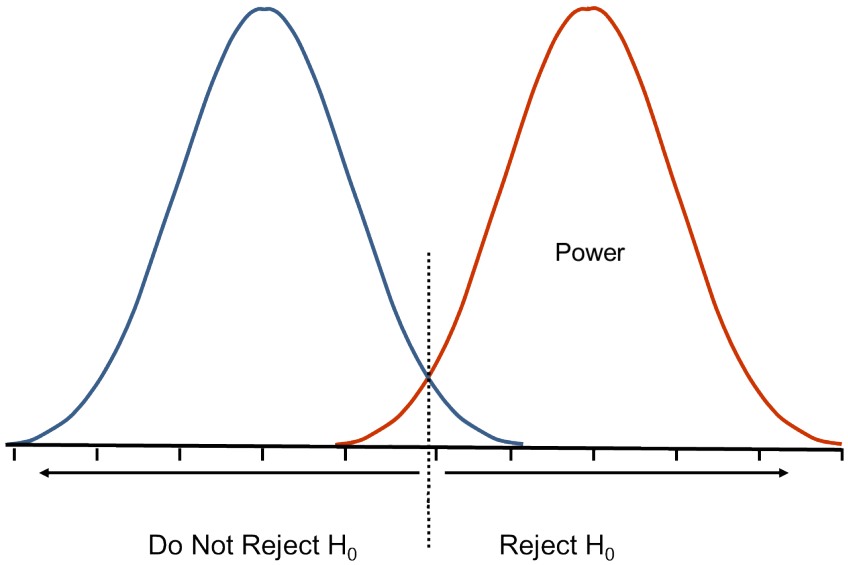
One also has to be aware of the underlying assumptions:

- In all the tests there is an assumption that the individuals are independent (unrelated) and from a homogenous (unstructured) population
- If these assumptions are violated you risk getting false positives!
- Hence Quality Control (QC) and appropriate modelling is crucial!

We will return to these issues a bit later.

# Study design

- Will your study answer your research question? **Key: power**
- Power is the probability that a true association is found when testing




Blue line: Distribution under $H_0$. Red line: Distribution under anticipated effects.

- Before you start your study: calculate power for your study and assess it Rule of thumb: power should be at least 0.8 (or the study is not really worth performing)!

# Power and power calculations

- Power depends on
  - the inheritance mode, e.g. recessive effect
  - the effect size, e.g. OR of 1.3 (the bigger the higher power)
  - the frequency of allele, e.g. 0.04 (the bigger the higher power)
  - **the rejection criterion**, e.g. $p < 0.05$ (the bigger the higher power)
  - **the number of samples** (the bigger the higher power)
  - **the test you use**
- Can often be calculated using "power-calculators"
- So before you start:
  Do power calculations to make sure you will have enough samples!
- To detect association we might not choose the model that is most correct, but instead choose the model that has the most power

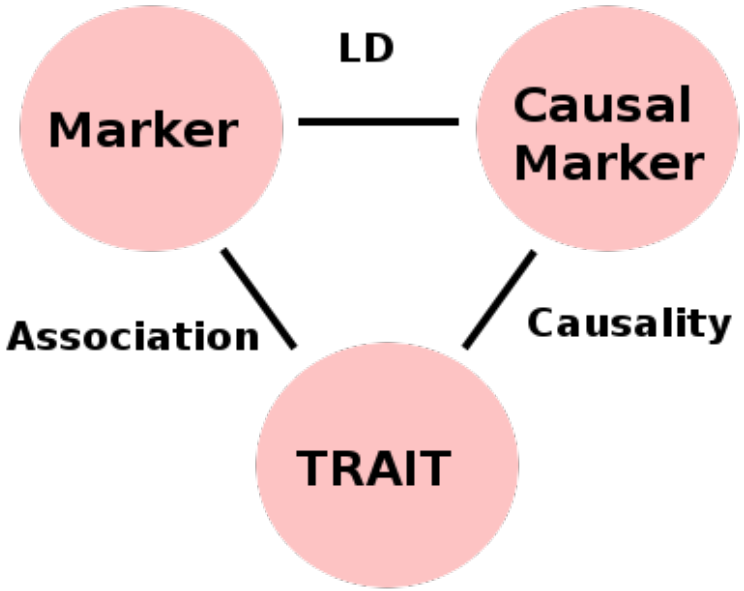
# Outline

# Types of association studies

- Candidate causative genetic variant
  - 1 SNP or deletion, duplication (evidence from other study).
- Candidate causative gene
  - 5-50 SNPs (evidence from other study or function)
- Candidate causative region
  - 100s of SNPs (evidence from other study)
- Genome-wide (GWAS)
  - $>$500,000 SNPs (no prior evidence required)

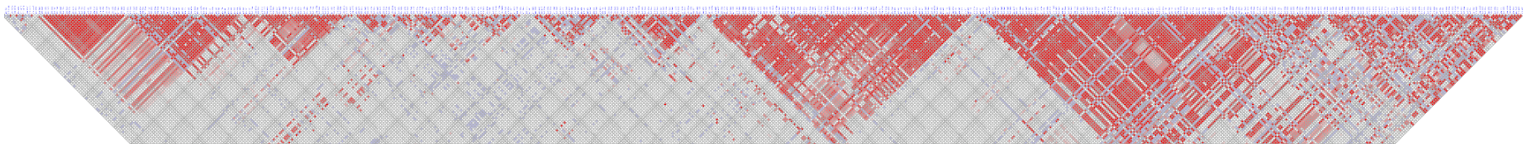
# Why GWAS?

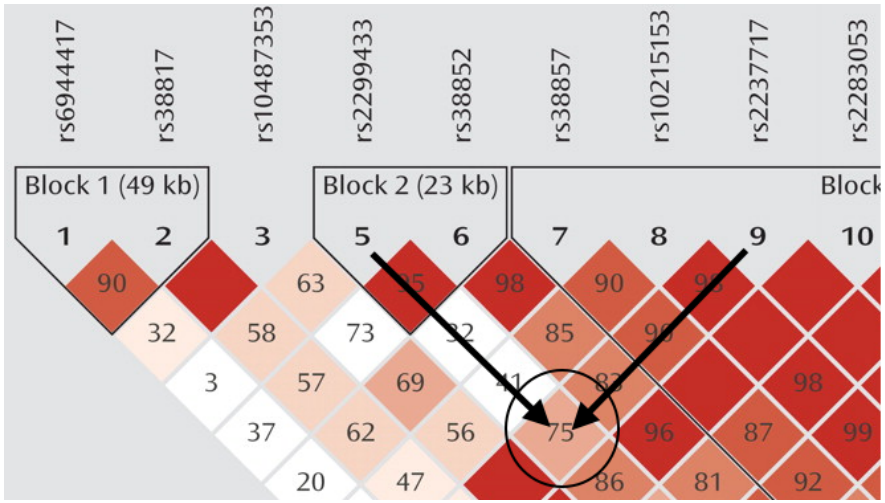
- If we look at 500.000 SNPs we are likely not to have the causal SNP!
- But, remember SNPs in high LD with a causal SNP will also be associated:



# Why GWAS?

- SNPs are in high LD in blocks along the human genome

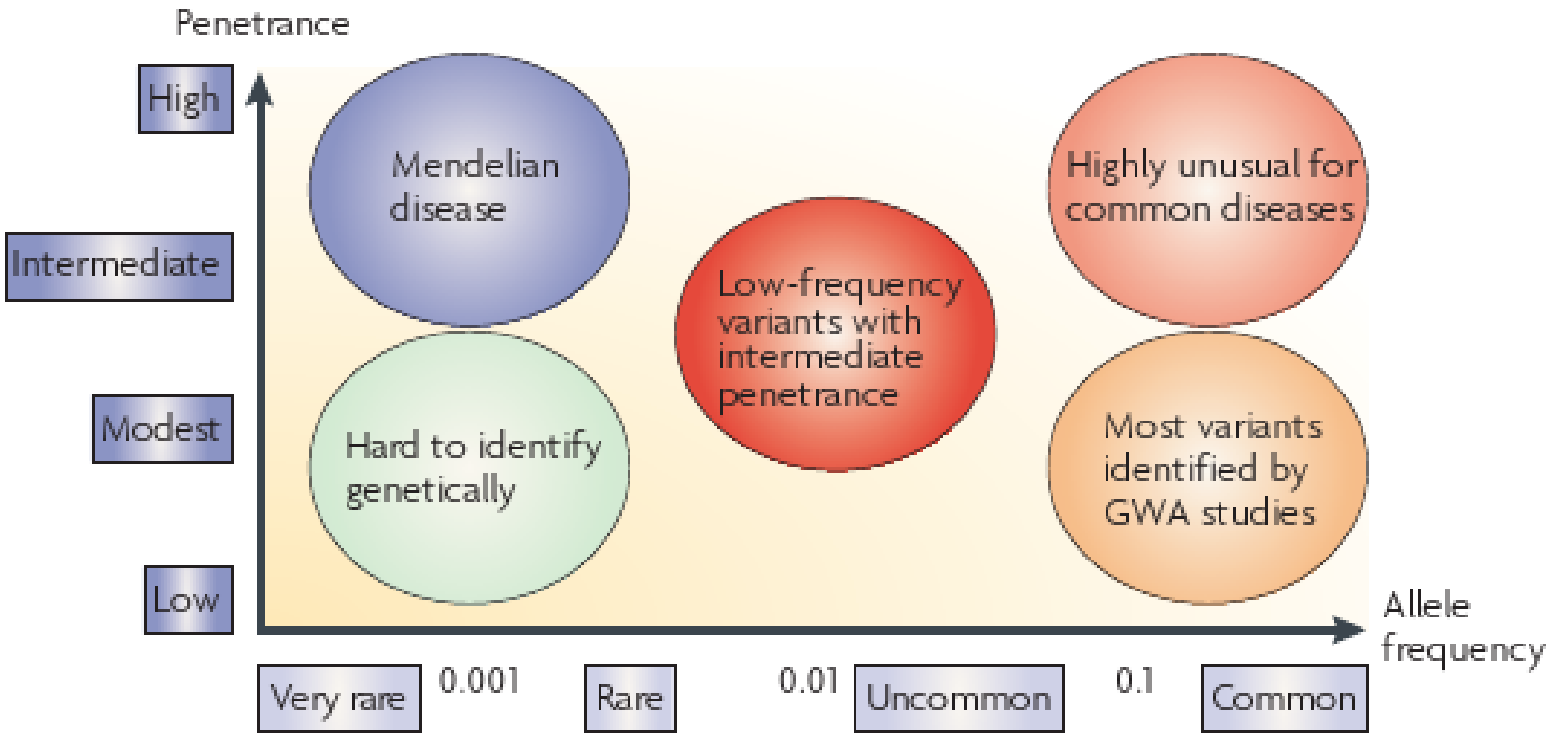




# Why GWAS?

- By testing a few SNPs in each block most common SNPs are indirectly tested
- We can test most common SNPs (indirectly) by using $\geq 500,000$ SNPs
- Pro: Cheap! (only need to genotype $\geq 500,000$ SNPs)
  Con: We are far from sure the identified SNPs (if any) are causal!

# When GWAS?




Strategies for locating disease loci

# How GWAS (step-by-step overview)

1. Collect samples and traits of interest (based on power calculations!)
2. Genotype samples at a number of SNP loci ($\geq$500,000)
3. Lots and lots of quality control (QC)!
4. Statistically test each SNP for association
5. Assess the results:
   - make sure things went OK
   - identify associated SNPs
6. Identify causal variant (if possible)
7. Replicate associations in a different dataset
8. Investigate what the underlying biological mechanism is
9. Ideal longterm goal/hope: better prevention or treatment

# GWAS step-by-step

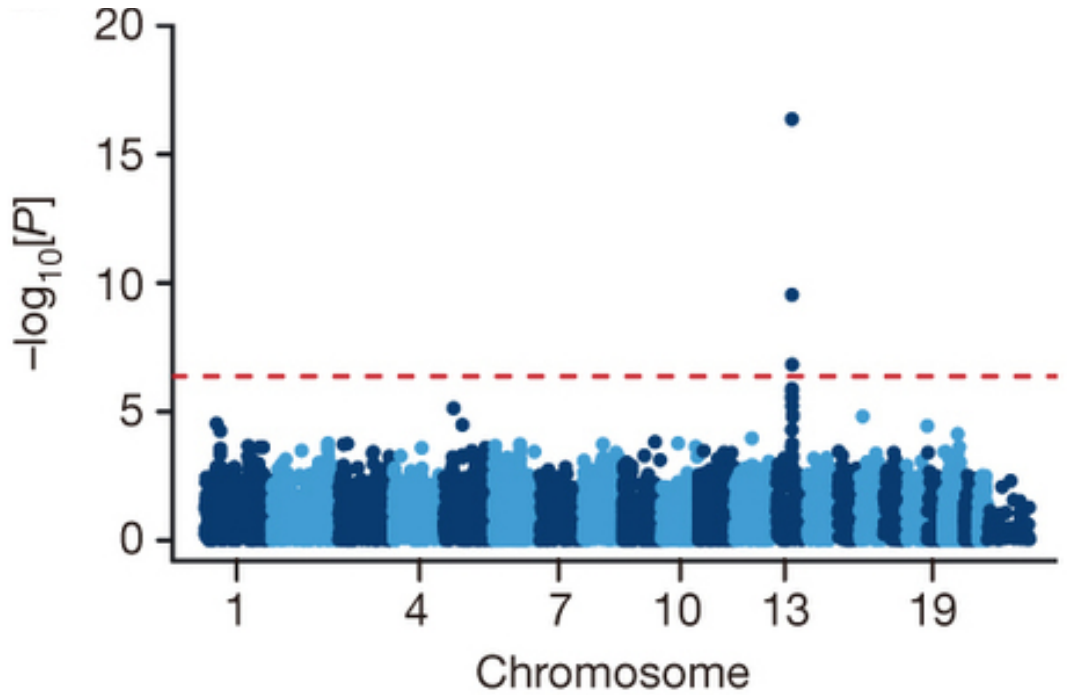
1. Collect samples and traits of interest (based on power calculations!)
2. Genotype samples at a number ($\geq$500,000) of SNP loci
3. **Lots and lots of quality control (QC)!**
4. **Statistically test each SNP for association**
5. **Assess the results:**
   - **make sure things went OK**
   - **identify associated SNPs**
6. Identify causal variant (if possible)
7. Replicate associations in a different dataset
8. Investigate what the underlying biological mechanism is
9. Ideal longterm goal/hope: better prevention or treatment

# Statistically test each SNP for association

- Use one of the tests you just learned how to perform
- There are programs like PLINK2 that will help you do this
- Can be done using one 1-line command
- Also offers functions for doing QC (we'll see that later)

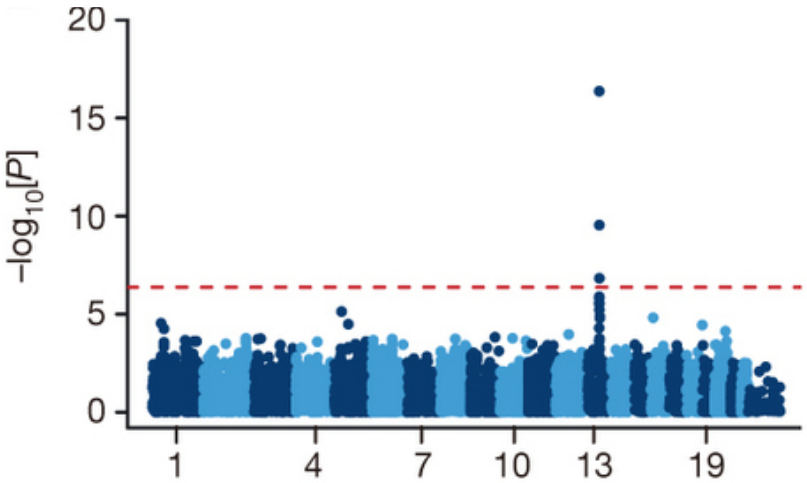
# Identify associated SNPs

## Manhattan plot



# What p-value threshold to use

- Usually for a single test we use a p-value threshold of $\alpha = 0.05$
- If you perform many tests with this $\alpha$ some will be falsely rejected
  With threshold 0.05 thousands of false positives!! (-log(0.05)=1.3)




So we have to **correct for multiple testing**

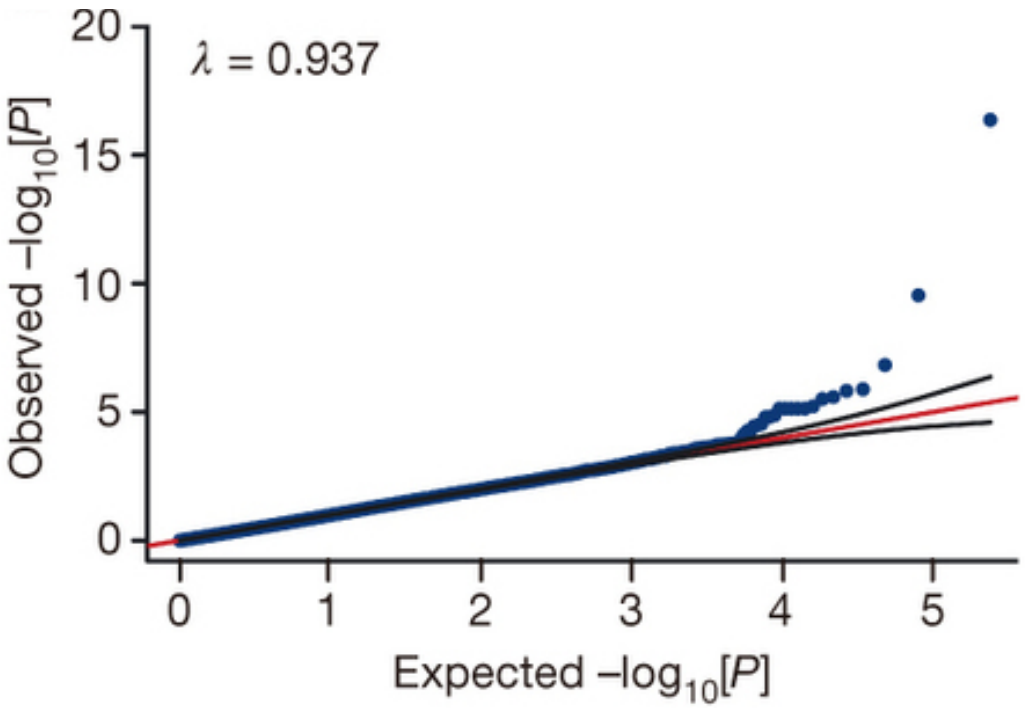
- Often **Bonferroni correction** is used; $\alpha$ is divided by the number of tests:
  - E.g. 100000 SNPs and $\alpha = 0.05$
  - Bonferroni corrected $\alpha = 0.05/100000 = 0.0000005 = 5 \times 10^{-7}$
  - Which on the Manhattan plot is $-log_{10}(5 \times 10^{-7}) = 6.3$

# Exercise

Solve exercise 2A, i.e. perform your first GWAS analysis :)

# Make sure things went OK!

## QQ-plots and genomic control inflation factor $\lambda$




**If so most of the dots will be on the x=y line and $\lambda \simeq 1$**

# Exercise

Solve exercise 2B, i.e. check if your results look OK...

# Lots and lots of QC

This shows why we usually do QC first ...! :)

Let's therefore return to that step
(we wont go through all QCs, but some important ones)

# Sample mislabling?

- One thing that can go wrong is the samples can be misslabled
- If so, genotypes won't match phenotypes
- This is difficult to catch
- But a simple check is to see of gender is correct
- If not the disease status is likely not to be either...
- We can check this using PLINK2

# Sample mislabling?

- One thing that can go wrong is the samples can be misslabled
- If so, genotypes won't match phenotypes
- This is difficult to catch
- But a simple check is to see of gender is correct
- If not the disease status is likely not to be either...
- We can check this using PLINK2
- **Exercise:** try checking it for your data (exercise 2C)

# Closely related individuals or duplicates?

- All association tests mentioned assume that the participants are **independent** samples from a population
- This would not be the case if some participants
  - are closely related
  - represented more than once
- One way to check if this is the case is to use PLINK2 (again)

# Closely related individuals or duplicates?

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- This would not be the case if some participants
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- One way to check if this is the case is to use PLINK2 (again)
- **Exercise:** try checking it for your data (exercise 2D)

# Batch biases/non-random genotyping error?

- Sometimes the data handling/generation process can lead to non-random genotyping errors
- E.g. if all cases were genotyped first and then all controls, then changes in genotyping procedure along the way may lead to non-random differences in genotypes between cases and controls
- This may lead the false positive association test results
- **Exercise:** try checking it for your data (exercise 2E+F)

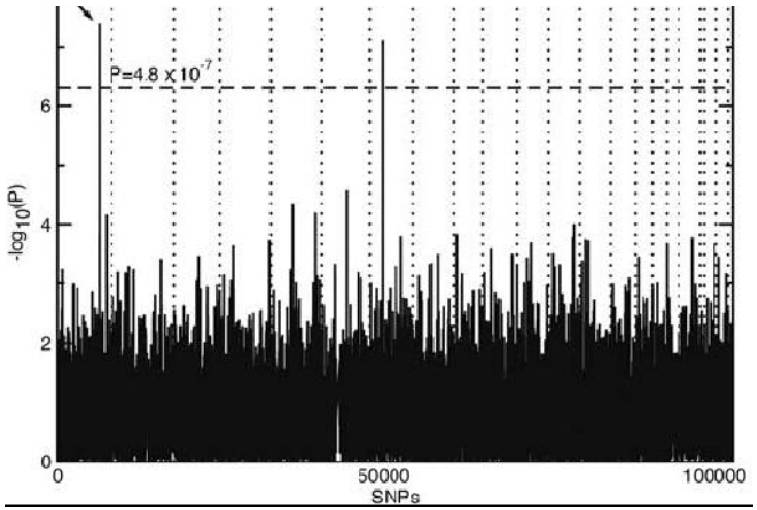
# Additional important checks?

- Other additional signs of something being wrong include:
  - high missingness in specific loci/individuals
  - loci (strongly) out of Hardy-Weinberg Equilibrium (why?)
- Furthermore, low frequency variants tend to be difficult to genotype
- Removing such loci/individuals can help a lot
- **Exercise:** try rerunning your analyses with these QC filters (exercise 2G)
- If you are done with the previous exercises, you can look into exercise 2F.

# Outline

# First study went extremely well!

- Study of age-related Macular Degeneration (Klein et al. 2005)
- 96 cases and 50 controls, 100K SNPs



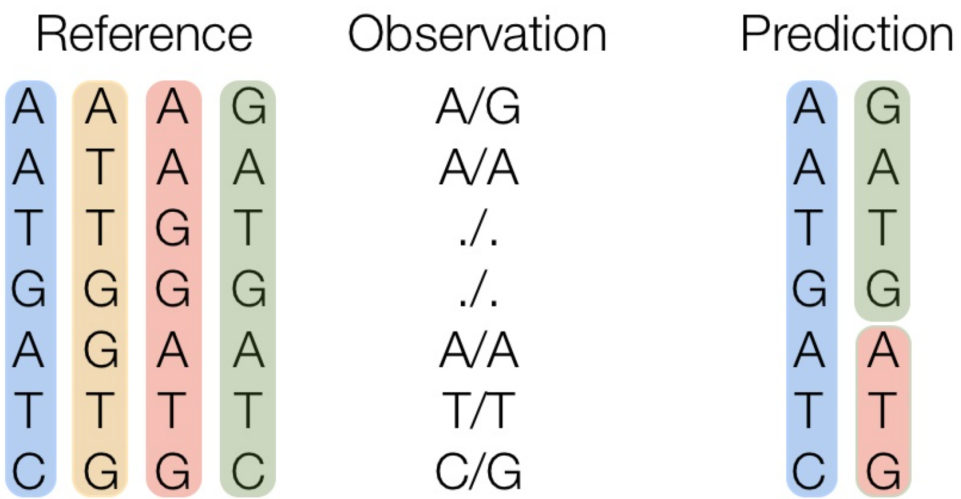

- SNP in *CFH* with large effect (OR=7.4) led to new biological insight

# Turned out to be unusual...

- MANY studies and more than 50,000 associations with $p < 5x10^{-8}$
- In the beginning few were replicated
  (underpowered, population structure, insufficient corr. for multiple tests)
- So later studies have many more samples and are much stricter
- Many studies find only small effect sizes and and some give only limited biological insight
- Some studies may be worth the effort and lead to:
  - Discovery of novel biological mechanisms
  - Clinical applications
  - Drug development and repurposing

# NGS enters the stage

- NGS allowed generation of large **reference panels**
  - The 1000 Genomes Project $>$ 2500 genomes
  - Haplotype Reference Consortium $>$ 65000 haplotypes
- Imputation:




- Summarised in **posterior genotype probabilities**:

$$P(AA), \quad P(AB) \quad \text{and} \quad P(BB)$$

# NGS enters the stage

**How do we asses effects of rare or study-specific variants?**

- NGS allows generation of **study specific reference panels**
- GWAS based directly on **NGS sequencing**
  - Example: Liu et al., 2018, Cell 175, 347−359
    $> 141K$ low-pass genomes $=>$ 16 novel associations
- Important to account appropriately lack of **full genotype information**
  - Increase **power**
  - Reduce **false positives**
- Summarised in **posterior genotype probabilities**:

$$P(AA), \quad P(AB) \quad \text{and} \quad P(BB)$$

# Dealing with uncertain genotypes in associations

- The easy solution: Dosage aka **expected genotype**, which use

$$E[g_i] = \sum_{g=0}^{2} g \; p(G_i = g|X)$$

  in the single-SNP tests described above.

- The complicated solution: **Maximum likelihood**, based on

$$p(y|X) = \prod_i \sum_g p(y_i|G_i = g)p(G_i = g|X)$$

- Here $p(y_i|G_i = g)$ is the **probability of disease** in the case-control studies or the **normal distribution density** in the quantitative trait studies.
- and $p(G_i = g|X)$ is the **posterior genotype probability**.

# Exercise

Solve exercise 3, How to run association tests on genotype probabilities using SNPTEST.