Hypothesis Testing
Correlation & Regression
as Bivariate Analyses

j3

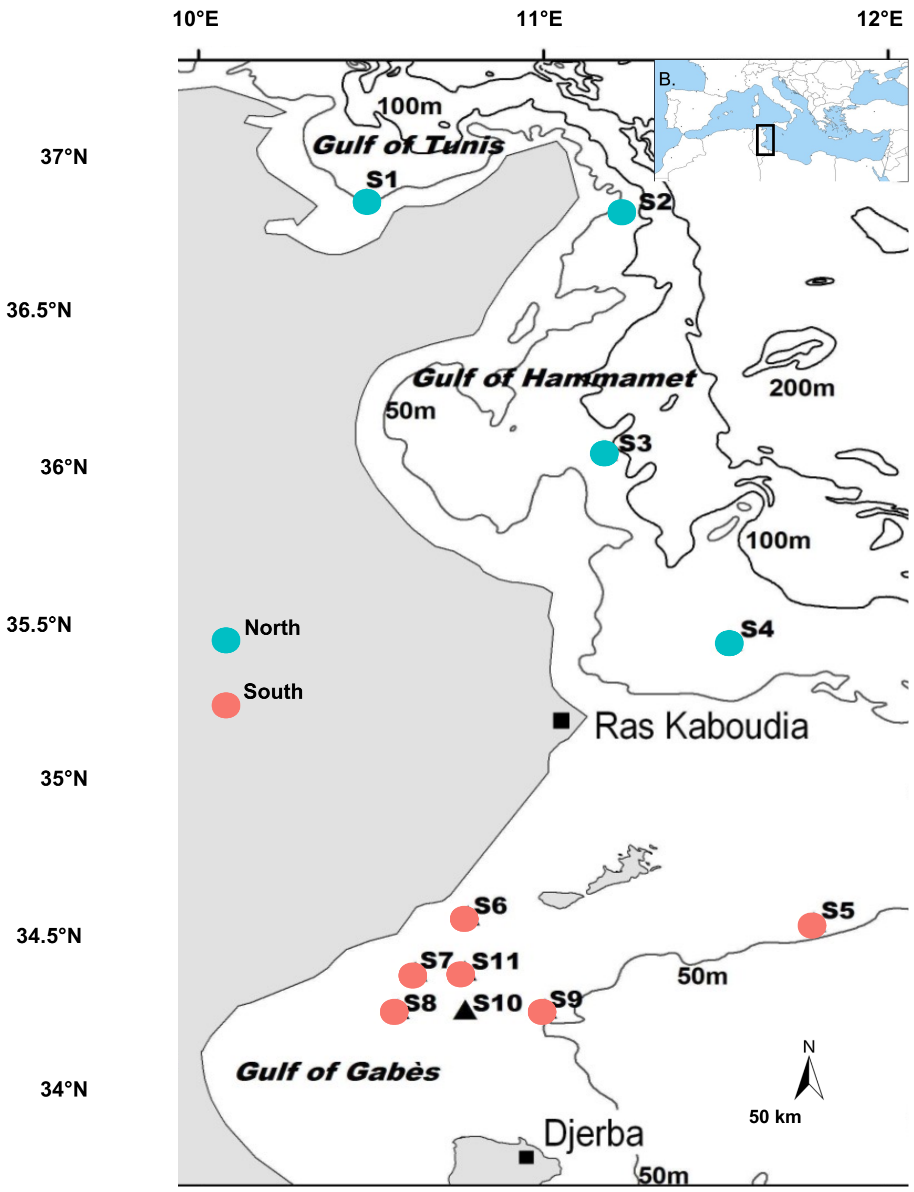
– 06.09.23 –

Institut Méditerranéen d'Océanologie

Institut de Recherche pour le Développement
FRANCE

ANF METABIODIV

Bio-informatique & Sciences de l'Environnement : Exploration de la Diversité Taxonomique des Ecosystèmes par Metabarcoding



Variability in species richness between North & South

Is there a real significative difference or just a coincidence ?

Using **statistics** to answer your question !!

Population VS samples

Population: set of individuals or objects of the same kind (very large or infinite)

→ We can't study an entire population: in statistics, we study a limited number of individuals, a part of the population: **a sample**

→ We try to **deduce properties** of the population from the sample

→ If we want to **study the variability** of a variable of interest in the population, we need a **representative sample** (drawn at random)

In a population, we can measure a characteristic: **a variable** that is the result of a random phenomenon.

- Qualitative
- Quantitative (continuous)

A **probability law** describes the random behavior of a phenomenon that depends on chance.

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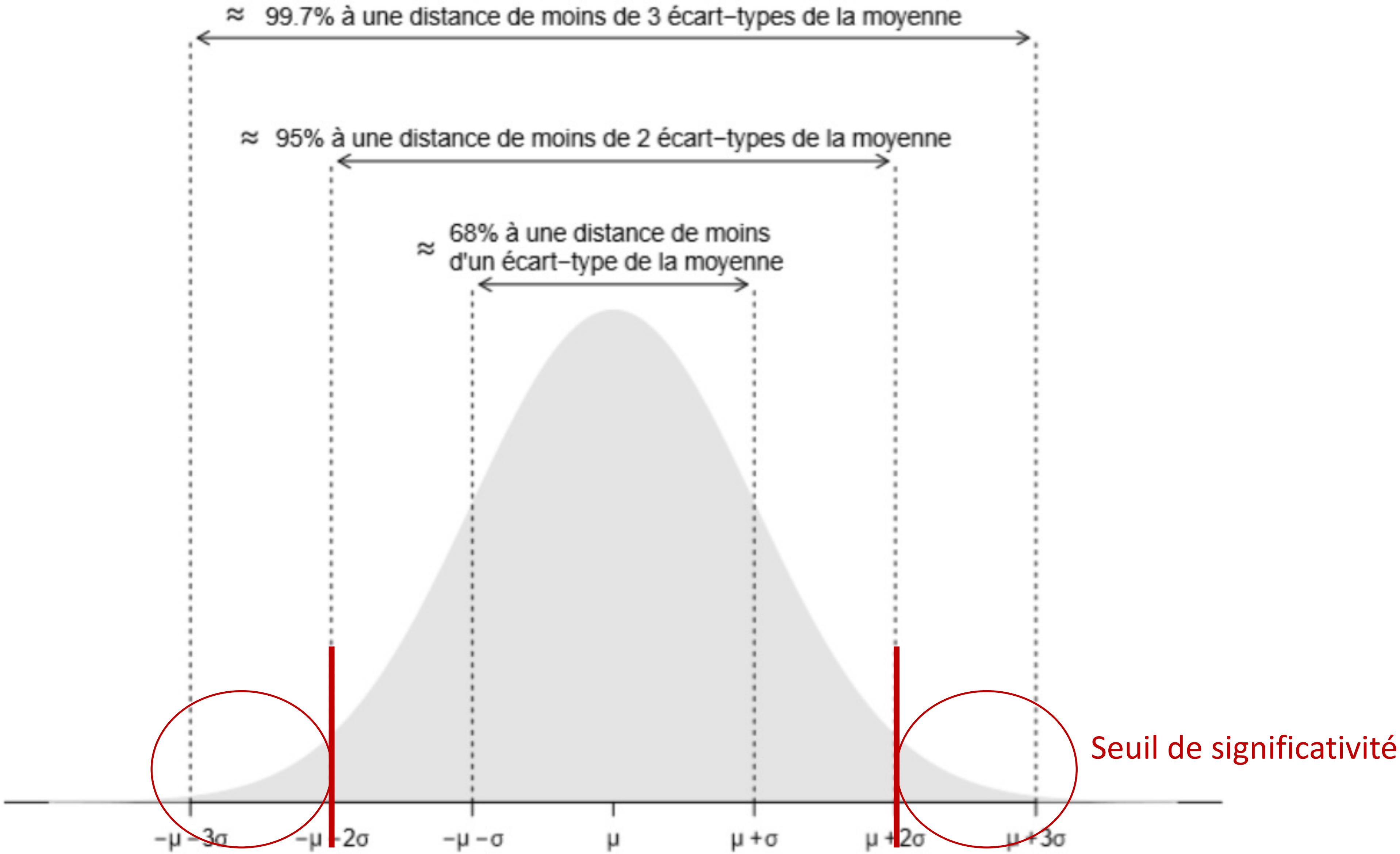
A **probability law** describes the random behavior of a phenomenon that depends on chance.

THE NORMAL LAW

If we have 1000 samples of a variable following a normal distribution, and plot the number of samples equal to each value, we obtain a "bell" curve / gaussian distribution

$X \sim N(\mu,\sigma2)$ with μ and $\sigma2$ the parameters of the distribution:
- μ: expectation of X
- σ: standard deviation of X = dispersion around the mean

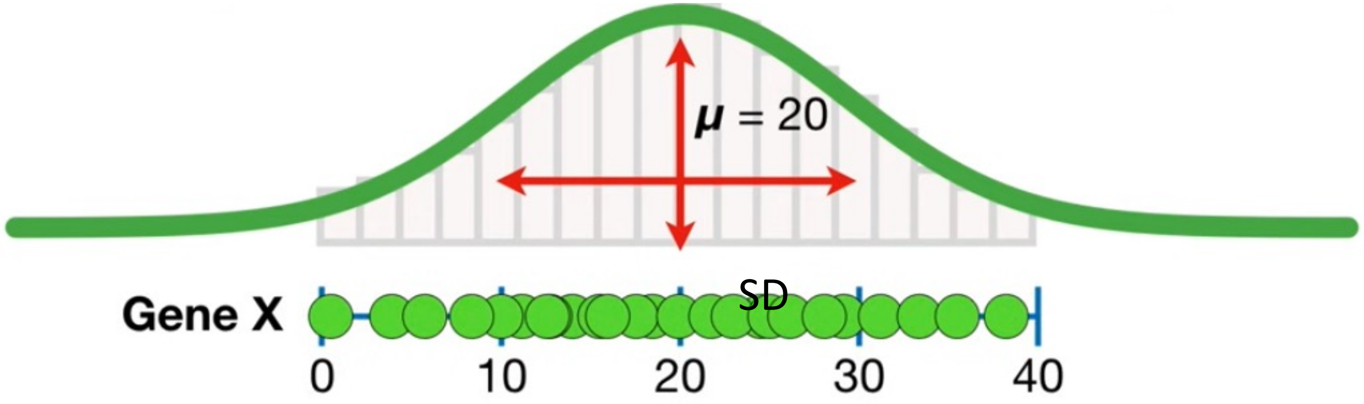
Répartition des valeurs autour de la moyenne



Remember : Descriptive statistics (Univariate analysis)

Merely describe, show and summarize collected data

- **Central tendency** (mean, mediane…)
- **Dispersion** (variance, standard deviation)
- **Frequency distribution** (count, relative, cumulative)



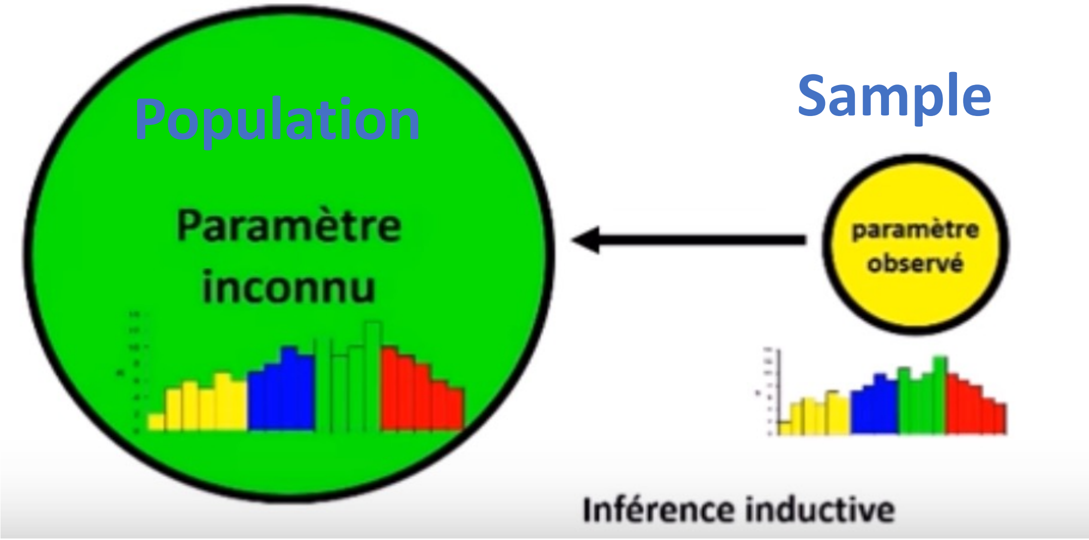
Identify the characteritics of data for each variable(s)

→ **Allows you to formulate hypotheses and guide statistical analyzes**

Inferential Statistics

Predictions - Generalizations

Sample



Make inferences about the population

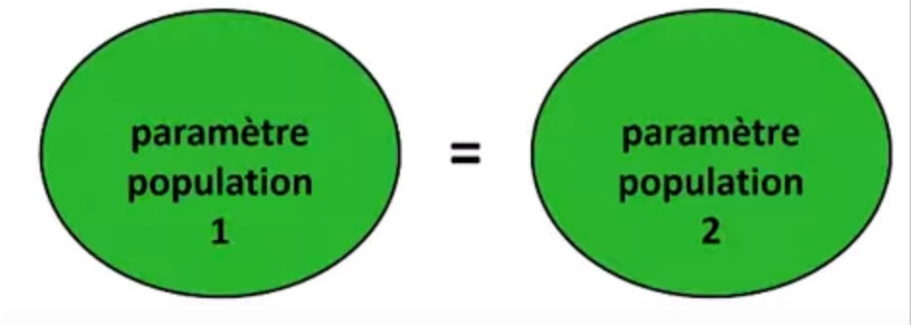
- How can I use my sample to make predictions about the population = Estimation
- How do I prove a theory about my data's behaviour (comparison) = Hypothesis Testing

Hypothesis testing approach

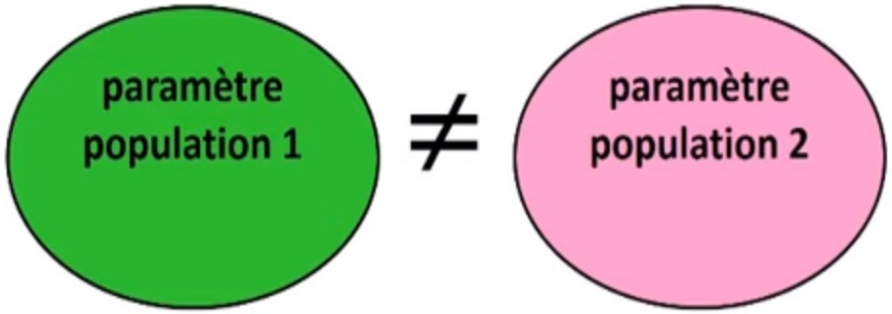
Trying to validate a hypothesis relating to a population parameter from a sample comparisons

Is there a real difference or just a coincidence (chance)

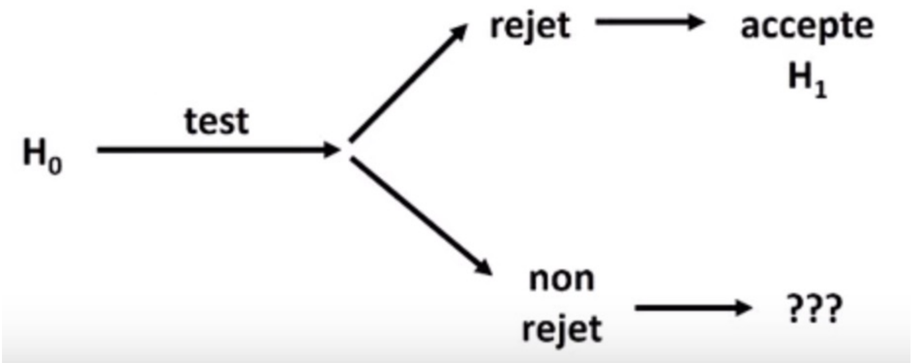
Null Hypothesis H0



Alternative hypothesis H1



We are testing the null hypothesis!



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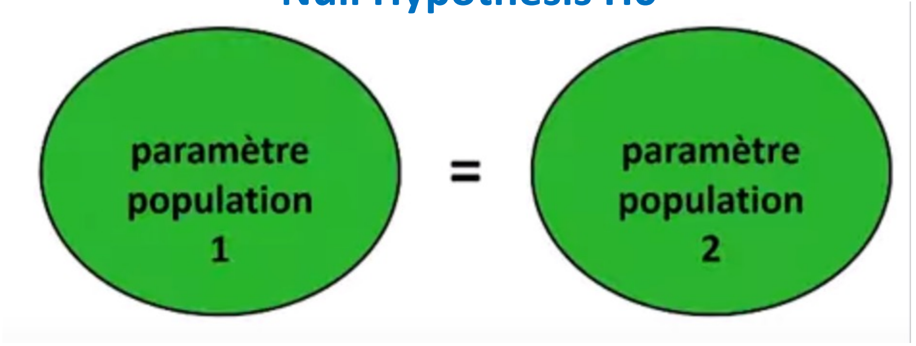

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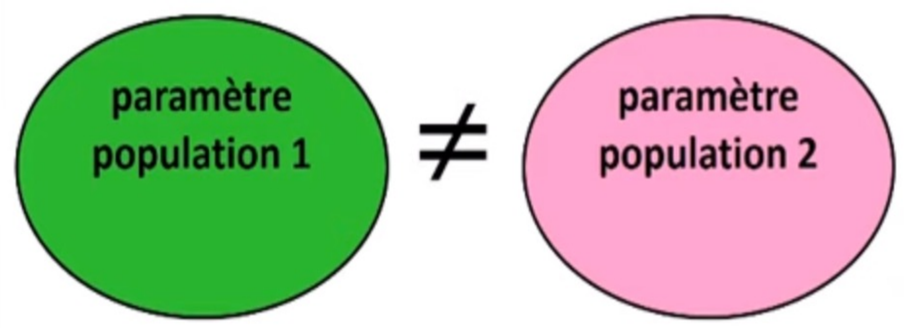
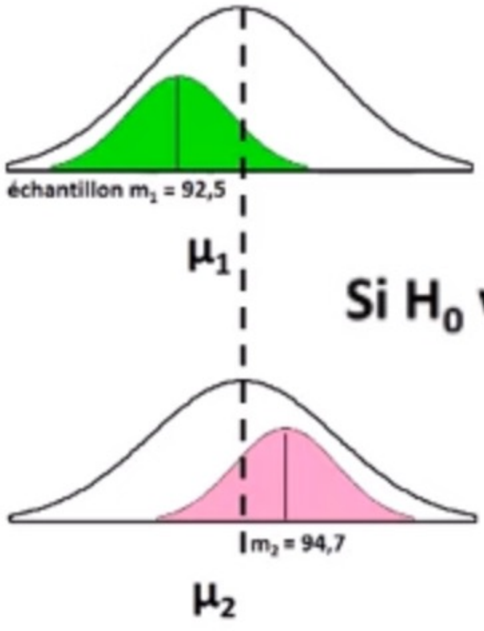


"Absence of Evidence is not Evidence of Absence"

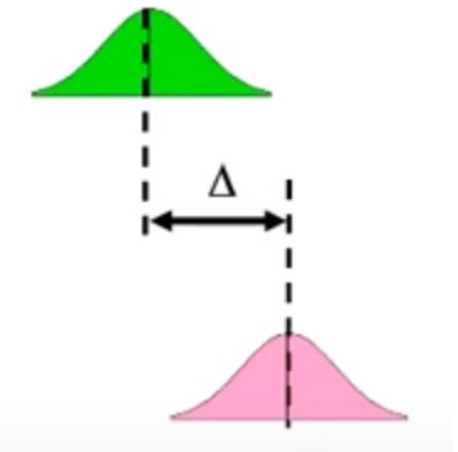
Hypothesis testing & mean comparison

If H0 true… no difference

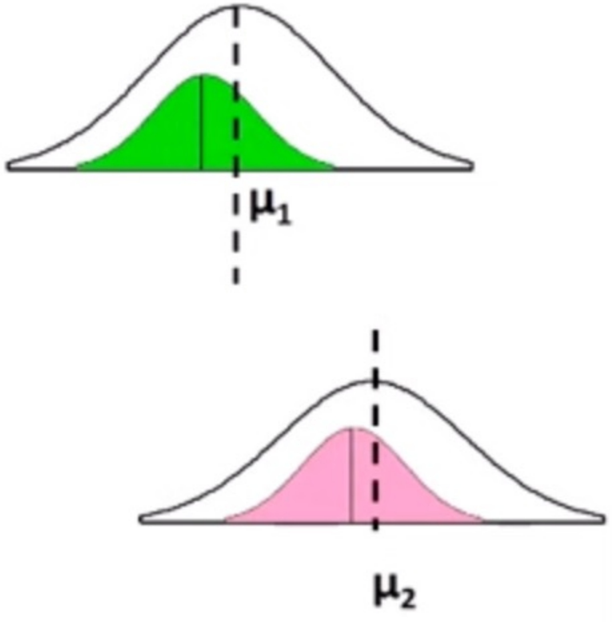


$$H_0 : \mu_1 = \mu_2$$

$$\text{Si } H_0 \text{ vraie} : \Delta = m_1 - m_2 \approx 0$$



If H0 rejected, H1 accepted



$$H_1 : \mu_1 \neq \mu_2$$

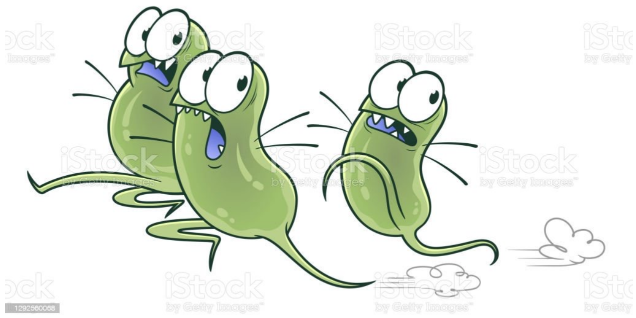
SAME distribution
→ Sampling fluctuation

Two different distributions

Inference Issue : Subjected to errors!!

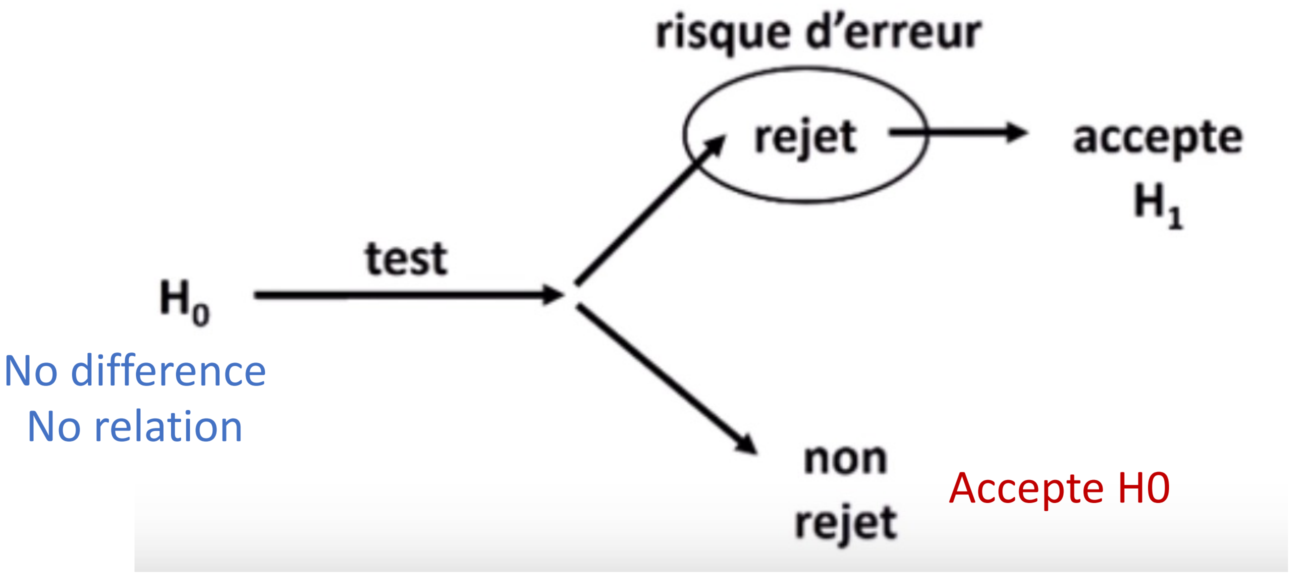
The risk is linked to the result of hypothesis testing

Because of your sampling!



The risk of Type I error α

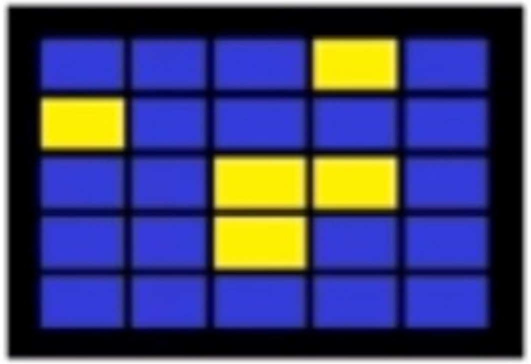
- A probability between 0 and 1, or 0 and 100%
- Is when a difference is affirmed but there is none (=False positive)!!



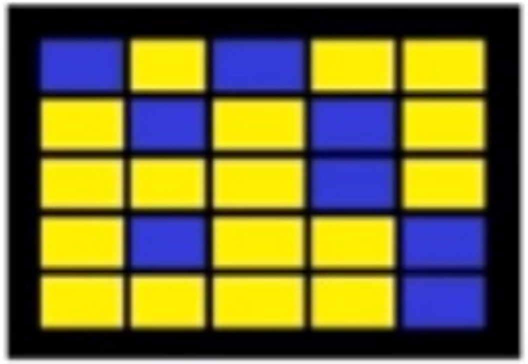
α= Risk to reject H0 if H0 is true

Sampling

25 tiles
→ 80% blue



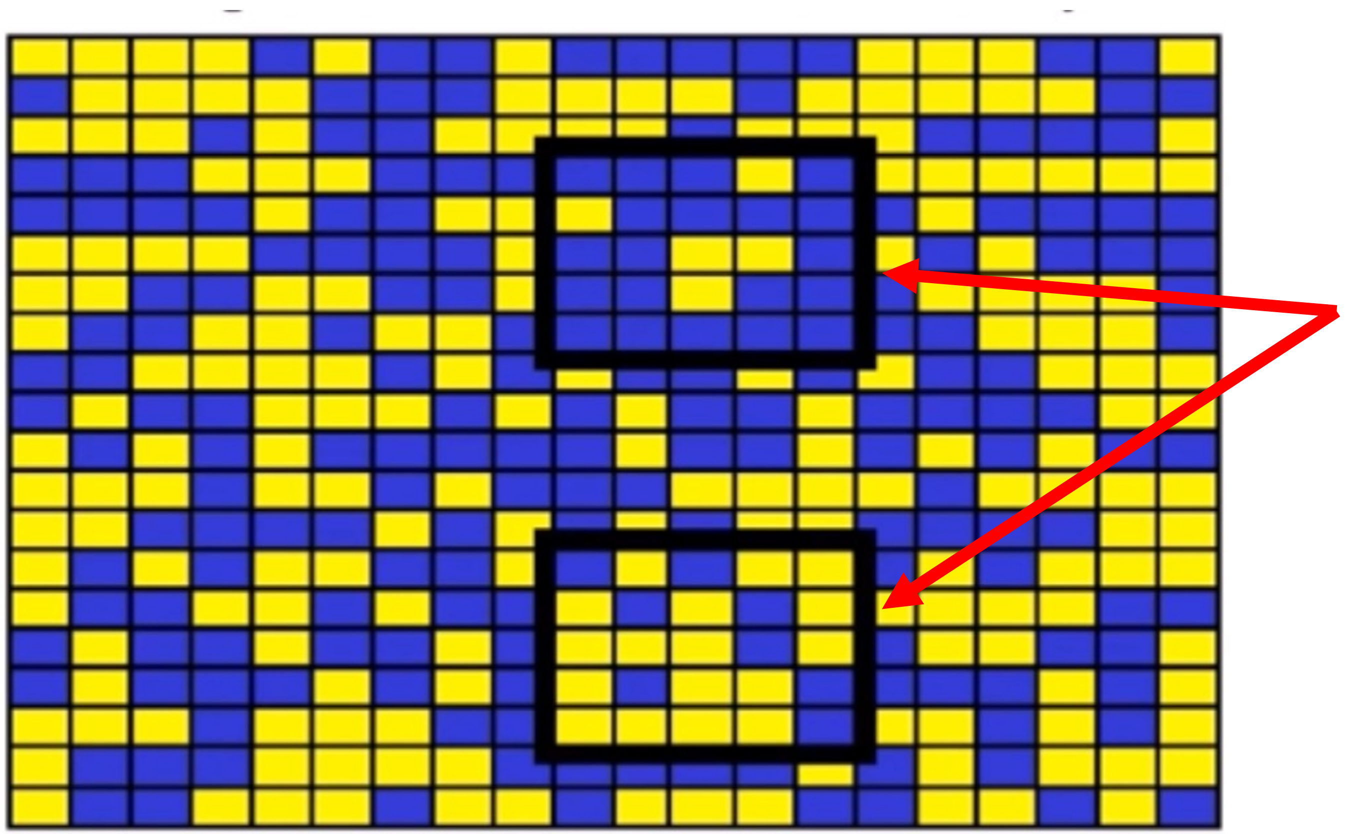
25 tiles
→ 32% blue



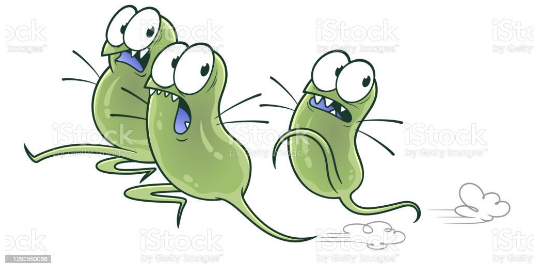
Do the two samples come from the same population? (same distribution)?

- H0 is rejected
- but let's go to the store...see the population

Come from the same population (50% blue, 50 % yellow)!!

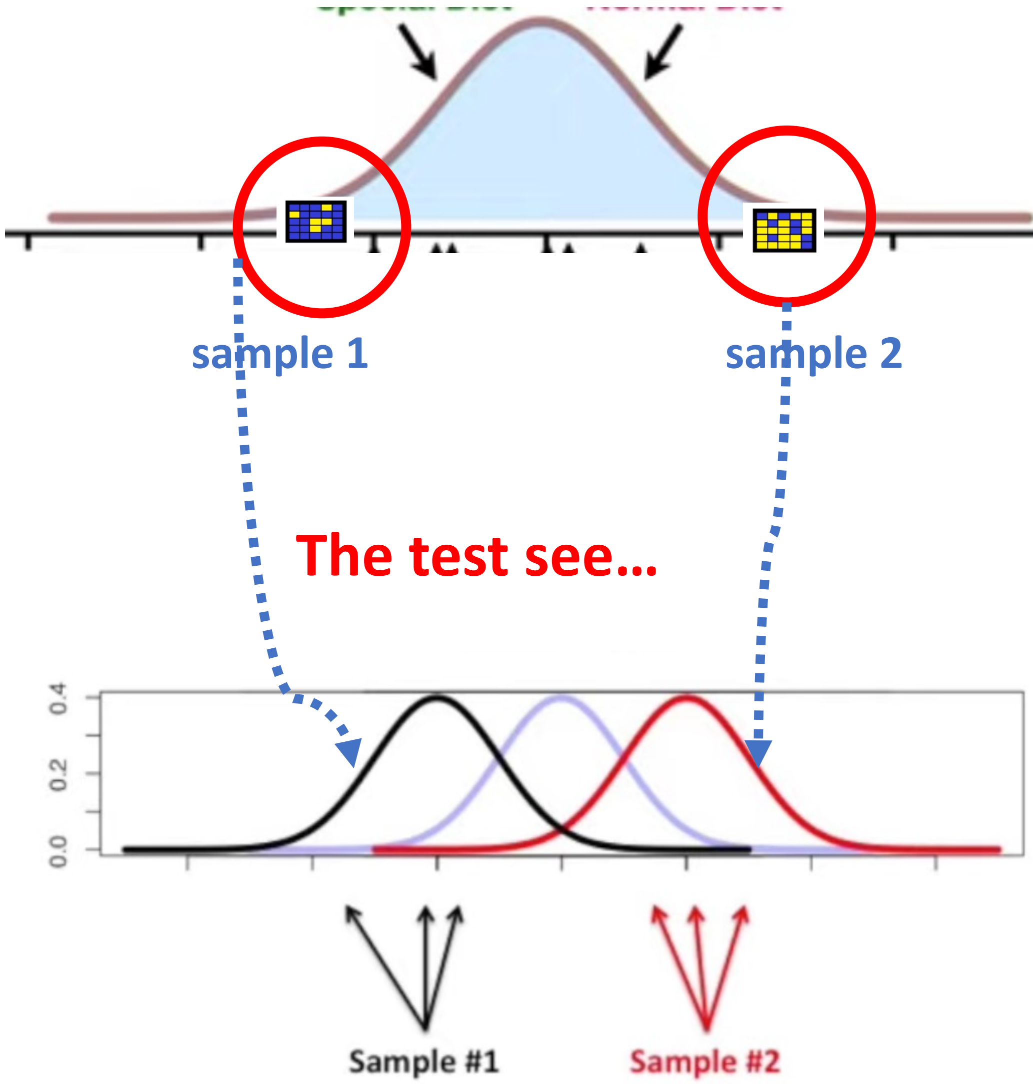


Rare sample type



Conclude on the basis of our samples that they came from two different distributions = Type I error

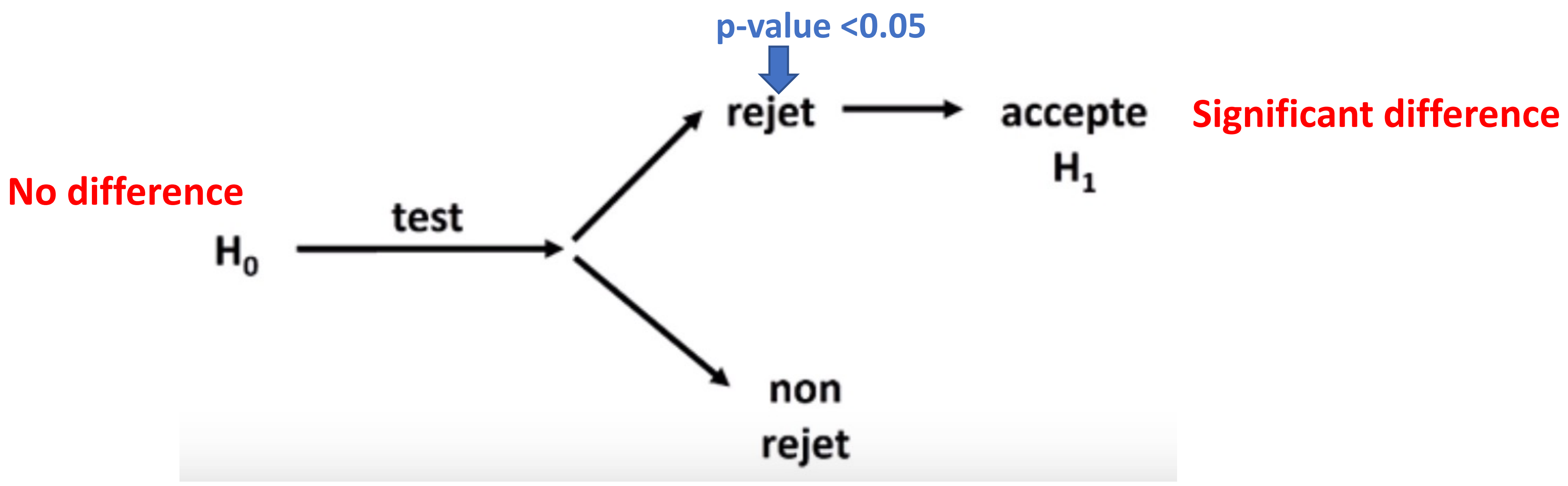
Data come from the same distribution but …



Two different distributions

- α is choosen before the test : Significance threshold

- α often set 5% (H0 wrongly rejected)
- In science the "almost no chance" translates to in less than 5% of cases where H0 is true = p-value < 0.05



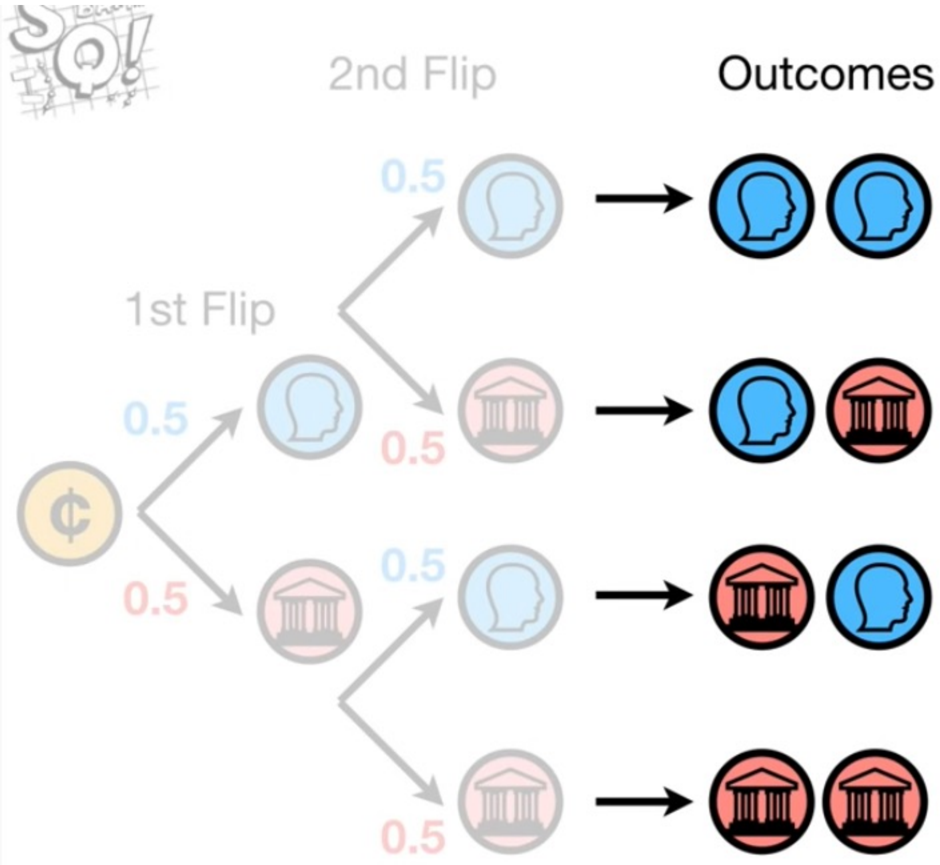
Concept of p-value…

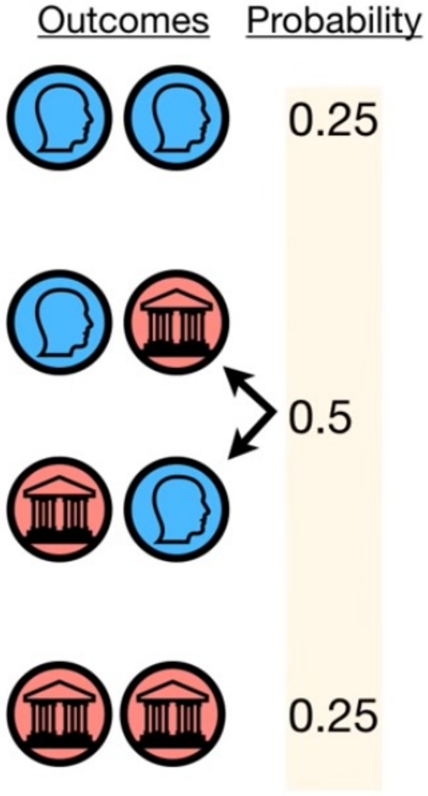
My Coin is special: Heads twice in a row!

The Null hypothesis H0: even though I got 2 Heads in a row my coin is not different from a normal coin!

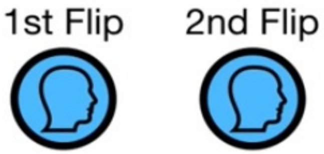
A small p-value will tell us to reject H0 (p-value <0.05)!

So let's test the hypothesis by calculating the p-value!





$$\frac{\text{The number of times we got } \textbf{2 Heads}.}{\text{The total number of outcomes.}}$$

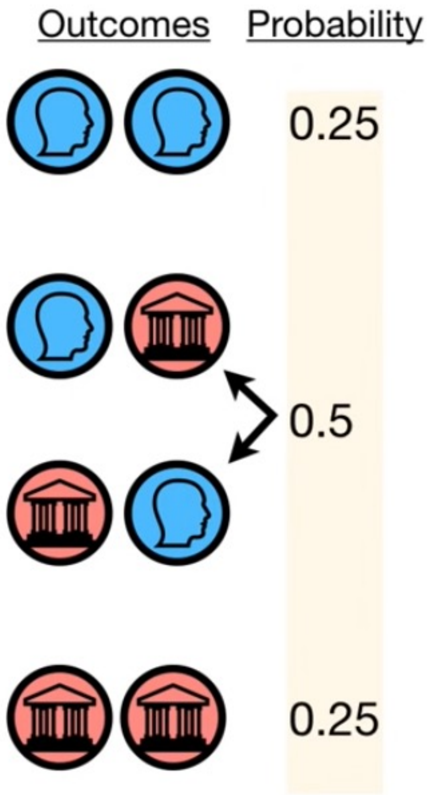


A ***p-value*** is composed of three parts:

1) The probability random chance would result in the observation.

2) The probability of observing something else that is equally rare.

3) The probability of observing something rarer or more extreme.



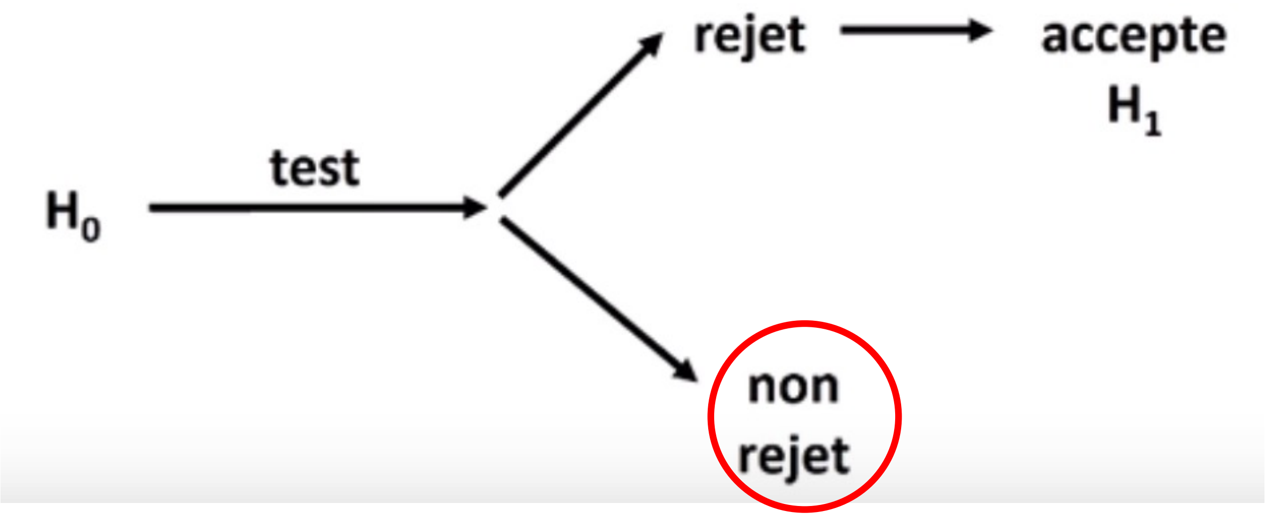
Nothing

P- value for 2 Heads (Sum of three parts)= 0.25+ 0.25 + 0 = 0.50!

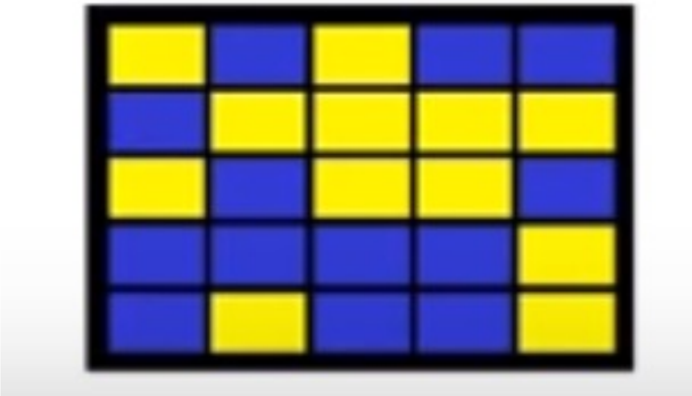
My coin is not special! p-value >>> 0.05!!!

Risk of Type II Error : β

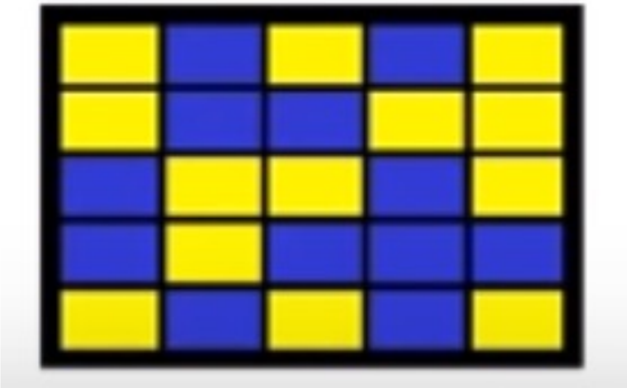
Failing to conclude **a difference when there is a true one** ("False Negative")
Probability of not rejecting H0, if H1 is true



β is not calculable

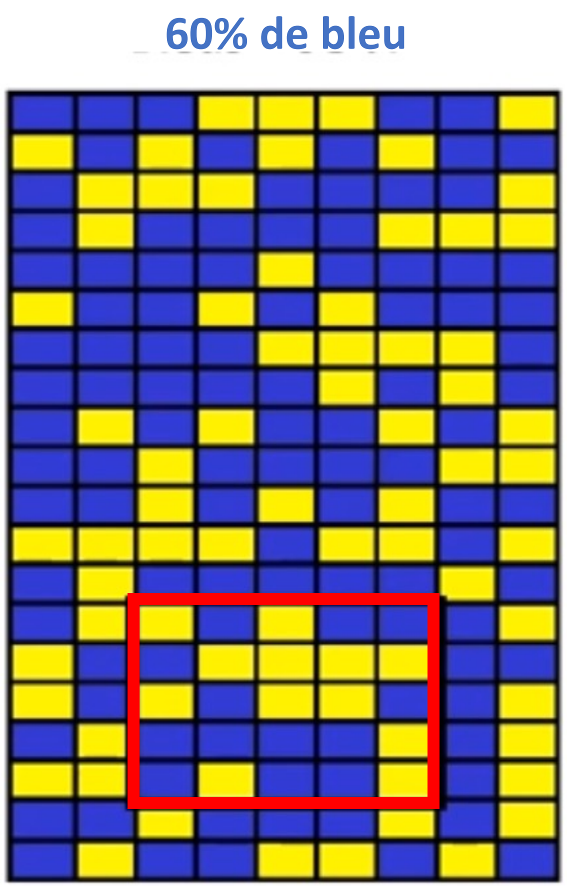


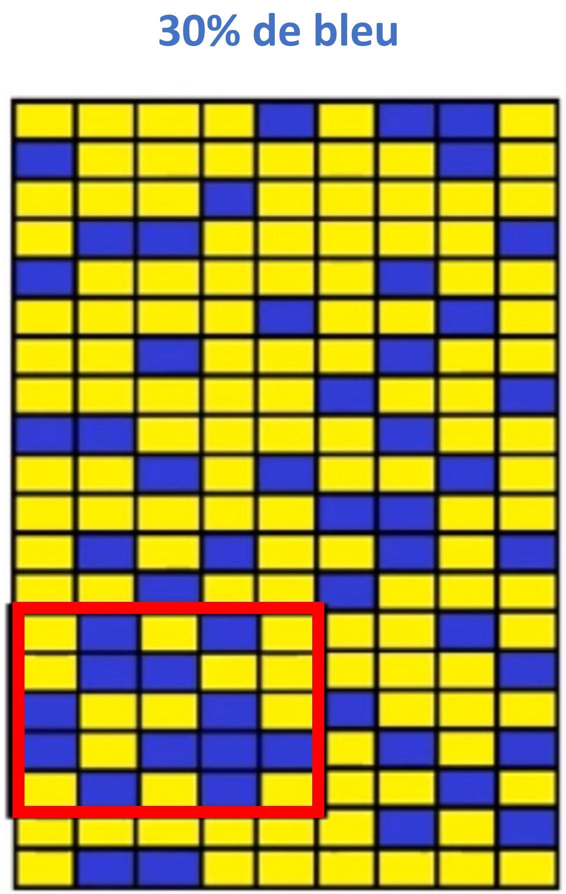
48% blue



52% blue

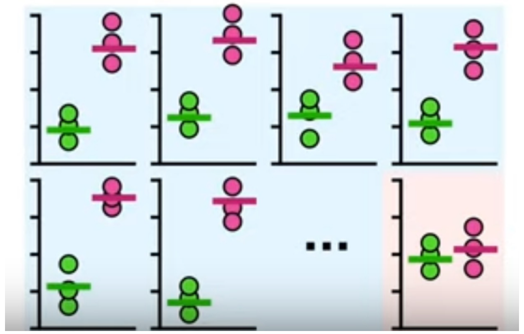
Do these two samples come from two different distributions or not?

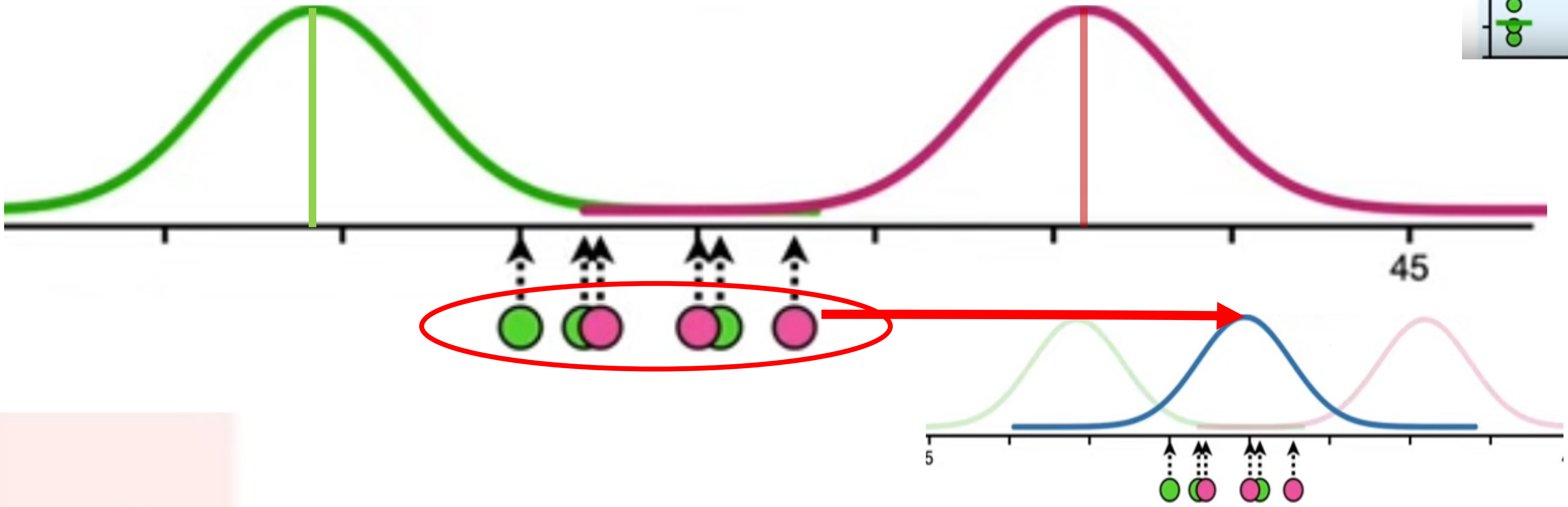


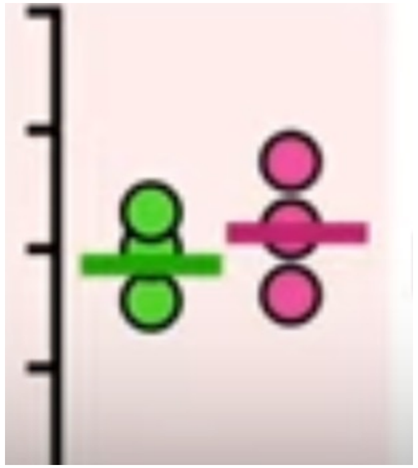


- 2 different tiles = 2 different populations, H0 should be rejected But that would not have been the case during the test with our sampling…

But sometimes…





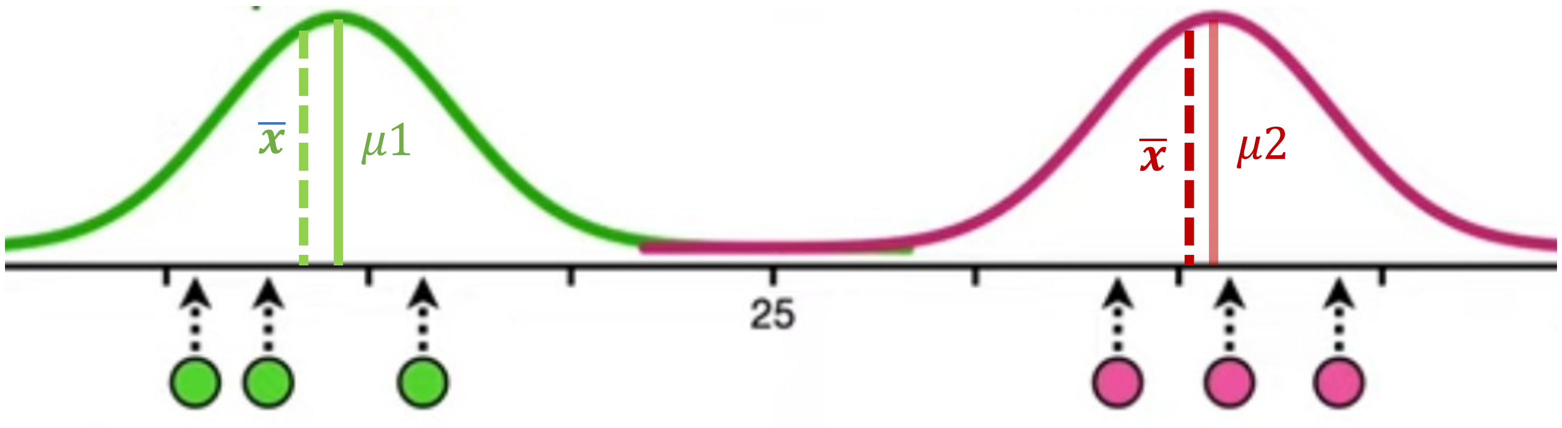


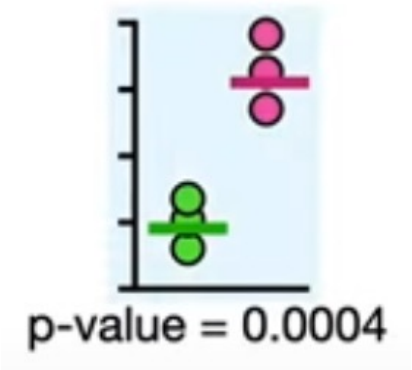
p=0.23!!!

Even if two different distributions (pop)...the test (your data) thinks they come from the SAME distribution!

Unable to correctly reject H0...

Scientifically … representative sampling of population





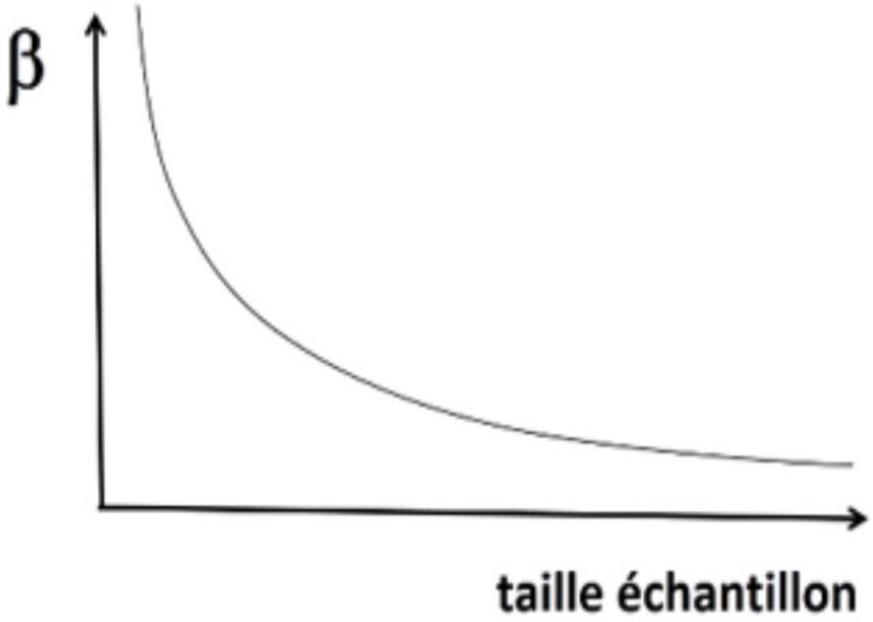
→ H0 correctly rejected

→ = Data do not belong to same distribution

→ Two different populations

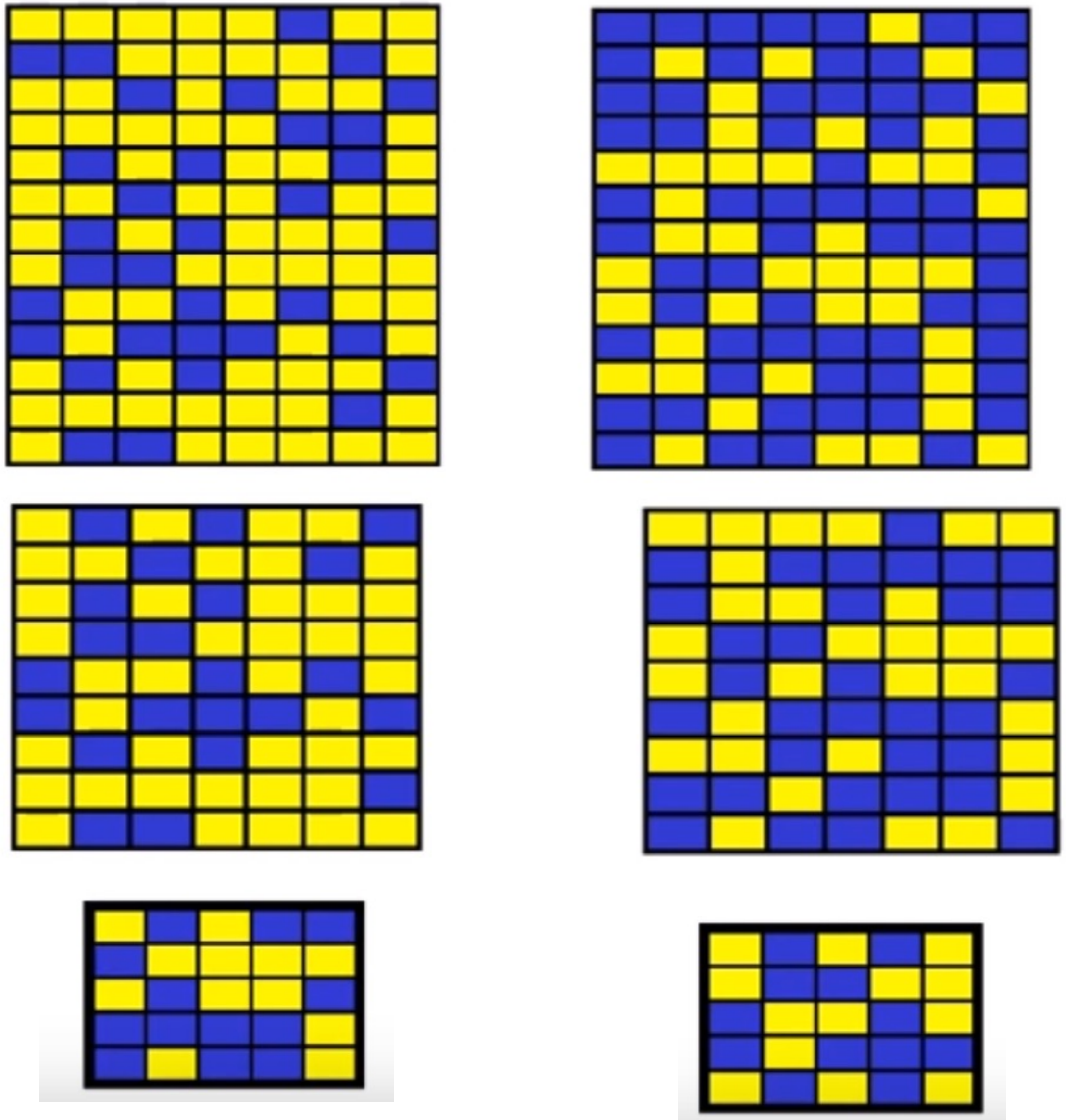
Fundamental relationship

Power =1 $- \beta$



Power: Probability of correctly reject the H0 hypothesis
Ability of a test to detect differences

The more the size increases, the more the differences appear! The power of the test increases!

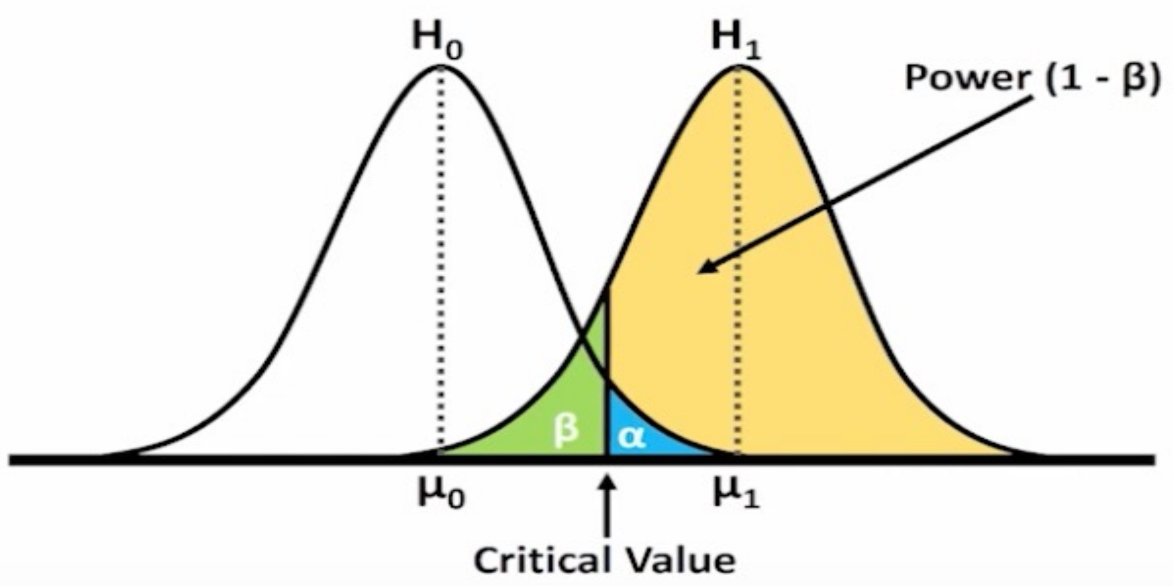


Summary

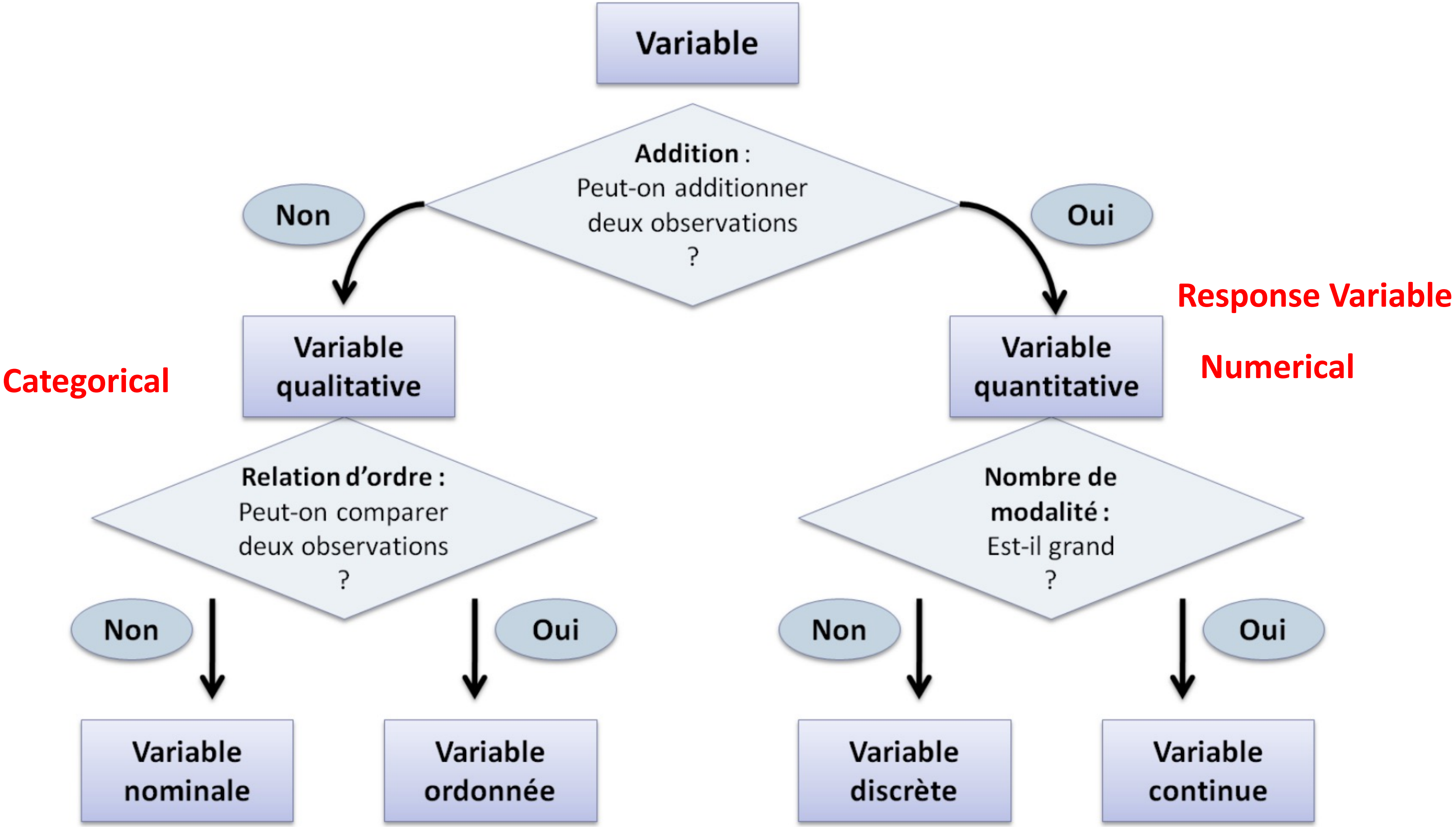
Population

TEST échantillons

	H_0 vraie	H_1 vraie
accepter H_0	OK	*erreur type 2* β Faux Négatif
rejeter H_0	*erreur type 1* α Faux positif	OK



Reminder on variables… important for statistical tests



- Maried, single…

→ No relation order

- Behaviour
- good, excellent…

Child in family (1,2,3..)
finite number of real values

Size, weight : infinite

Bivariate Hypothesis Testing

- Seek to **quantify the association** between a **variable to be explained** (response/Quantitative) and an **explanatory variable** (factor/categorical)

- **Make statistical inferences about the relationship between two variables,** **One quantitative variable (response) & one qualitative (explicative)!**

→ Can variations in species richness (response variable) be explained by the explanatory variable (factor) Treatment
→ **Comparison of mean between groups**

- **Parametric or non parametric test??**
- **which test?? significance ? (p-value)**
- **How many groups??**
- **Post hoc test required ??**

Which test for independent samples?
ONE categorical variable (H/F) & ONE continuous variable (numerical)

Normalité des données?
Shapiro, Q-Q plots

Which test for independent samples?
ONE categorical variable (H/F) & ONE continuous variable (numerical)

Normalité des données?
Shapiro, Q-Q plots

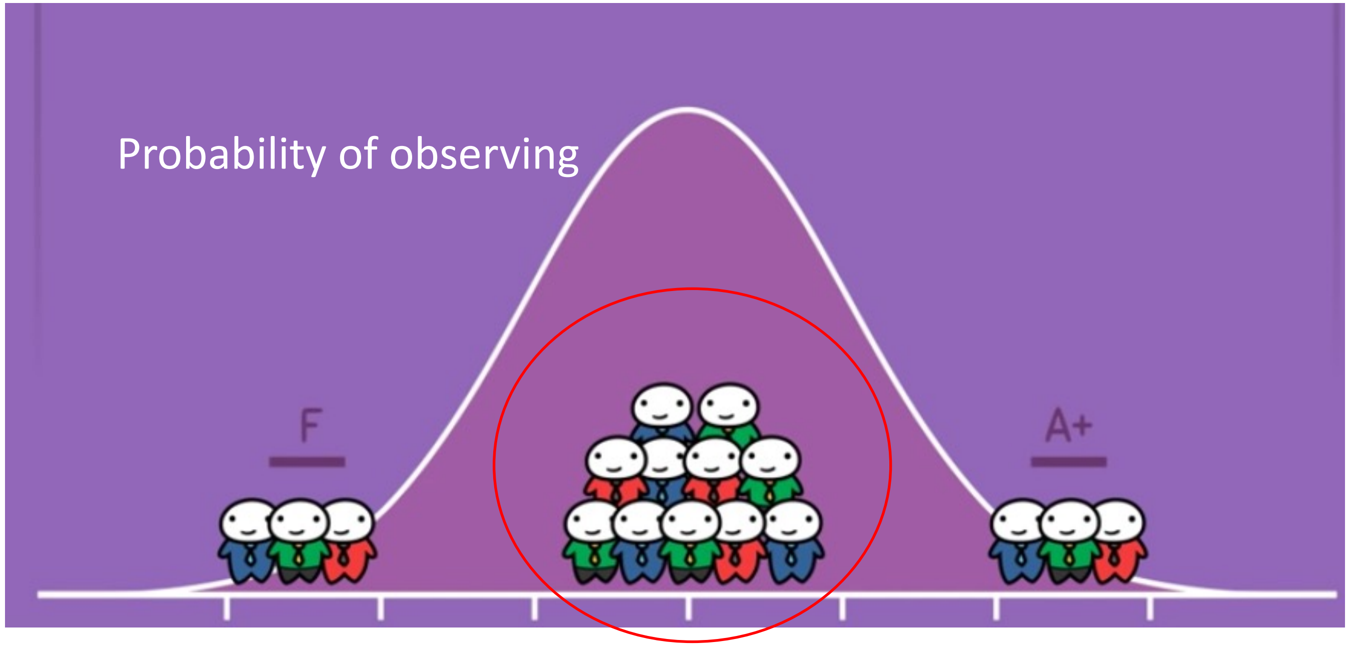
Yes

Parametric test

Features of Normal distribution

Symmetric, unimodal

- Center around the mean
- Dispertion around the mean: Standard deviation (SD)
- 95% data -/+ 2 SD



rare

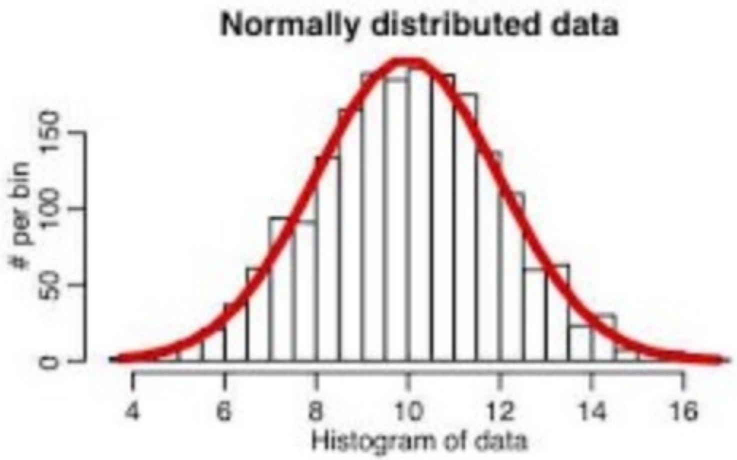
majority

rare

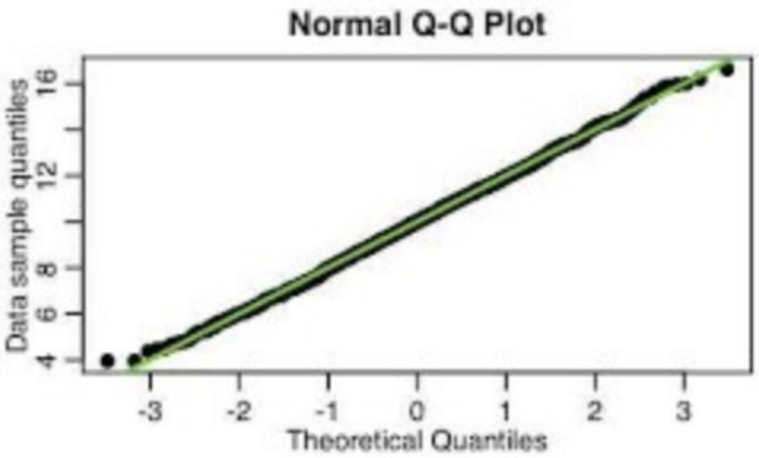
Check normality of data: Shapiro Test & QQ-plots!!

Q-Q plot normale: Compare your distribution with a normal distribution

Do my data follow a normal distribution ?



Your Data

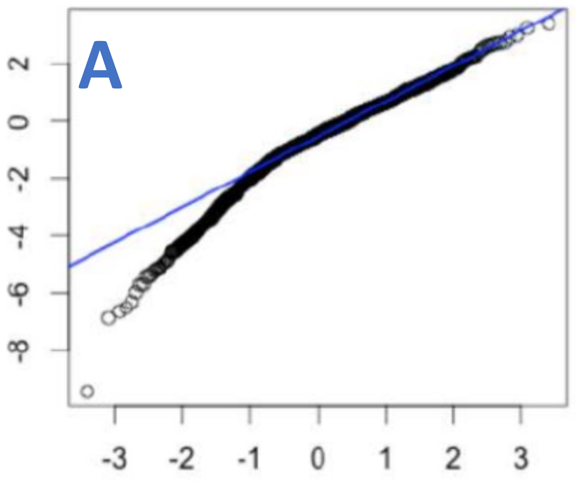


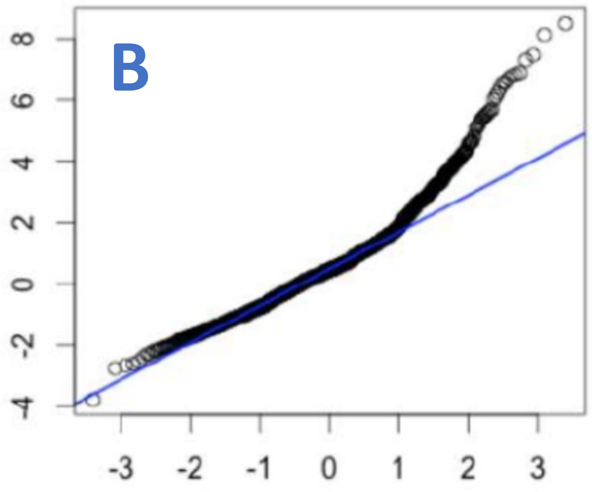
Normal Data (u= 0, SD=1)

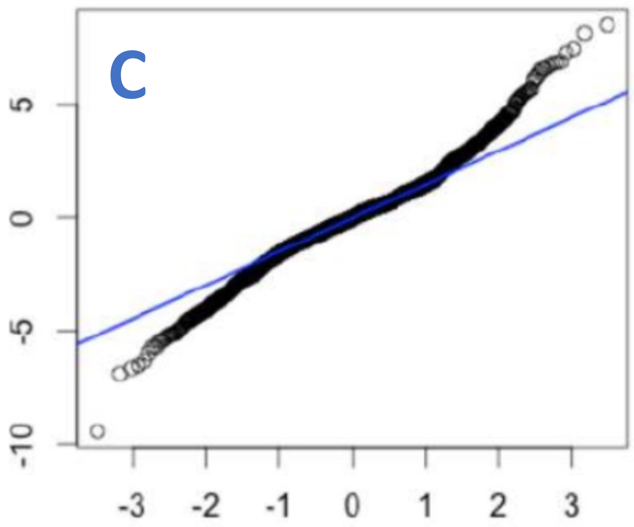
Conclusion?

The line draws by QQ-Plot indicates the position that the points must have to follow a normal distribution

What are the distributions (bottom) corresponding to these QQ-plots?

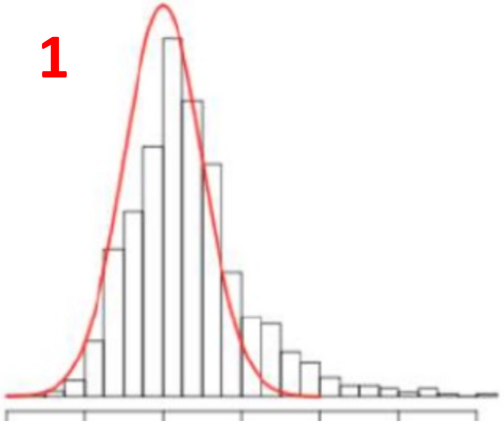






???????????

1



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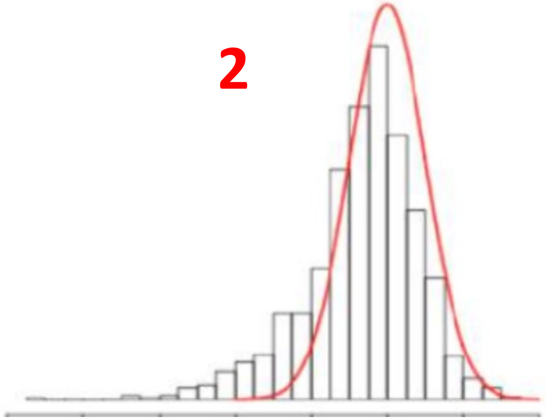

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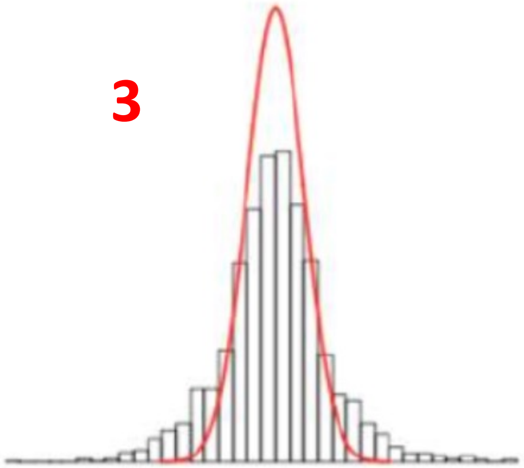
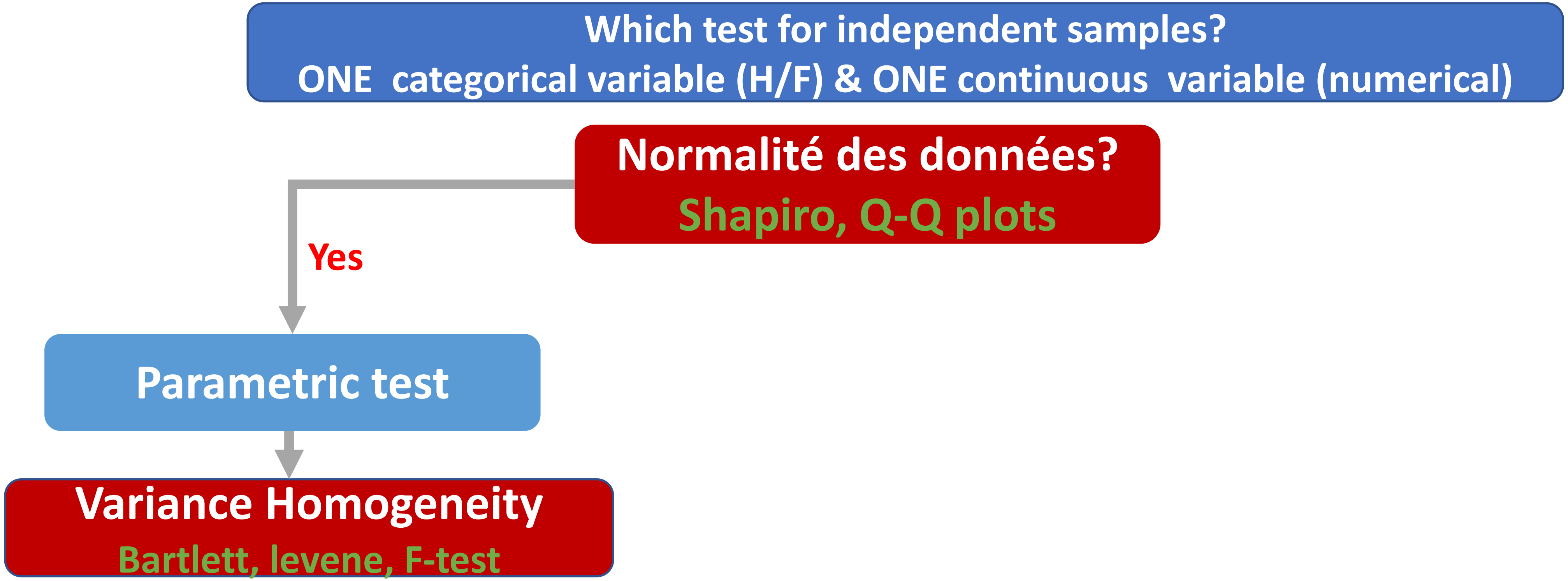


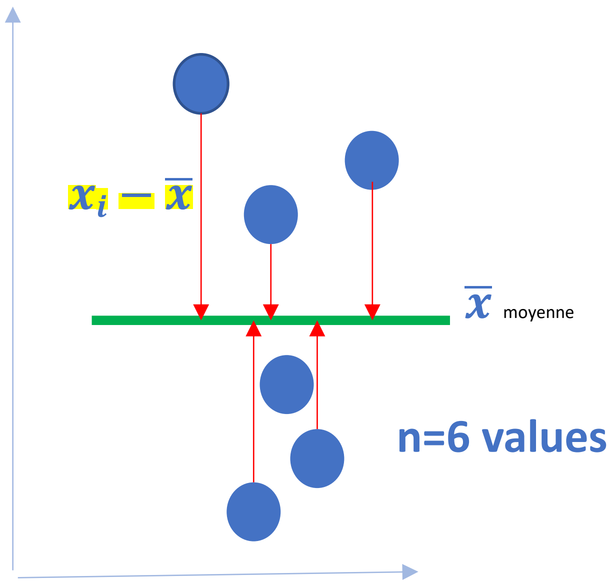

Which test for independent samples?
ONE categorical variable (H/F) & ONE continuous variable (numerical)
Normalité des données?
Shapiro, Q-Q plots
Yes
Parametric test
Variance Homogeneity
Bartlett, levene, F-test

Variance= S^2/σ^2

- Variance measures the degree of dispersion of a data set around the mean
- Arithmetic mean of squared deviations from the mean! ☹

→ square unit

$$S^2 = \sum_{i=1}^{n} \frac{(x_i - \overline{x})^2}{n-1}$$



Standard Deviation=S/ σ

$$S = \sqrt{S^2}$$

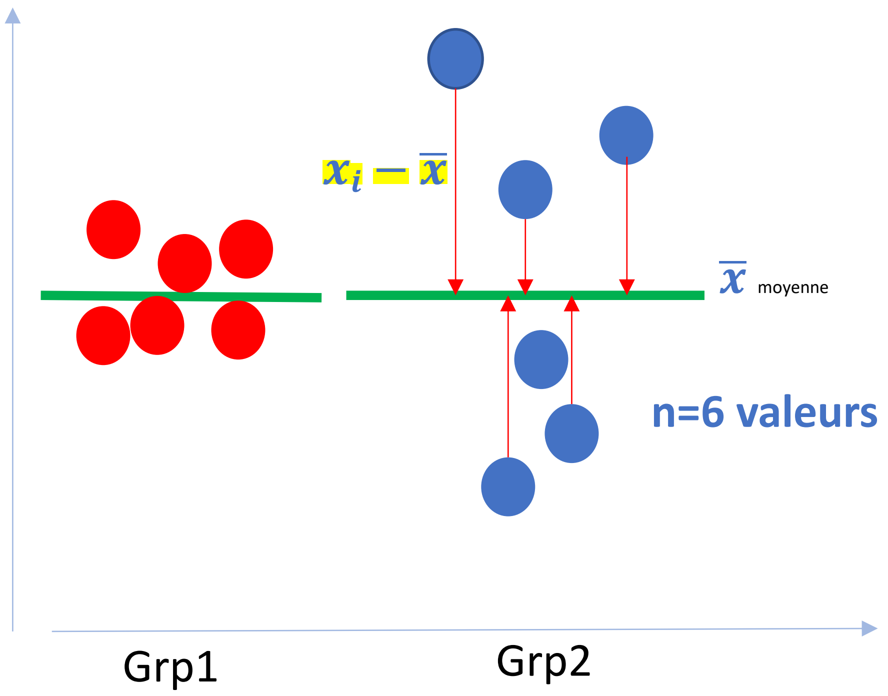
The advantage of the standard deviation : expressed in the same unit as the data series

$$S^2 = \sum_{i=1}^{n} \frac{(x_i - \overline{x})^2}{n-1} = \frac{Sum\ of\ Squares\ (SS)}{n-1}$$

SS will be greater in the sample….??

Results of test using variance :

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
groupe	3	13.03	4.343	0.211	0.887
Residuals	14	288.75	20.625		



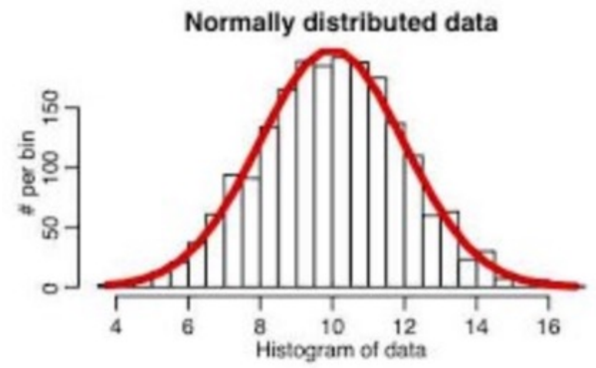
- ***Sum of Squares*** **(= SS, Sum Sq) in your results!**

→ Numerator of variance!!

- ***Mean Square (= Mean Sq= VARIANCE formula!!!)***

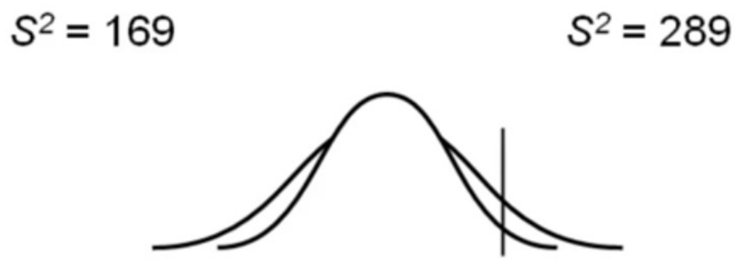
Requirement for parametric test… check-list!

- Check normality of data: Shapiro Test & QQ-plots!!
- Shapiro: H0 is «data follow normal distribution»



- Check variance Homogeneity : F-test (2 groups), Bartlett’s & Levene’s tests

H0: « No difference »

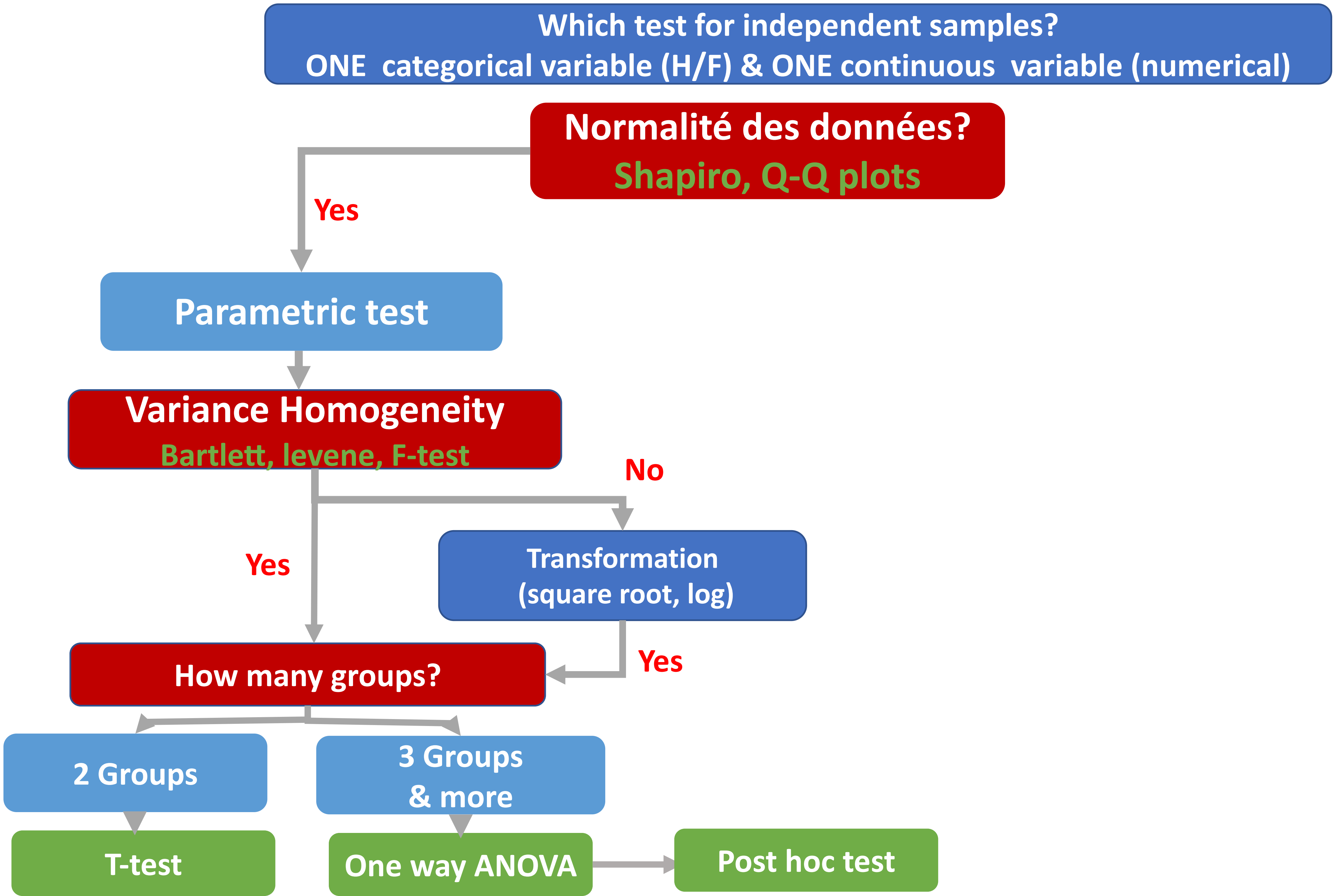


Parametric Tests

Follow a known distribution (Normal distribution) → **Position** parameters
Dispersion parameters

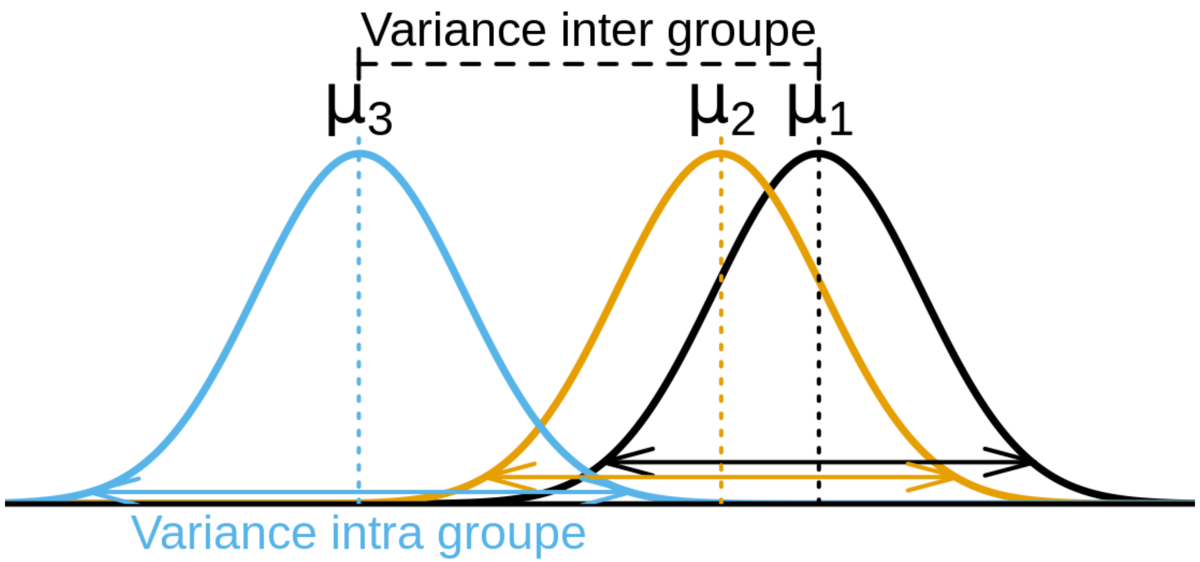
Conditions are required (variance homogeneity)

- **T-test (paired or unpaired):** Compare of the means from 2 sample groups for one variable
- **One way Anova** (variance analysis) : compare the means of three or more sample groups for one variable


Which test for independent samples?
ONE categorical variable (H/F) & ONE continuous variable (numerical)
Normalité des données?
Shapiro, Q-Q plots
Yes
Parametric test
Variance Homogeneity
Bartlett, levene, F-test
No
Yes
Transformation
(square root, log)
Yes
How many groups?
2 Groups
3 Groups
& more
T-test
One way ANOVA
Post hoc test

ANOVA: ANalysis Of VAriance (One way Anova= Univariate) (3 groups at least)

- Compare the variance of the group means to that within groups (i.e. intra-group variance) for a single explanatory variable (qualitative)



ANOVA: ANalysis Of VAriance (One way Anova= Univariate)

- ***Postulate*** = The **VARIATIONS** observed between the **MEANS** of the different groups (AT LEAST 3) are so small that they are easily explained by chance!!!

- **Evaluation : Compare the variance of the group means to that within groups (i.e. intra-group variance)**

- **ANOVA → variations through the Variance quantity**

Factor effect!

- **Statistic F** = $\frac{Inter-group\ Variance}{Intra-group\ Variance}$

Chance /fluctuation

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
groupe	3	13.03	4.343	0.211	0.887
Residuals	14	288.75	20.625		

Idea :

if the factor really has an effect, the part of the variations that can be attributed to it = Inter-group variance will be significantly higher than the part of the variations that cannot be attributed to it = Intra-group variance!

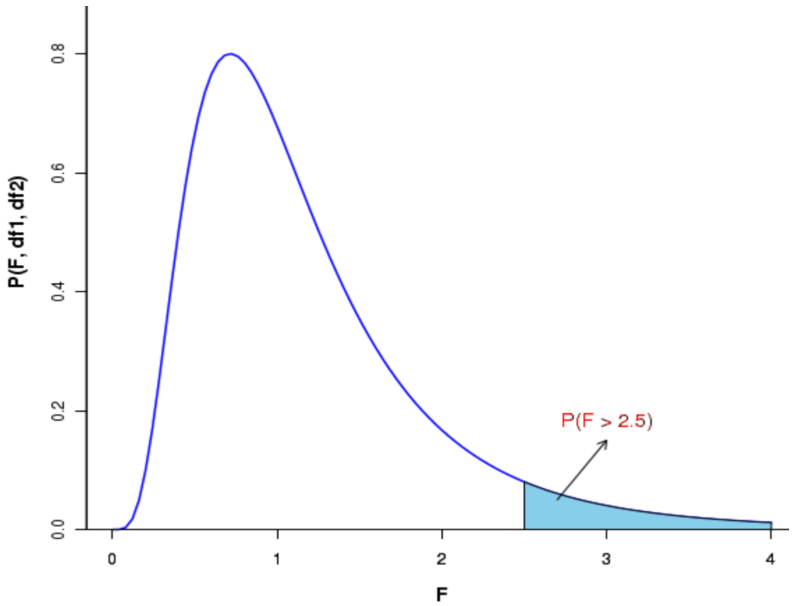
Statistic F Follows a so-called **Fisher-Snedecor** law:

= **Distribution F used for test of variances,** distribution of variances not being normal

- Relation of an observed value of F with the a priori probability of encountering such a value (> or =) by chance!
- → probability given by the law = p-value!
- !

Denominator S^2 Numerator S^2 S^2

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
groupe	3	13.03	4.343	0.211	0.887
Residuals	14	288.75	20.625		



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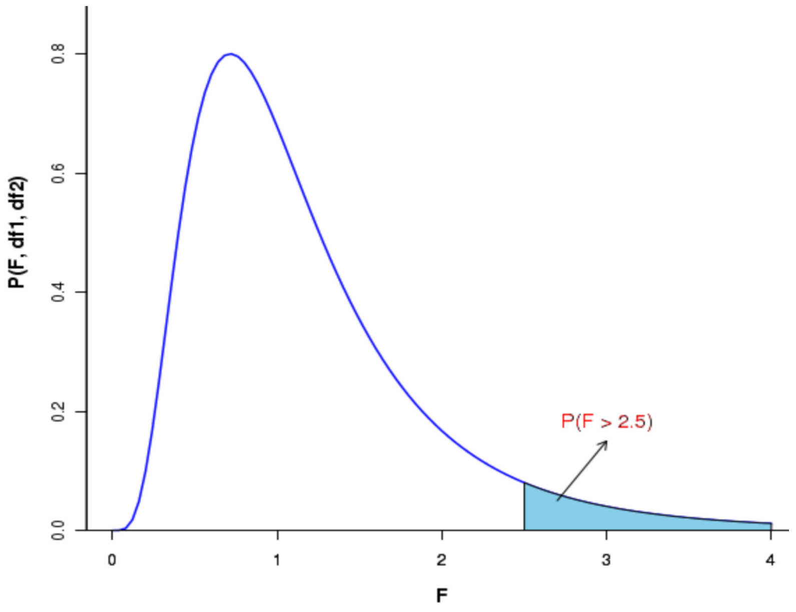
- → probability given by the law = p-value!
- !



Denominator S^2 Numerator S^2 S^2

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
groupe	3	13.03	4.343	0.211	0.887
Residuals	14	288.75	20.625		

variances		ddl	F
entre k groupes	v_k	k-1	v_k / v_r
résiduelle	v_r	N - k	

Degré de liberté

- Two-ways ANOVA : Influences of TWO qualitative variables on ONE quantitative variable

Exple: Influence of soil type and degree of humidity (ordinal variable) on plant yield

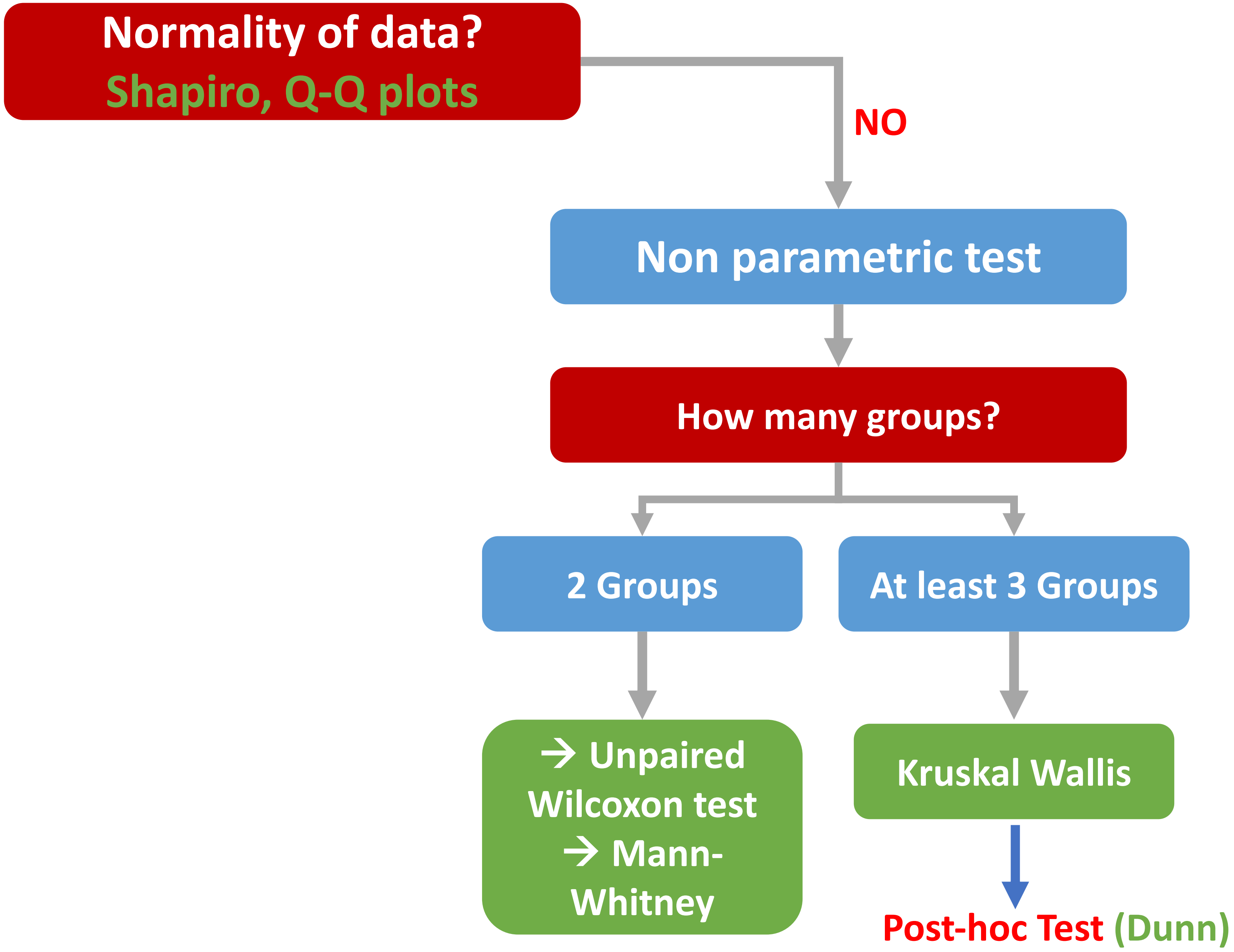
Non-parametric tests

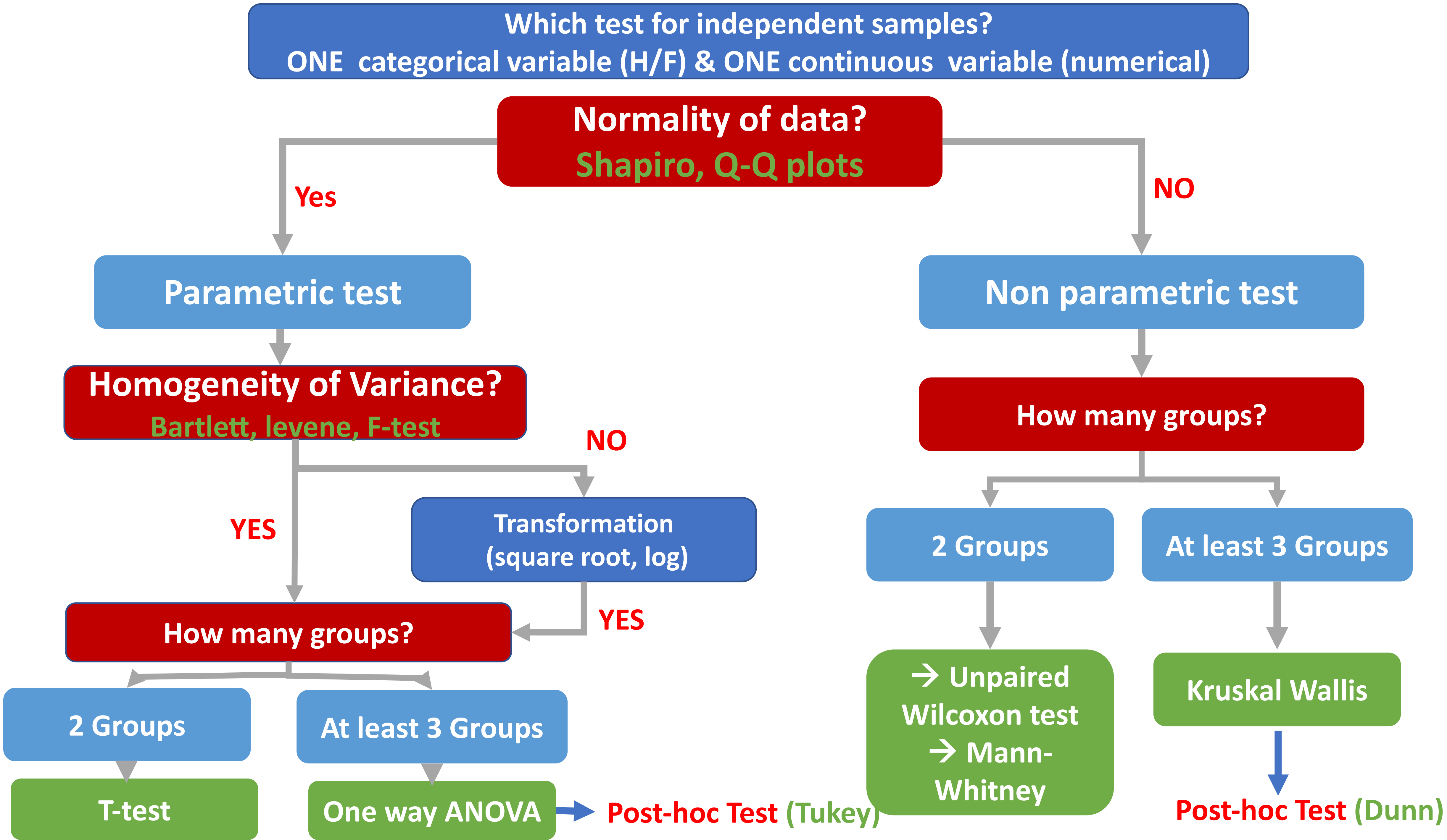
No assumptions are made for the distribution of data:
Distribution-free tests, they are alternative to parametric tests

- **Wilcoxon Rank test : samples are paired/unpaired, 2 sample groups**
- **Mann-Withney test: Independent samples, 2 sample groups**
- **Kruskal wallis test : Independant samples, Three or more groups**

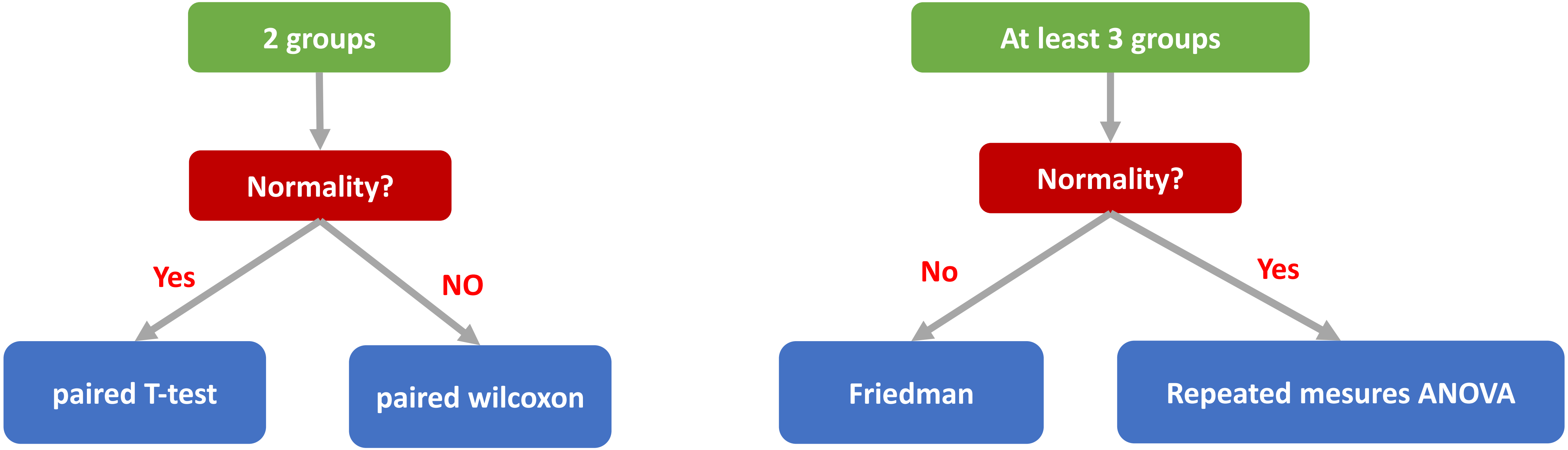
→ Based on the average ranks: we classify the values, we replace by a position (1,2 etc), Compares the average of the ranks between the groups

Which test for independent samples?
ONE categorical variable (H/F) & ONE continuous variable (numerical)




Which test for independent samples?
ONE categorical variable (H/F) & ONE continuous variable (numerical)
Normality of data?
Shapiro, Q-Q plots
Yes
NO
Parametric test
Non parametric test
Homogeneity of Variance?
Bartlett, levene, F-test
NO
YES
Transformation
(square root, log)
YES
How many groups?
2 Groups
At least 3 Groups
T-test
One way ANOVA
Post-hoc Test (Tukey)
How many groups?
2 Groups
At least 3 Groups
→ Unpaired Wilcoxon test
→ Mann-Whitney
Kruskal Wallis
Post-hoc Test (Dunn)

Repeated measurements – paired samples
Exple= time series, Before-After Treatment…



Post-hoc Test

Statistical tests with at least 3 groups!
After ANOVA, Kruskal-wallis

→ The result of an ANOVA test is an Overall p-value

Exple: You are comparing the effect of 3 soil types (A,B,C) on plant growth
ANOVA returns a p-value of 0.03
It does not tell you which pair of groups are significantly differents!!!!

→ Post-hoc Test! Multiple comparisons (eg: Gp A vs. Grp. B; GrpB vs. Grp C; Grp C vs. Grp A!)

- Parametric Post-hoc test (ANOVA) → **Tukey Test**
- Non-parametric Post-hoc test (Krukal wallis) → **Dunn Test**

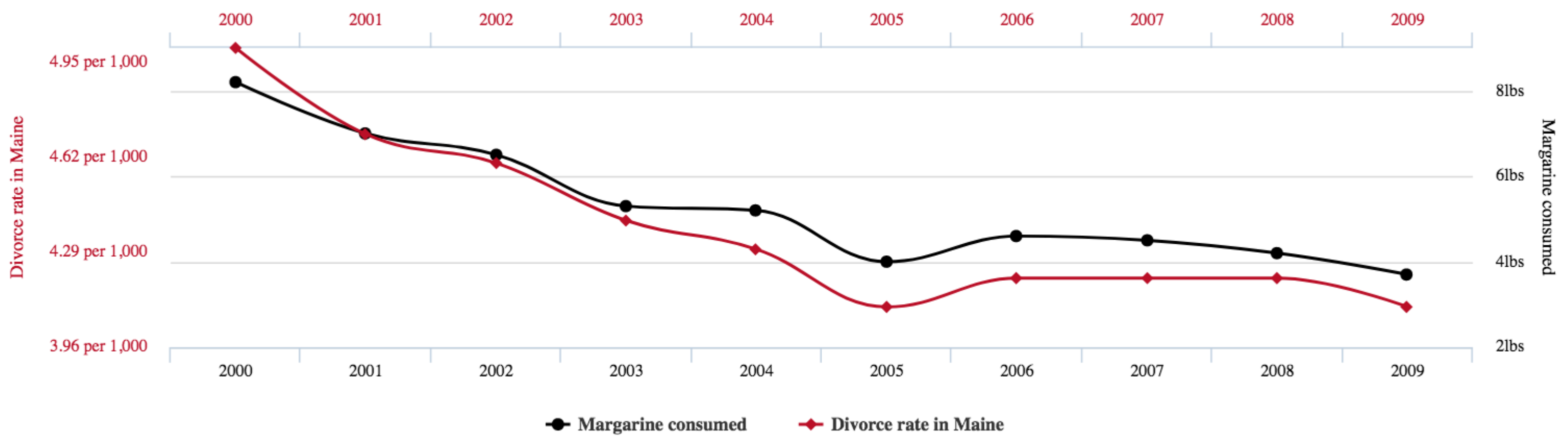
Linear Regression & Correlation (Bivariate analysis)

Objective : Analyze the **link** that may exist between **two variables** (here: quantitatives)
(Two qualitative variables -> Khi2 test)

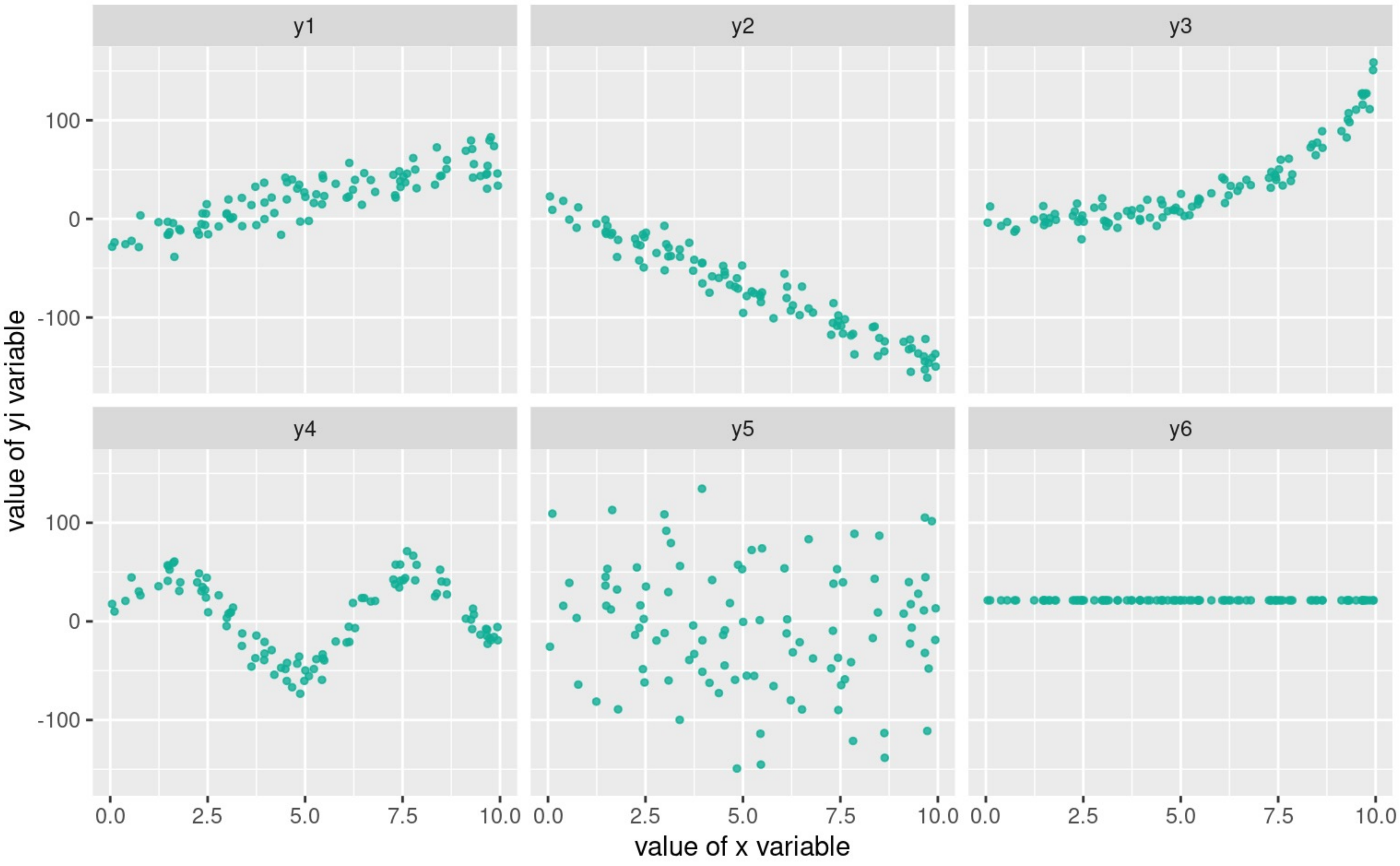
Link/relationship/dependence between the variables

→ The values of two variables **do not evolve independently** but on the contrary, present a certain form, a certain regularity

→ Intensity of the association does not indicate causality …



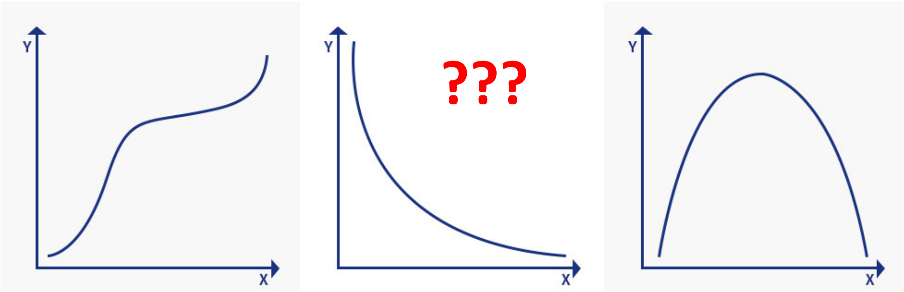
What are the relationship between the variables in each graph?

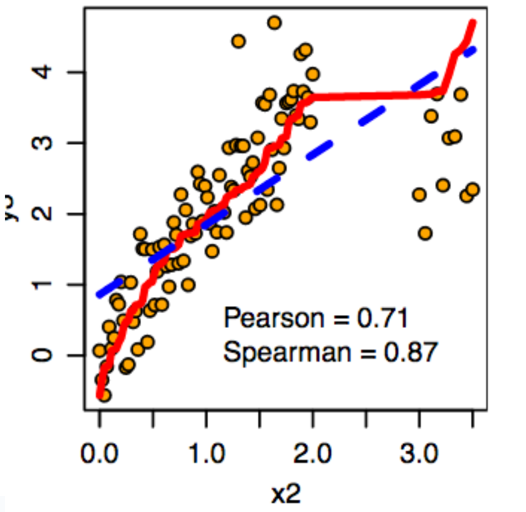


Association: Correlation Coefficient r
Intensity & Direction of the association between two variables

- **Strict Linear Relationship : Pearson** (**r**, parametric)
- **Monotonous relationship : Spearman** (**Rho**, non-parametric, rank-based)

  Kendall (**Tau**, non-parametric), Alternative to Spearman (small sampling)

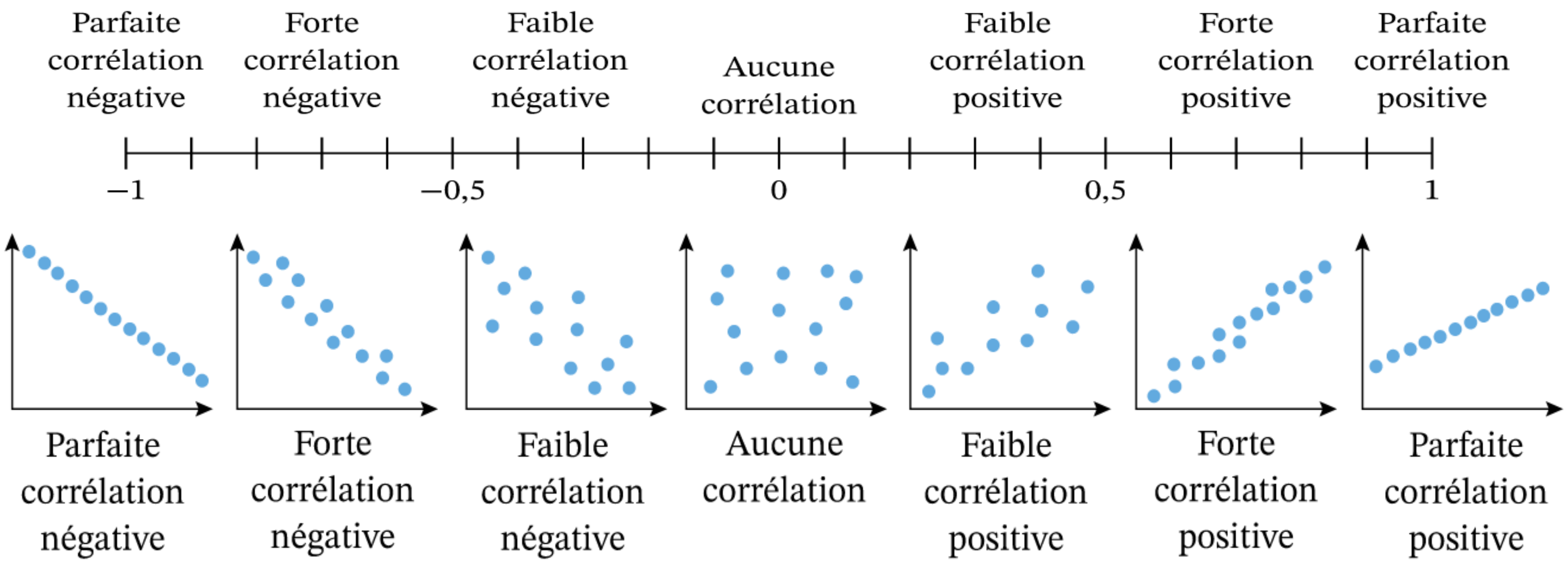




Coefficient r range between -1 et 1

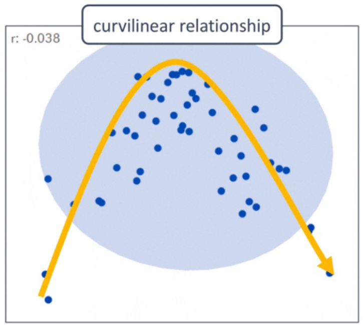
- **Positive correlation** : The values of both variables tend to increase together
- **Negative correlation** : The values of one variable tend to increase and the values of the other variable decrease
- **Zero** : no **LINEAR** association **(Pearson)**

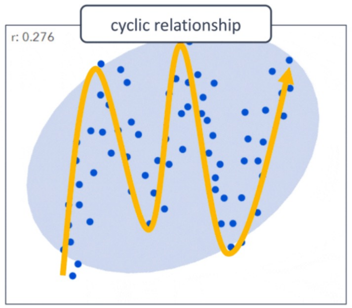
For information!!!



Because inspecting your results is never useless...

- r close to Zero: no association??

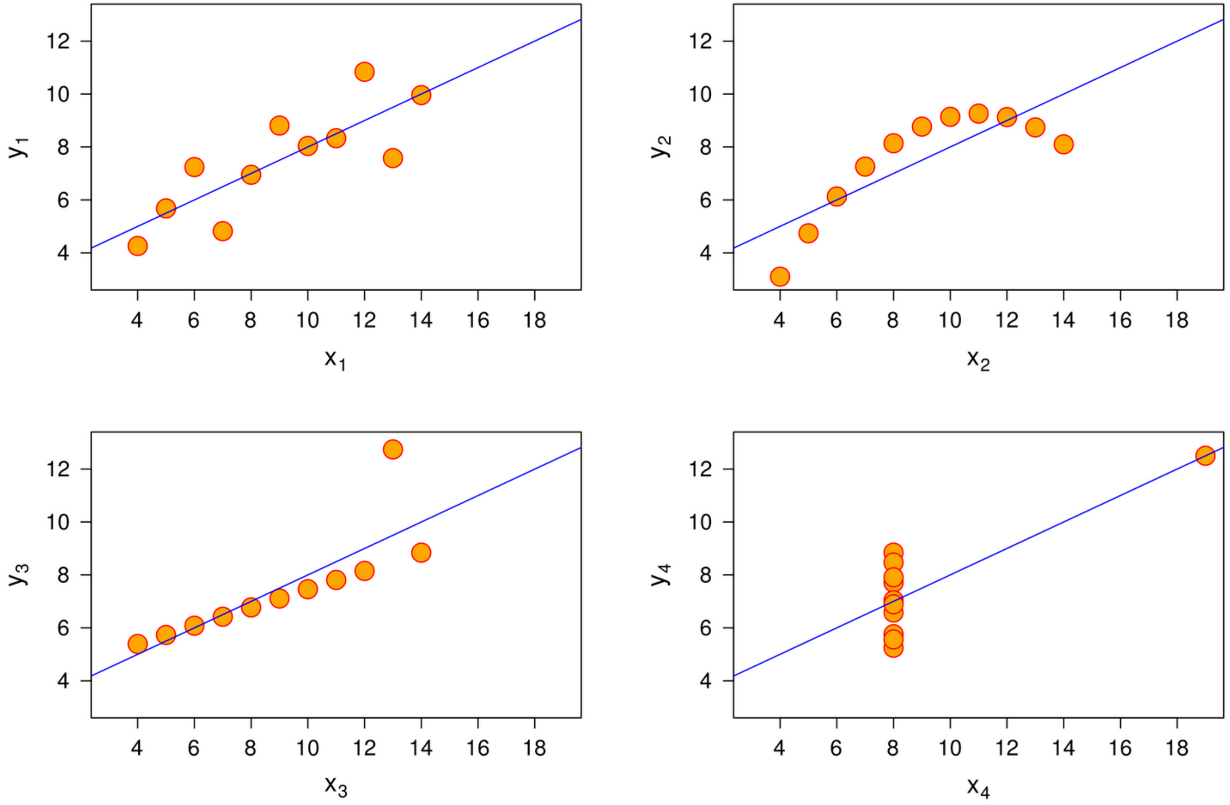




Really not useless : Anscombe…

- **4 dataset with same descriptive stats**

Propriété	Valeur
Moyenne des *x*	9,0
Variance des *x*	10,0
Moyenne des *y*	7,5
Variance des *y*	3,75



r =?

☺

0.3?
0.5?
0.8?

- **Distribution law of r under the Ho hypothesis: No statistical link between X and Y**

→ Access to p-values

Simple Linear Regression

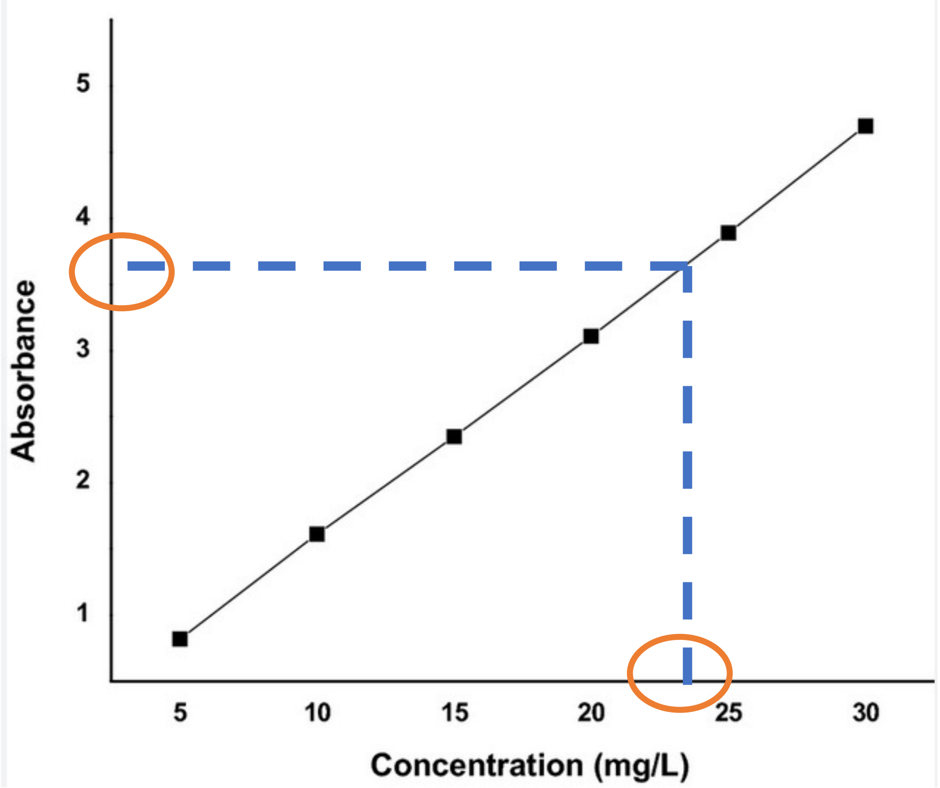
- Only for quantitative variables

- Plot the scatter plot Is there a relationship?
- Is it linear?
- What orientation (positive, negative)?
- If the association is **linear** → Make a **regression**

Requierement

- Normal distribution
- Variance homogeneity

Your favorite linear regression… calibration curve!!!

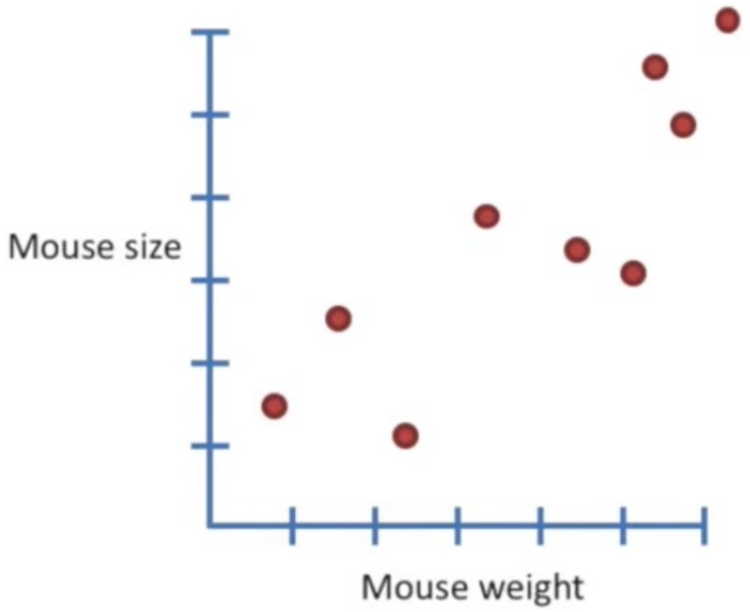


Explain and predict!

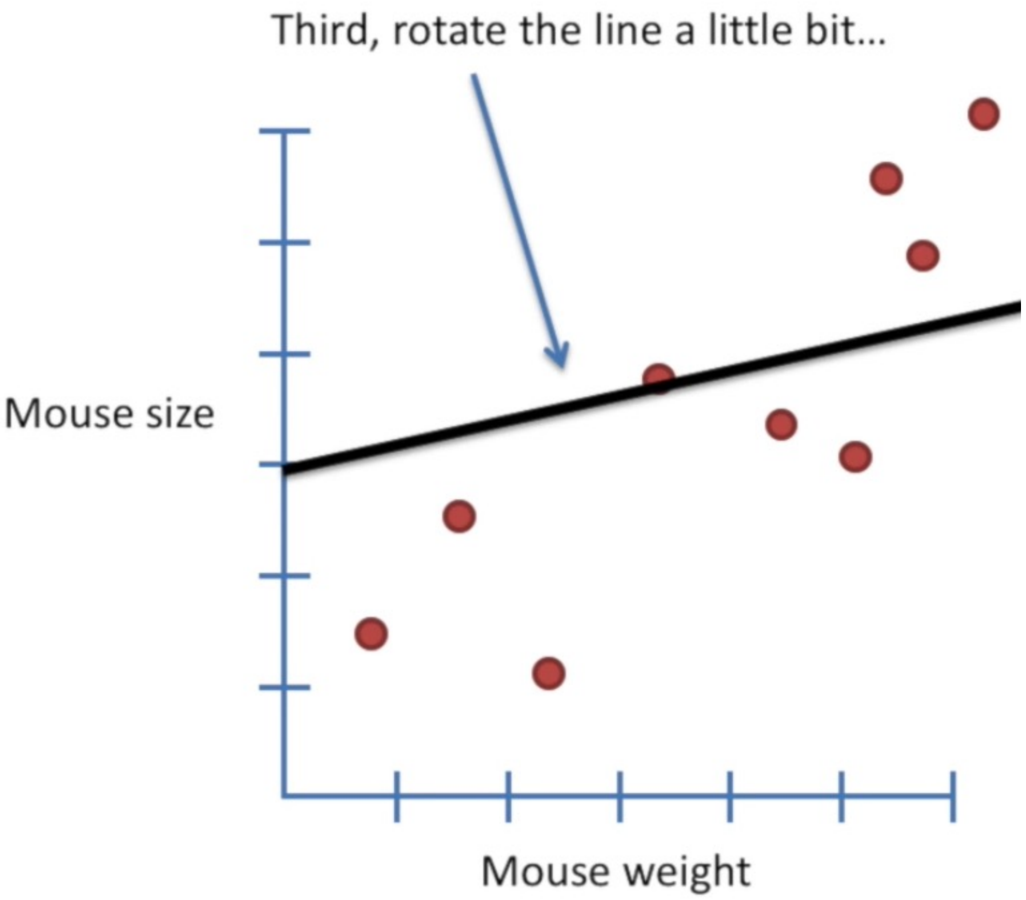
Models a linear type relationship (Y=aX+b)

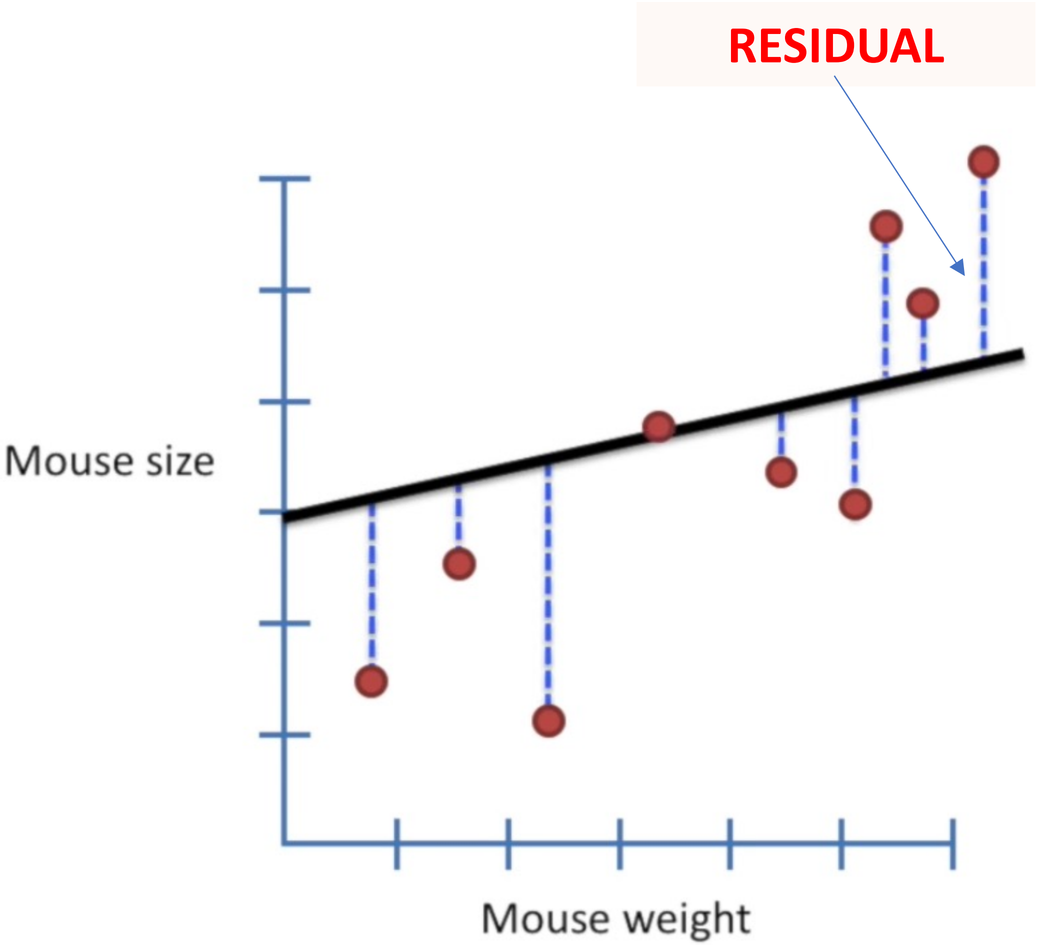
Model seeking to establish a linear relationship between a variable, called explained/dependent (Y), and another called explanatory/independent (X)

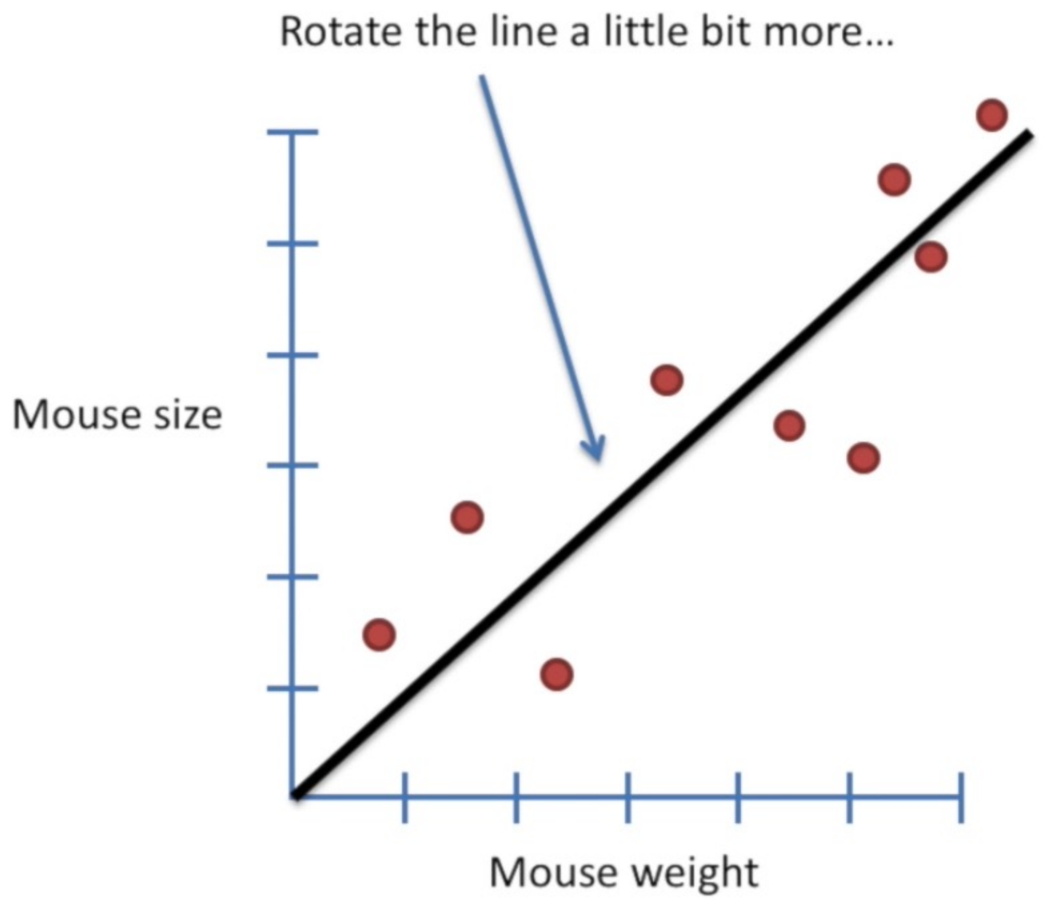
Can mouse Weight predict Size correctly? (R^2)
Relationship is due to chance? (p-value)

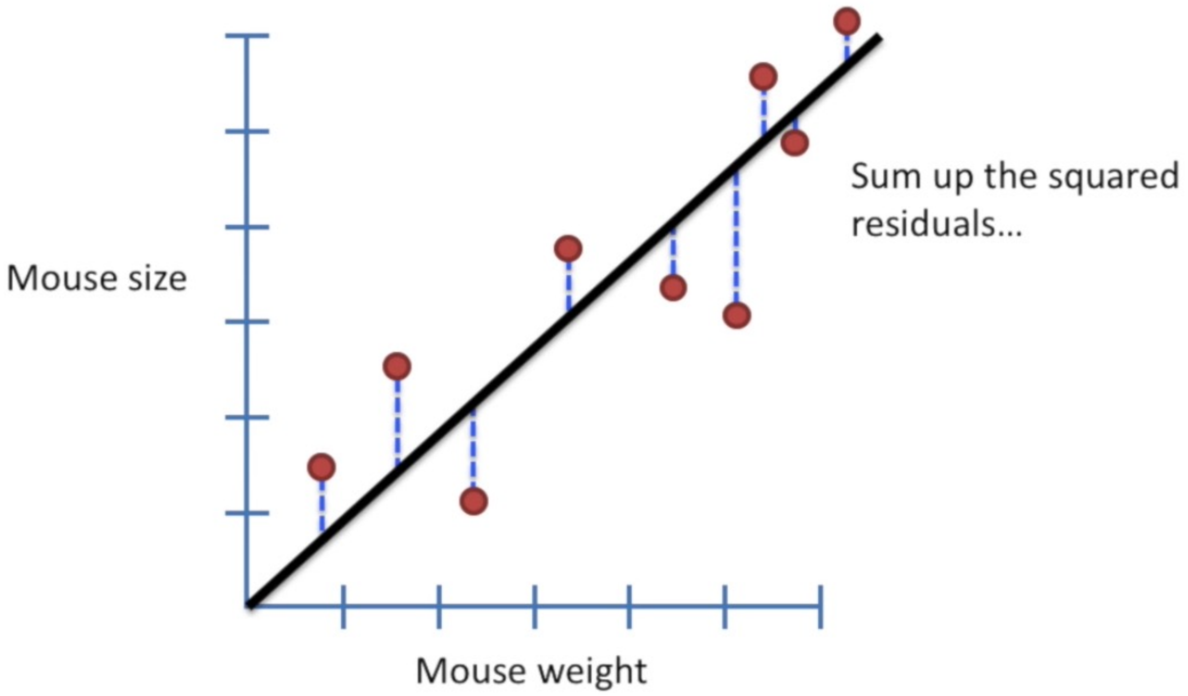


Least square method



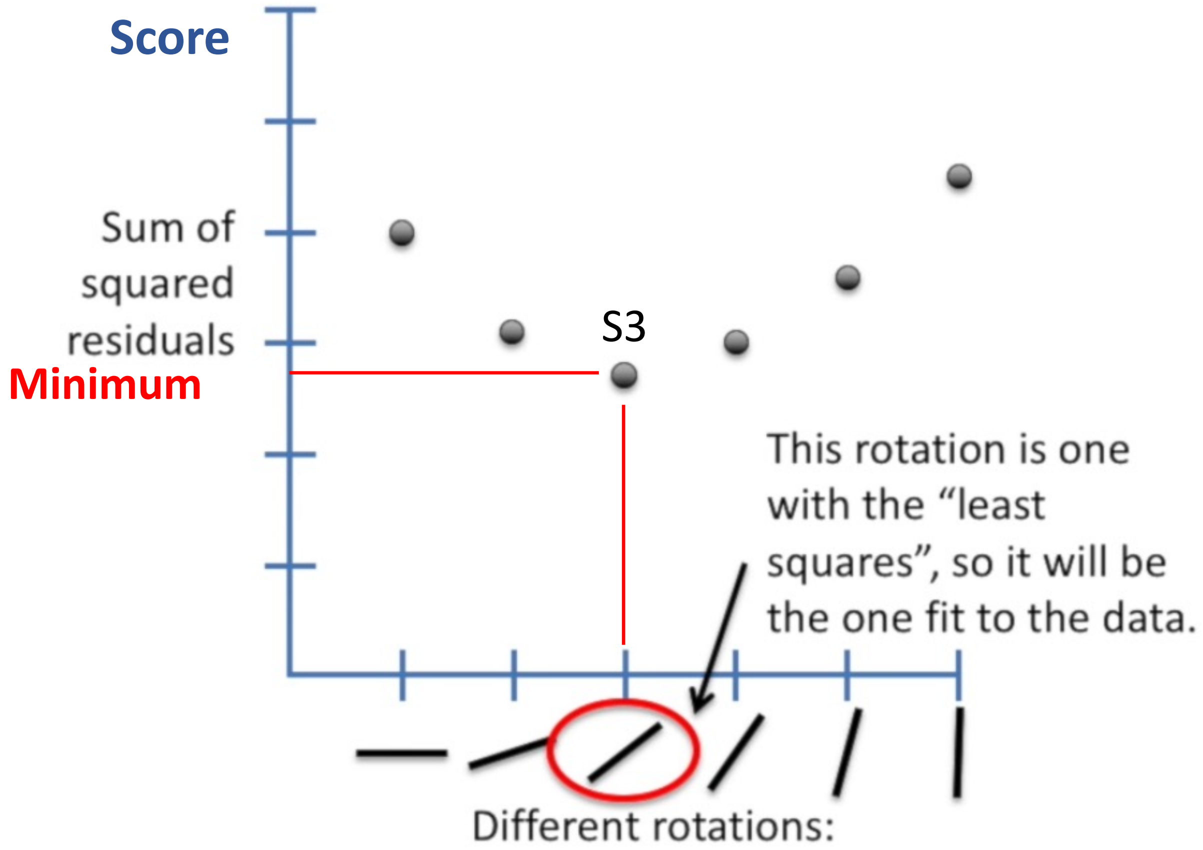




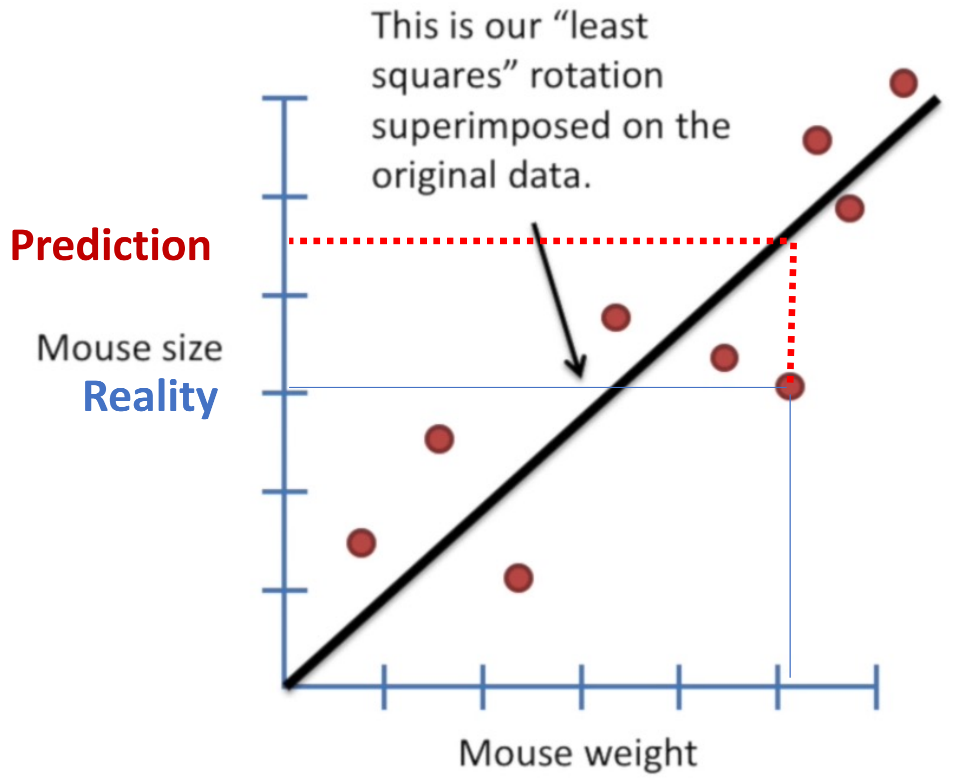


Again & again, recalculate

Resume : Sums of squared residuals for each rotation



Best rotation (=line position), the one which minimize the score of Sums of squared residuals !!!!



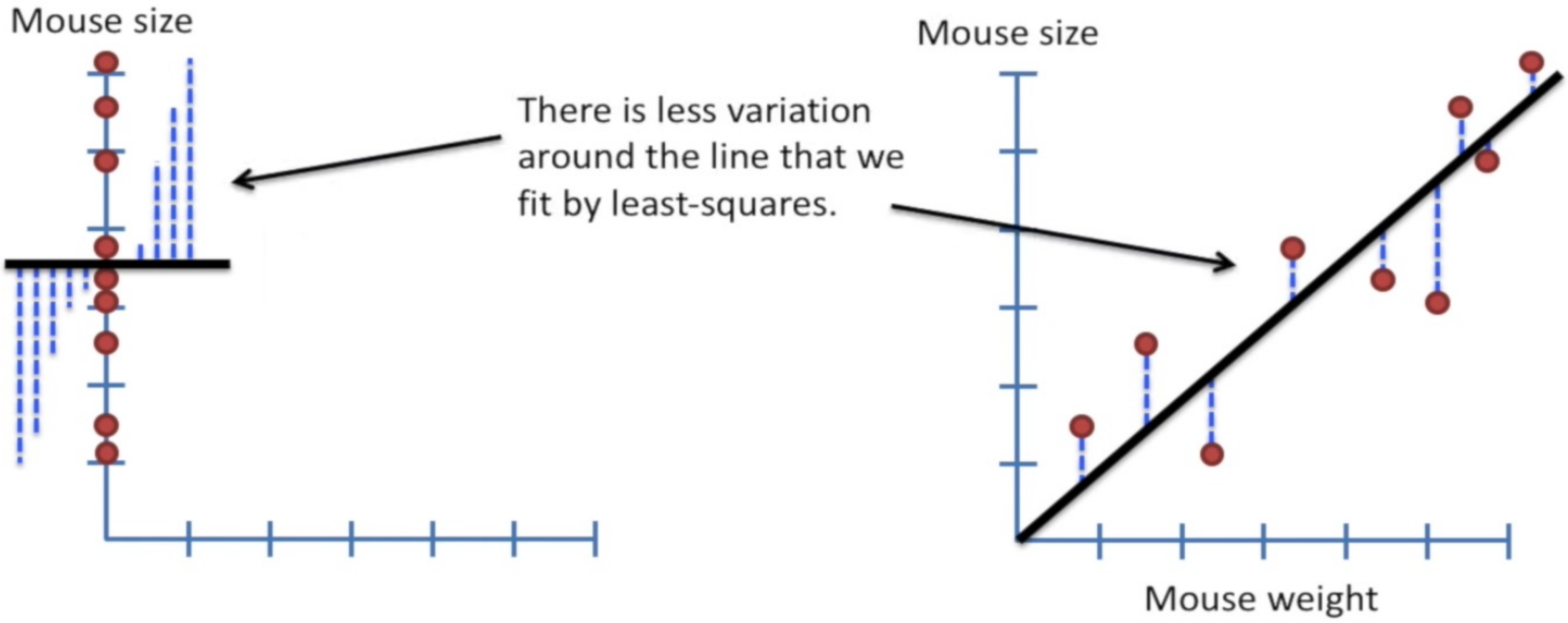
$$y = 0.1 + \boxed{0.78x}$$

Dependence to « Mouse weight »

Coefficient R^2 = prediction quality

how good is the model to predict Mouse size taking into account Mouse weight!!

R^2 : Determination Coefficient



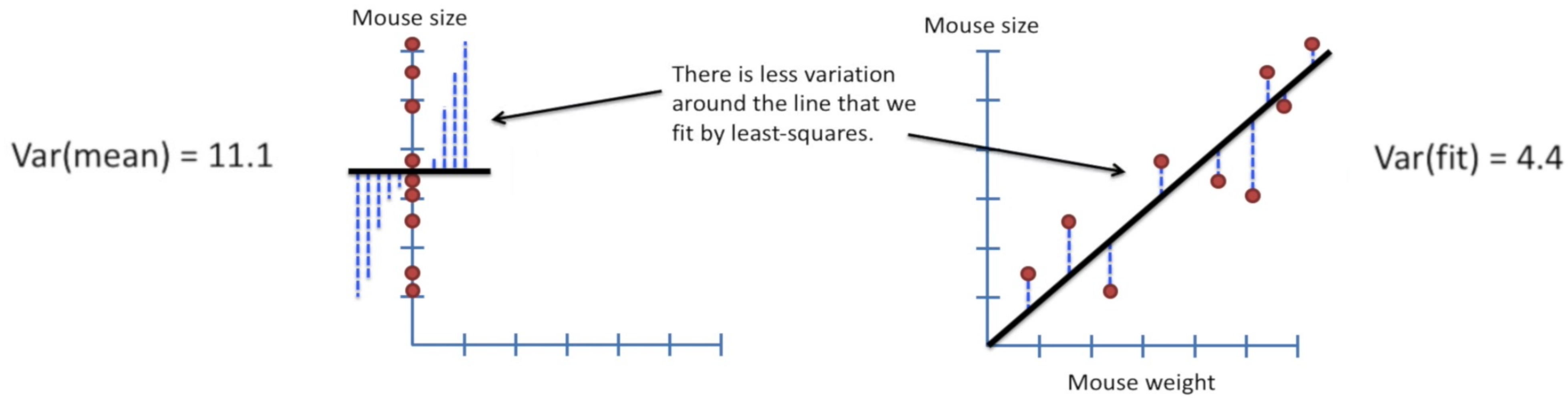
$$\text{Var(mean)} = \frac{\text{SS(mean)}}{n}$$

$$\text{Var(fit)} = \frac{(\text{data - line})^2}{n}$$

- **Taking into account « weight », less variations?? (SSfit < SSMean)!**

R^2 = % variation of the response variable explained by a linear model (weight variable)

$$R^2 = \frac{Var(mean) - Var(fit)}{Var(mean)}$$



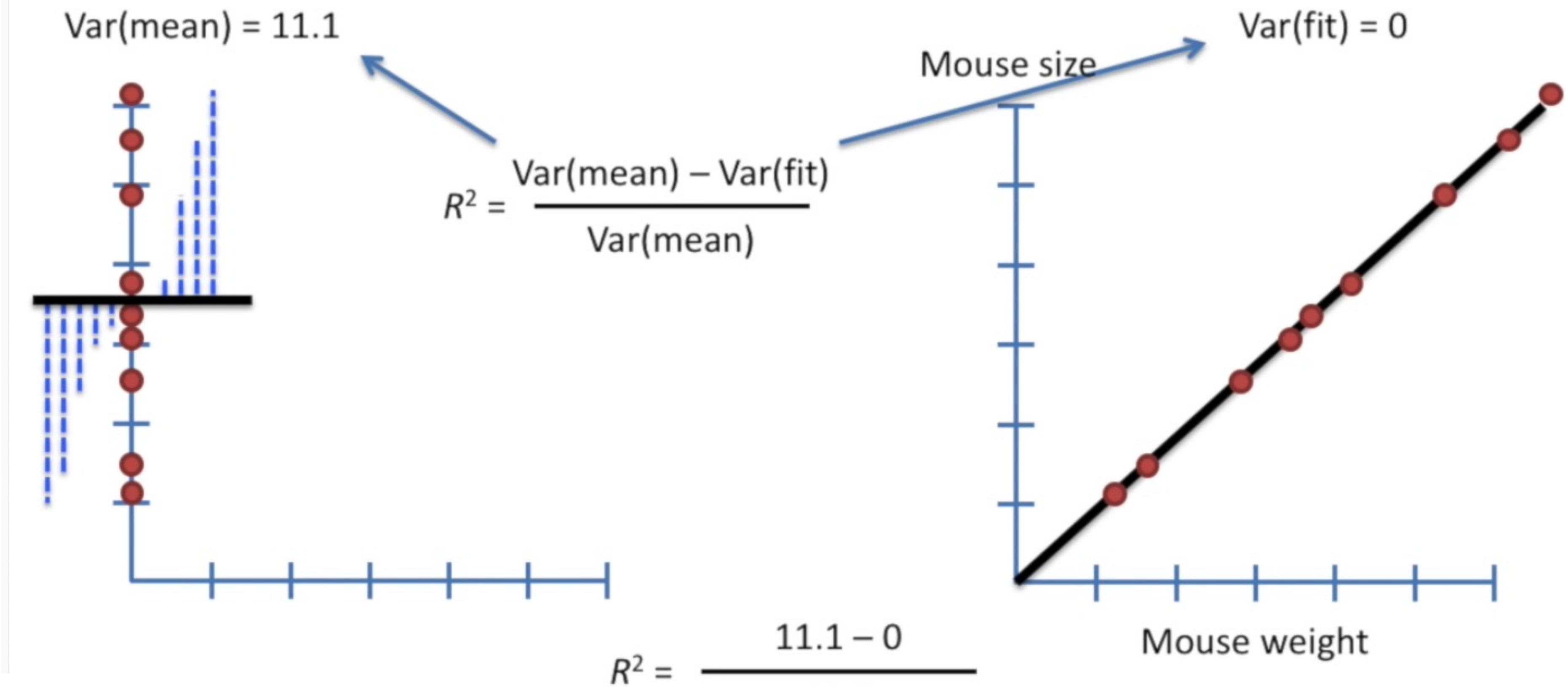
$$R^2 = \frac{11.1 - 4.4}{11.1} = 0.6 = 60\%$$

$$R^2 = \frac{Variation\ expliquée}{Variation\ totale}$$

→ **The established model explains 60% of the variability/variance of the "Mouse size"**

→ **R^2 between 0 and 1**

TO be sure …



$$R^2 = \frac{11.1 - 0}{11.1}$$

$R^2 = 1 = 100\%$

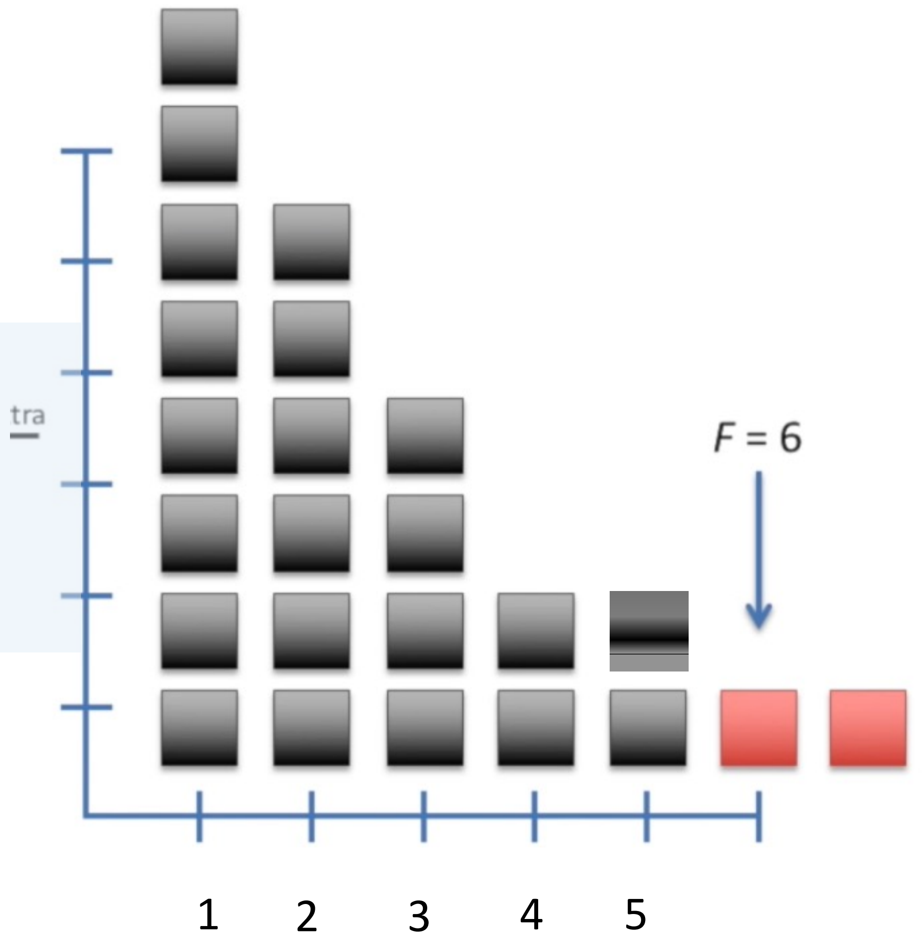
R^2 & significance?

- **Need a p-value…**
- **Variance … so p-value is given by the ratio F & distribution F**

$$F = \frac{\text{The variation in mouse size explained by weight}}{\text{The variation in mouse size not explained by weight}}$$

The p-value is provided by distribution F

- Random subsets from data
- Calculation of F for these subsets
- Calculation of F for the initial data (F=6)
- **G**enerates the distribution F



$$\text{p-value} = P_{F6} + P_{equal} + P_{more\ extreme}$$

Relation between r & R^2

Correlation coefficent of Pearson r can be linked to linear regression R^2
Its square is the explained variance by the regression (**R^2**)

r =0.5 -> R^2 = 0.25 -> 25% of the Y variance explained by X variable… ☹

Multiple Testing Issue: increasing the risk…

Test is based on probabilities, so there is always a risk of drawing the wrong conclusion!

→ No hypothesis test is 100% reliable

Performing hypothesis testing:

- You have two hypotheses :
- H0: Null hypothesis = the reference hypothesis : No difference
- H1: Alternative hypothesis: There is a difference

- You encounter: Type I error : α = Risk alpha

α = 0.05 Is the probability (significance threshold) to incorrectly reject H0!
In other words, an acceptable chance of a false positive!!

Differential abundance : Multiple testing!!

ONE TEST : $P_{\text{False Positive}} = P_{\text{error}} = \underline{\alpha} = 0.05$

Complementary Prob $P_{\text{no\_error}} = 1 - \underline{\alpha} = 0.95$

TWO TEST without making error : $P_{\text{no\_error in two tests}} = (1 - \underline{\alpha}) * (1 - \underline{\alpha}) = (1 - \underline{\alpha})^2$

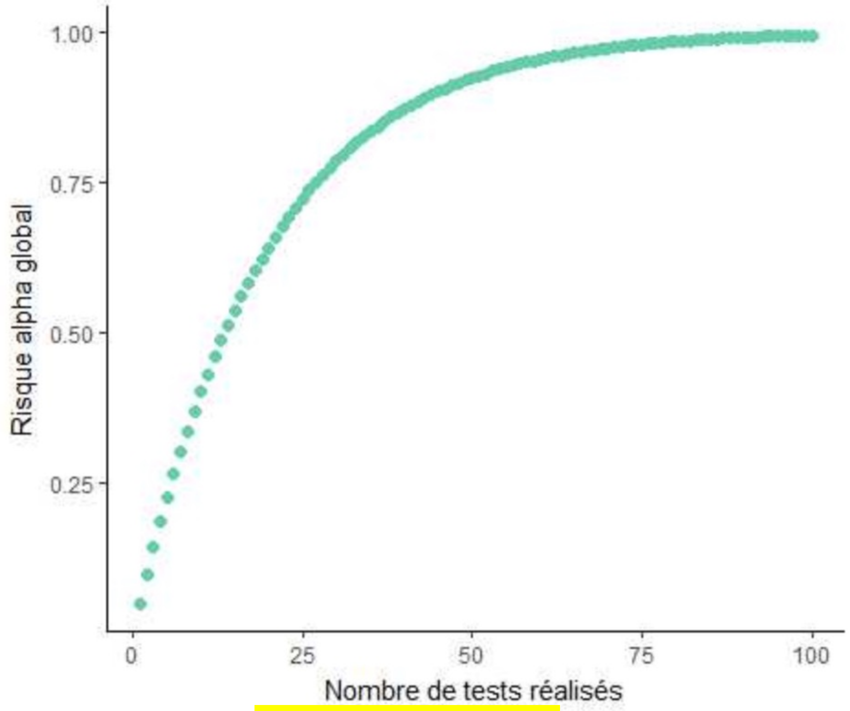
Complementary Prob $P_{\text{at\_least\_ONE\_error in two tests}} = 1 - (1 - \underline{\alpha})^2$

Generalization to n TESTS $P_{\text{at\_least\_ONE\_error in\_n\_tests}} = 1 - (1 - \underline{\alpha})^n$

It's called the global $\underline{\alpha}$ risk

What does it means…

- You test ONE ASVs (n=1) for differential abundance: $1-(1-\underline{\alpha})^n = 1-(1-0.5)^1 = 0.05$
- You test 3 ASVs (n=3): $1-(1-0.05)^3= 0.14$
- You test 100 ASVs (n=100): $1-(1-0.05)^{100}= 0.9941$



The global risk $\underline{\alpha}$ reach 0.9941=99.41%!!!!

→ 99% to wrongly reject the H0 at least One times

Need to ajusted this phenomen by using p-value adjusted!

FDR : False Discovery Rate : Benjamini-Hochker

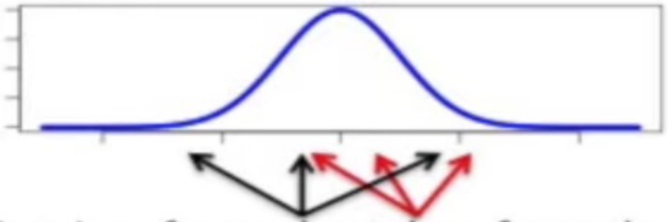
The idea : Discard bad data that looks good!!!

Benjamini-hocherk **adjusts p-values**
to limit the number of **false positives**
that are reported as **significant** (pvalue < 0.05)

Adjusts p-values
means that it makes them **larger!**

Using FDR cutoff < 0.05
means less than 5% of the significant results will be false positives

Mathematical approach FDR-Benjamini-Hochker



10 pairs of samples taken from the same distribution. (i.e. 10 genes that were not effected by the drug).

p-values:									
0.91	0.11	0.71	0.31	0.51	0.41	0.61	0.21	0.81	0.01

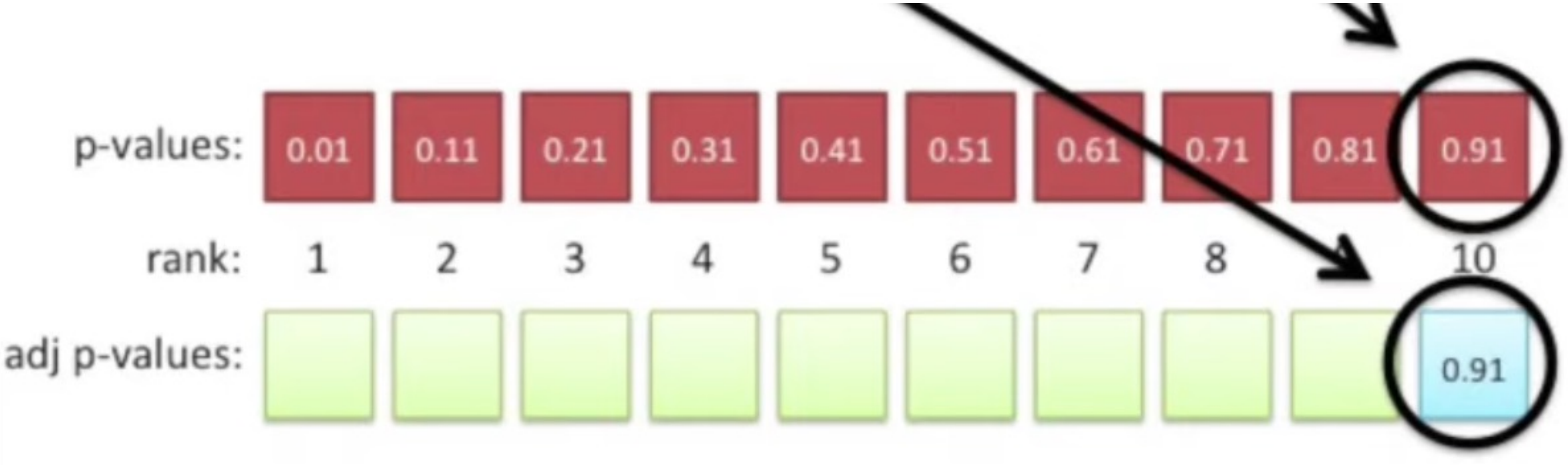
Notice that one of the p-values is a false positive (that is to say, less than 0.05)

p-values									
0.01	0.11	0.21	0.31	0.41	0.51	0.61	0.71	0.81	0.91

smallest ⟷ largest

1- Ranking pvalue

Prepare space for adjusted p-value



2- Largest adjusted pvalue and larger pvalue are same

Next adjusted pvalue ....

p-values:	0.01	0.11	0.21	0.31	0.41	0.51	0.61	0.71	0.81	0.91
rank:	1	2	3	4	5	6	7	8	9	10
adj p-values:										0.91

The smallest of the two options

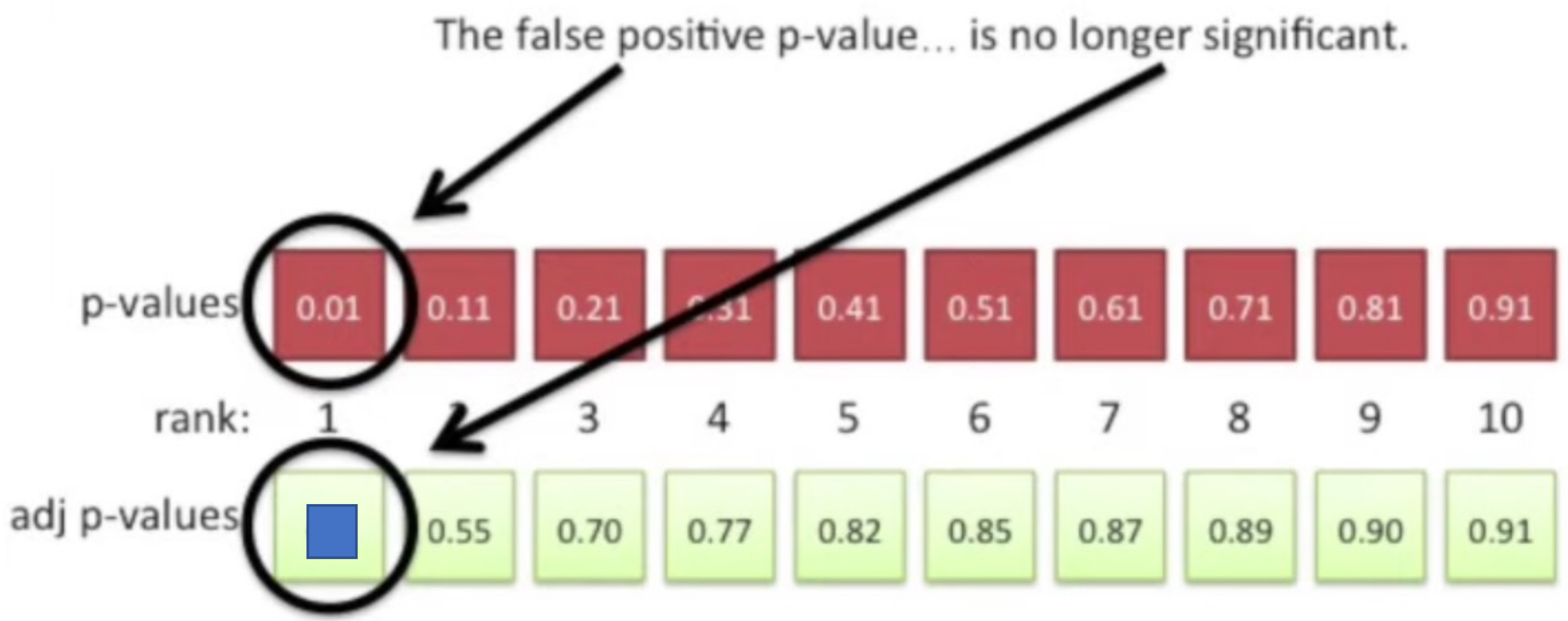
b: the current p-value $* \left(\frac{\text{total \# of p-values}}{\text{p-value rank}} \right)$

a: The previous adjusted p-value = 0.91

b: $0.81 * \left(\frac{10}{9} \right) = 0.90$

p-values:	0.01	0.11	0.21	0.31	0.41	0.51	0.61	0.71	0.81	0.91
rank:	1	2	3	4	5	6	7	8	9	10
adj p-values:									0.90	0.91

Finally...


The false positive p-value... is no longer significant.
p-values
0.01
0.11
0.21
0.41
0.51
0.61
0.71
0.81
0.91
rank:
1
3
4
5
6
7
8
9
10
adj p-values
0.55
0.70
0.77
0.82
0.85
0.87
0.89
0.90
0.91