



Faculty of Health Sciences



# Day 2: Hypothesis testing, tests for continuous responses, multiple testing

Paul Blanche

Section of Biostatistics, University of Copenhagen

February 18, 2026



# Outline/Intended Learning Outcome (ILOs)

## Hypothesis testing

ILO: to describe the principles and logic of hypothesis testing

## One and two sample tests for continuous responses: t-test

ILO: to identify when, how and why to use a t-test

ILO: to define a p-value and contrast its use with that of a confidence interval

## Power and Sample size calculation

ILO: to identify why and how to make power and sample size calculations

ILO: to analyse their strengths and limitations

## Multiple testing

ILO: to describe the multiple testing problem and employ basic remedies

## Nonparametric test: Wilcoxon

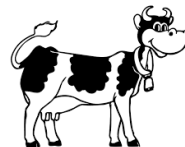
ILO: to contrast pros and cons of Wilcoxon vs t-test



# Case: cow milk data

► **Research question:**

Should cows be fed with **Barley** or **Lupin**, to produce the best milk?



► **Outcome:**

protein level of the milk (%) at 12 weeks after calving.



**Statistical aim:** provide a yes/no answer about the **population** supported by the observed data (**sample**) while controlling the risks of a “false finding”, via a **Hypothesis test**.<sup>1</sup>

---

<sup>1</sup>**Note:** important complementary information is given by the confidence interval of the effect size. It will be emphasized shortly.

# Research question and Null hypothesis

- ▶ A hypothesis test aims to answer a very **precise & specific** research question.

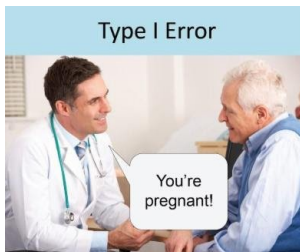
**Case:** Is there a **difference in (population) mean** level of protein between cows fed with lupin and barley, at 12 weeks?

- ▶ The **null hypothesis**  $\mathcal{H}_0$  of the test should reflect it and state the **opposite of what you aim to prove**.
  - ▶ **Scientific hypothesis:** there is a difference.
  - ▶ **Null hypothesis:** there is **no** difference.

Choosing the opposite is important to appropriately control the **risk of wrong conclusion**.



# Hypothesis testing and risks of false conclusions



## Case:

- ▶ **Type-I error:** conclude a difference although it does not exist.
  - False positive finding
- ▶ **Type-II error:** do not conclude to a difference although it exists.
  - False negative finding

# Hypothesis testing and risk control

**We want** to ensure that the risk of wrongly rejecting the null hypothesis ( $\alpha$ ) is small (often 5%), i.e. **a small risk of a false scientific finding**.

**Reasoning:** the data need to be convincing enough to support the (new) research finding.

**Limitation:** it might be difficult to have enough data to support a (new) finding ( $\rightarrow$  power).



# The logic of hypothesis testing

1. **Assume** that the data have been generated in a world in which the **null hypothesis is true**.
  2. Under this assumption, **calculate how unlikely it is to obtain some results that contradict the null hypothesis** as least as much as those obtained with your data (i.e., compute the p-value).
  3. Reject the null hypothesis if this is unlikely 'enough'.
- ▶ Similar to a proof by contradiction.
  - ▶ Computation in step 2. depends on the type of observed data.



# Outline/Intended Learning Outcome (ILOs)

## Hypothesis testing

ILO: to describe the principles and logic of hypothesis testing

## One and two sample tests for continuous responses: t-test

ILO: to identify when, how and why to use a t-test

ILO: to define a p-value and contrast its use with that of a confidence interval

## Power and Sample size calculation

ILO: to identify why and how to make power and sample size calculations

ILO: to analyse their strengths and limitations

## Multiple testing

ILO: to describe the multiple testing problem and employ basic remedies

## Nonparametric test: Wilcoxon

ILO: to contrast pros and cons of Wilcoxon vs t-test





# Case: cow milk data

Data from  $n = 25$  (Barley)+27 (Lupin) cows:

```
protein  Diet
3.28 lupins
3.04 barley
3.07 barley
2.92 barley
3.29 lupins
3.18 lupins
```



	Barley	Lupin
Mean (SD):	3.43 (0.31)	3.21 (0.27)

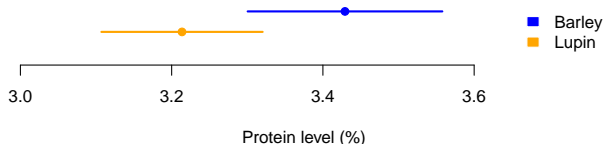
etc...

- Is the **difference** observed in the data **sample** large enough to conclude to a difference in the **population**?

# First approach: not optimal for testing for a difference

Comparison of 95% confidence intervals:

- ▶ Lupin: [3.11;3.32]
- ▶ Barley: [3.30;3.56]



We cannot conclude on the significance of the difference

(see slides lecture 1).

But the two CI can be interesting to report anyway.

# A better approach

## Compute:

- ▶ p-value for the difference in mean.
- ▶ confidence interval for the difference in mean.



## Two-sample t-test (1/2)

**Model assumptions:** (1 & 2 are important, 3 not always)

1. The two samples are **independent** (no pairing).
2. Observations from each sample are **independent**.
3. Observations are normally distributed.

To test the null hypothesis  $\mathcal{H}_0 : \mu_1 = \mu_2$ , i.e. the population means are the same in the two populations, we compute the **t-statistic**.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s.e.(\bar{x}_1 - \bar{x}_2)}$$

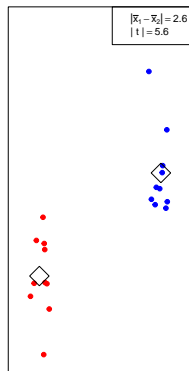
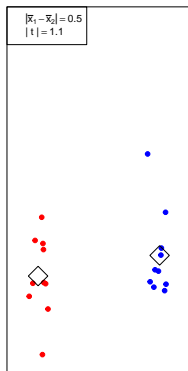
where the standard error is  $s.e.(\bar{x}_1 - \bar{x}_2) = \sqrt{s_1^2/n_1 + s_2^2/n_2}$ .

The value  $t$  quantifies how large the (sample) difference  $(\bar{x}_1 - \bar{x}_2)$  is **relative** to the amount of **information** provided by the data  $(s.e.(\bar{x}_1 - \bar{x}_2))$  and is used to compute a p-value.



## Appendix: t-statistic intuition (1/3)

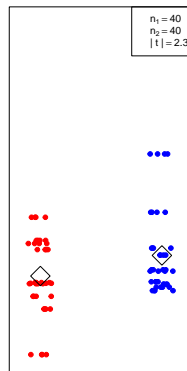
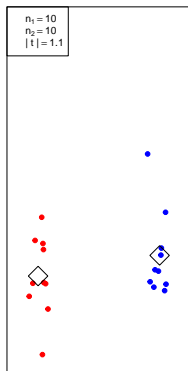
- For given standard deviations  $s_1$  and  $s_2$  and sample sizes  $n_1$  and  $n_2$ , the larger the difference in mean  $\bar{x}_1 - \bar{x}_2$  and the larger the t-statistic (absolute values).



- **It makes sense:** the right plot is more convincing than there is a difference in mean than the left plot.

## Appendix: t-statistic intuition (2/3)

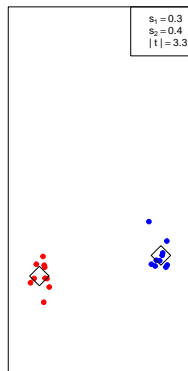
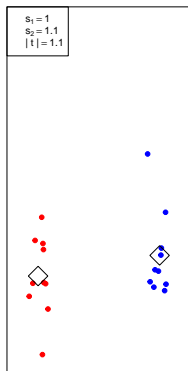
- For given standard deviations  $s_1$  and  $s_2$  and difference in mean  $\bar{x}_1 - \bar{x}_2$ , the larger the sample sizes  $n_1$  and  $n_2$  and the larger the t-statistic (absolute values).



- **It makes sense:** the right plot is more convincing that there is a difference in mean than the left plot.

## Appendix: t-statistic intuition (3/3)

- For given difference in mean  $\bar{x}_1 - \bar{x}_2$  and sample sizes  $n_1$  and  $n_2$ , the smaller the standard deviations  $s_1$  and  $s_2$  and the larger the t-statistic (absolute values).



- **It makes sense:** the right plot is more convincing than there is a difference in mean than the left plot.

## Two-sample t-test (2/2)

The key idea to use the  $t$ -statistics is that under the model assumption, it follows a specific distribution<sup>2</sup> whatever the value of the (population) means ( $\mu_1, \mu_2$ ) and standard deviations ( $\sigma_1, \sigma_2$ ) in each group.

Hence we can assume  $\mu_1 = \mu_2$  and calculate how **unlikely** it is to obtain a  $t$  value that **contradicts the null hypothesis as least as much** as that obtained with your data, that is, we can compute a **p-value**.

**The larger  $|t|$  the more the data contradict  $\mathcal{H}_0 : \mu_1 = \mu_2$ .**

p-value =  $P(|T| > |t|)$ , where  $T$  is a random variable that follows the  $t$ -distribution.

---

<sup>2</sup>the  $t$ -distribution, aka Student's distribution, which depends on the two sample sizes  $n_1$  and  $n_2$ ; already encountered in Lecture 1.





# The p-value (1/2)

## Informal definition:

The p-value is *“the probability of seeing a result as extreme as your observed result, when the null model is true.”*<sup>3</sup>

- ▶ “result”= test statistic, i.e., a single value that (“cleverly”) summarizes the data.
- ▶ “extreme”= unlikely/unexpected.

## Interpretation:

1. If the **p-value is small** the data are at odds with the null hypothesis<sup>4</sup> and the finding is said to be statistically **significant**.
2. If the **p-value is large**, the finding is said to be not statistically **significant**.

---

<sup>3</sup>Westfall & Henning. Understanding advanced statistical methods. CRC Press, 2013 (page 405).

<sup>4</sup>i.e. either the null hypothesis is not true or a very rare event has occurred.



# The p-value (2/2)

## Interpretation:

3. We imagine a large number of repetitions of the study with the null hypothesis being true and define the p-value as the proportion of these studies which provide less support for the null hypothesis than the data actually observed.

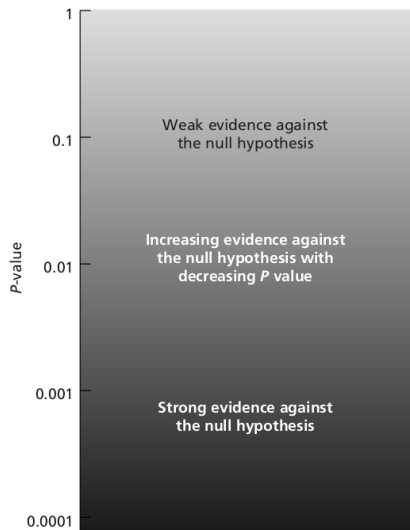
## Recommendations:

- ▶ Traditionally the value  $p=5\%$  has been used to divide “significant” from “non-significant” results, but good practice is to report the actual p-value.
- ▶ The choice of the threshold to claim significance (often  $p=5\%$ ) should be prespecified.



# p-value and strength of evidence

The smaller the p-value, the stronger the evidence against the null hypothesis.



## Case: Two-sample t-test

- ▶  $\bar{x}_1 = 3.43, \bar{x}_2 = 3.21$
- ▶  $\bar{x}_1 - \bar{x}_2 = 0.22$
- ▶  $n_1 = 25, n_2 = 27$
- ▶  $s_1 = 0.31, s_2 = 0.27$
- ▶  $s.e.(\bar{x}_1 - \bar{x}_2) = 0.081$
- ▶  $t = 2.66$
- ▶  $p\text{-value} = P(|T| > |t|) = 0.011$

We conclude that there is a **significant difference** in mean protein level of the milk between cows fed with barley and lupin ( $p=0.011$ ).



# Don't misinterpret p-values!

Widespread misunderstandings called for clarifications:

- ▶ The p-value is **not** the probability of  $\mathcal{H}_0$  being true.

*"Researchers often wish to turn a p-value into a statement about the truth of a null hypothesis, or about the probability that random chance produced the observed data. The p-value is neither."*<sup>5</sup>

This is because  $P(\text{extreme result}|\mathcal{H}_0) \neq P(\mathcal{H}_0|\text{extreme result})$ , which relates to the concept of "invalid inversions".<sup>6</sup>

<sup>5</sup> Wasserstein & Lazar. "The ASA statement on p-values: context, process, and purpose." Am. Stat. 70.2 (2016): 129-133.

<sup>6</sup> Similarly, "The probability that a woman has breast cancer is small. The probability that someone with breast cancer is a woman is large. Obvious— but some invalid inversions are not so easily seen." Senn, S.J. (2013). Invalid inversion. Significance 10 (2): 40-42.

<sup>7</sup> Held, Pawel & Schwab (2020), Significance 17(6), 10-11; Goodman (1992) Statistics in Medicine, 11(7), 875-879.



# Don't misinterpret p-values!

Widespread misunderstandings called for clarifications:

- ▶ The p-value is **not** the probability of  $\mathcal{H}_0$  being true.

*"Researchers often wish to turn a p-value into a statement about the truth of a null hypothesis, or about the probability that random chance produced the observed data. The p-value is neither."*<sup>5</sup>

This is because  $P(\text{extreme result}|\mathcal{H}_0) \neq P(\mathcal{H}_0|\text{extreme result})$ , which relates to the concept of "invalid inversions".<sup>6</sup>

- ▶ Observing a p-value  $\leq 5\%$  does not mean that the positive finding is easy to reproduce!<sup>7</sup>

As an example, suppose you conduct a study and find a significant p-value of  $p = 5\%$ . You try to replicate your positive finding by conducting the exact same study again. What is your chance to get a significant p-value ( $\leq 5\%$ ) again? To calculate that, you need to know the true treatment effect " $\mu_1 - \mu_2$ ", which you do not know, of course. But what about if we assume that it is what you have estimated in your (first) study, i.e.  $\bar{x}_1 - \bar{x}_0$ ?

<sup>5</sup> Wasserstein & Lazar. "The ASA statement on p-values: context, process, and purpose." Am. Stat. 70.2 (2016): 129-133.

<sup>6</sup> Similarly, "The probability that a woman has breast cancer is small. The probability that someone with breast cancer is a woman is large."

<sup>7</sup> Obvious— but some invalid inversions are not so easily seen." Senn, S.J. (2013). Invalid inversion. Significance 10 (2): 40–42.

Heid, Pawel & Schwab (2020), Significance 17(6), 10–11; Goodman (1992) Statistics in Medicine, 11(7), 875–879.



# Don't misinterpret p-values!

Widespread misunderstandings called for clarifications:

- ▶ The p-value is **not** the probability of  $\mathcal{H}_0$  being true.

*"Researchers often wish to turn a p-value into a statement about the truth of a null hypothesis, or about the probability that random chance produced the observed data. The p-value is neither."*<sup>5</sup>

This is because  $P(\text{extreme result}|\mathcal{H}_0) \neq P(\mathcal{H}_0|\text{extreme result})$ , which relates to the concept of "invalid inversions".<sup>6</sup>

- ▶ Observing a p-value  $\leq 5\%$  does not mean that the positive finding is easy to reproduce!<sup>7</sup>

As an example, suppose you conduct a study and find a significant p-value of  $p = 5\%$ . You try to replicate your positive finding by conducting the exact same study again. What is your chance to get a significant p-value ( $\leq 5\%$ ) again? To calculate that, you need to know the true treatment effect " $\mu_1 - \mu_2$ ", which you do not know, of course. But what about if we assume that it is what you have estimated in your (first) study, i.e.  $\bar{x}_1 - \bar{x}_0$ ? **Only 50%!**

<sup>5</sup> Wasserstein & Lazar. "The ASA statement on p-values: context, process, and purpose." Am. Stat. 70.2 (2016): 129-133.

<sup>6</sup> Similarly, "The probability that a woman has breast cancer is small. The probability that someone with breast cancer is a woman is large."

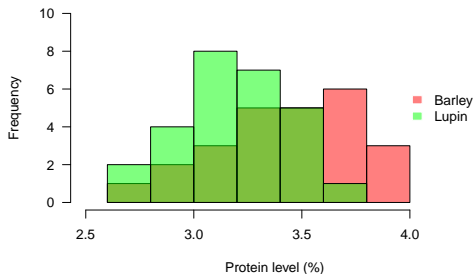
<sup>7</sup> Obvious— but some invalid inversions are not so easily seen." Senn, S.J. (2013). Invalid inversion. Significance 10 (2): 40–42.

Heid, Pawel & Schwab (2020), Significance 17(6), 10–11; Goodman (1992) Statistics in Medicine, 11(7), 875–879.



# Normality assumption

Normality should be checked for **each sample separately** (using histograms or qqplots).



But, when sample sizes  $n_1$  and  $n_2$  are both large enough (say  $> 15$ ) normality is **not important**<sup>8</sup>.

However, **skewed data can be transformed** to facilitate the interpretation and reduce the influence of outliers (this should be pre-specified).





# Confidence interval of the difference

**Good practice:** report an estimate of the mean difference and a **95% confidence interval**.

$$\bar{x}_1 - \bar{x}_2 \pm t_{df} \cdot s.e.(\bar{x}_1 - \bar{x}_2)$$

- ▶  $df$ : degree of freedom  $\approx n_1 + n_2 - 2$  when  $n_1 = n_2$  and  $s_1 = s_2$ .
- ▶  $t_{df} \approx 1.96$  when  $n_1$  and  $n_2$  are large (say  $\geq 15$ ).
- ▶ software will take care.

**Case:** mean difference of -0.22 (CI-95% = [0.05;0.38]; p-value = 0.011).



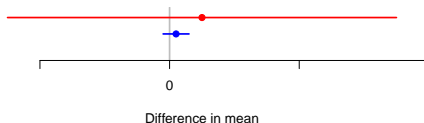
# Confidence interval vs p-value

- ▶ if 0 is  $\left\{ \begin{array}{c} \text{in} \\ \text{not in} \end{array} \right\}$  the CI, then the difference  $\left\{ \begin{array}{c} \text{is not} \\ \text{is} \end{array} \right\}$  significant.
- ▶ We can tell if the test is significant from looking at the CI, but we can't guess the CI from knowing the p-value.



# Confidence interval vs p-value

- ▶ if 0 is  $\left\{ \begin{array}{c} \text{in} \\ \text{not in} \end{array} \right\}$  the CI, then the difference  $\left\{ \begin{array}{c} \text{is not} \\ \text{is} \end{array} \right\}$  significant.
- ▶ We can tell if the test is significant from looking at the CI, but we can't guess the CI from knowing the p-value.
- ▶ A **wide** 95% that includes 0 suggests “**lack/absence of evidence**”.
- ▶ A **narrow** 95% that includes 0 suggests “**evidence of absence**” of difference (or existence of a “tiny one”, if any).



Altman & Bland (1995). *Statistics notes: Absence of evidence is not evidence of absence*. BMJ, 311(7003), 485.



# A common incorrect interpretation!

*"Based on the experience of reviewing statistics in more than 1000 manuscripts submitted to The Lancet Group of journals over the past 3 years, this Correspondence provides guidance to commonly encountered statistical deficiencies in reports and how to avoid them (panel)." <sup>9</sup>*

Panel: Basic recommendations for accurate reporting of statistics

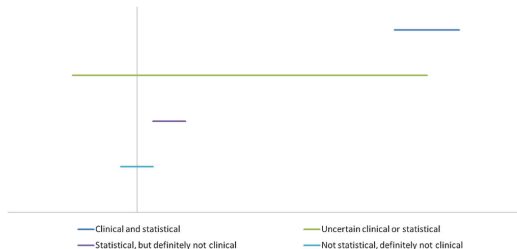
- Do not report results as showing no effect, unless all effects inside the interval estimate are clinically unimportant.

*"In particular, large p values should not be interpreted as showing no association or no effect: absence of evidence is not evidence of absence. Only a very narrow interval estimate near the null value (0 for differences, 1 for ratios) warrants inferring that the study found no important association or effect."*



# Clinical relevance versus statistical significance

**Fig. 1** A comparison of clinical and statistical significance is presented. The vertical line indicates the “no change” region of a measured effect. The horizontal distance from the line measures strength of the effect. Any confidence interval crossing that vertical line is not statistically significant, and any confidence interval near that line may not be clinically significant.



Clinical relevance  $\neq$  statistical significance !

**Source:** Maltenfort & Díaz-Ledezma. Statistics In Brief: Minimum Clinically Important Difference—Availability of Reliable Estimates. Clinical Orthopaedics and Related Research 475(4):p 933-946, 2017.

## Appendix: Need for CIs (1/2)

p-Values are only one tool for assessing evidence. When reporting the results of a clinical trial, CIs should always be reported to identify effect sizes that can be “ruled out” (i.e., effect sizes that are inconsistent with the data). If a p-value is significant, implying an effect, then the next natural question is “what is the effect?” CIs directly address this question. If a p-value is not significant, implying that you were not able to rule out the possibility of “no effect,” then the next natural question is “what effects could be ruled out?”

CIs again directly address this question. The under-reporting of CIs is a serious flaw in the medical literature.

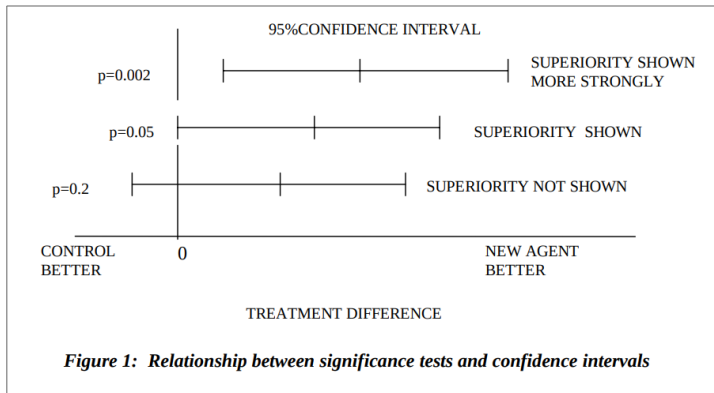
CIs are not a *replacement* for p-values but instead should be provided *with* p-values. p-values are still very useful tools particularly when assessing trends and interactions.

"Fundamental Concepts for New Clinical Trialists", by Evans and Ting (2016), pages 216–217, Section 8.3.4.2 "Need for CIs".

*“Avoid relying solely on statistical hypothesis testing, such as P values, which fail to convey important information about effect size and precision of estimates”* in "Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals", Updated January 2025, see <https://www.icmje.org>.



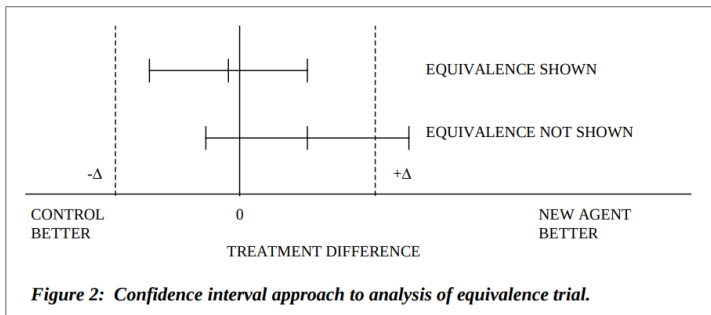
## Appendix: Need for CIs ( 2/2)



EMA scientific guidelines "Points to consider on switching between superiority and non-inferiority" (2000), [https://www.ema.europa.eu/en/documents/scientific-guideline/points-consider-switching-between-superiority-and-non-inferiority\\_en.pdf](https://www.ema.europa.eu/en/documents/scientific-guideline/points-consider-switching-between-superiority-and-non-inferiority_en.pdf)

# Appendix: CIs for evidence of “clinical equivalence”

E.g.:  $\Delta = 5$  mmHg in blood pressure

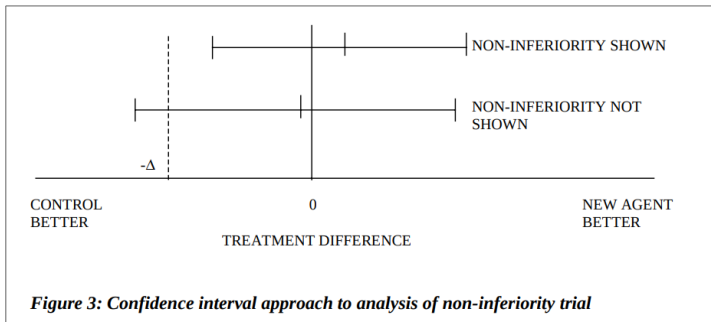


EMA scientific guidelines “Points to consider on switching between superiority and non-inferiority” (2000), [https://www.ema.europa.eu/en/documents/scientific-guideline/points-to-consider-switching-between-superiority-and-non-inferiority\\_en.pdf](https://www.ema.europa.eu/en/documents/scientific-guideline/points-to-consider-switching-between-superiority-and-non-inferiority_en.pdf)



# Appendix: CIs for evidence of “clinical non-inferiority”

E.g.:  $\Delta = 5$  mmHg in blood pressure



EMA scientific guidelines “Points to consider on switching between superiority and non-inferiority” (2000), [https://www.ema.europa.eu/en/documents/scientific-guideline/points-consider-switching-between-superiority-and-non-inferiority\\_en.pdf](https://www.ema.europa.eu/en/documents/scientific-guideline/points-consider-switching-between-superiority-and-non-inferiority_en.pdf)

# Two versions of the two-sample t-test

## “Classical” Student’s t-test (not recommended):

- ▶ Original t-test, described in many basic textbooks.
- ▶ Additional assumption<sup>10</sup> of equal standard deviations  $\sigma_1 = \sigma_2$ .
- ▶ Different formula for s.e. and degrees of freedom ( $df = n_1 + n_2 - 2$ ).

## Welch’s t-test (the presented one, recommended):

- ▶ No assumption of equal standard deviations: less restrictive.
- ▶ Formula for degrees of freedom more complicated, but software take care.
- ▶ Default in R.

---

<sup>10</sup> Note: with equal group sizes, i.e. when  $n_1 = n_2$ , which is typically the case in experimental research, then this assumption is **not** important at all. See e.g. Julious SA. *Why do we use pooled variance analysis of variance?*. Pharmaceutical Statistics. 2005 Jan;4(1):3-5.



# One-sample example

## Research question:

Is the mean protein level of the milk similar at 1 and 12 weeks after calving, for cows fed with Barley?

## Data $(t_1 - t_{12})$ :

Cow	Diff
B01	-0.08
B02	-0.03
B03	1.06
B04	0.48
B05	0.49
B06	0.74

etc...

## Null hypothesis:

The mean difference between protein level at 1 and 12 weeks is zero ( $\mathcal{H}_0 : \mu = 0$ ).

**One-sample** test because only one group of ( $n=25$ ) cows (barley).



# One-sample t-test

The **t-test statistic** measures the distance between the sample mean and the assumed population mean  $\mu$  under  $\mathcal{H}_0$  in units of the standard error:

$$t = \frac{\bar{x} - \mu}{s/\sqrt{n}}$$

If  $|t|$  is large, the data “contradict” the null hypothesis.

$$\text{p-value} = P(|T| > |t|)$$

where  $T$  is a random variable that follows the t-distribution with  $n - 1$  degrees of freedom.

- ▶ similar to the computation of the confidence intervals for the mean.
- ▶  $\text{p-value} \leq 5\% \iff \mu \text{ not in } 95\% \text{ CI.}$



# One-sample t-test: example results

- ▶  $\bar{x} = 0.46$
- ▶  $n = 25$
- ▶  $s = 0.31$
- ▶  $t = 7.43$
- ▶ p-value =  $P(|T| > |t|) < 0.001$  (for  $\mathcal{H}_0 : \mu = 0$ )



We conclude that there is a **significant difference** in mean protein level of the milk at 1 and 12 weeks after calving, for cows fed with barley ( $p < 0.001$ ).

**Reminder** (see Lecture 1):

we compute the 95% CI as  $\bar{x} \pm t_{n-1} \cdot s / \sqrt{n}$ , which here leads to  $[0.33; 0.58]$  (and does not include 0).

**Note:** this one-sample t-test corresponds to a **paired t-test**<sup>11</sup>.



# Outline/Intended Learning Outcome (ILOs)

## Hypothesis testing

ILO: to describe the principles and logic of hypothesis testing

## One and two sample tests for continuous responses: t-test

ILO: to identify when, how and why to use a t-test

ILO: to define a p-value and contrast its use with that of a confidence interval

## Power and Sample size calculation

ILO: to identify why and how to make power and sample size calculations

ILO: to analyse their strengths and limitations

## Multiple testing

ILO: to describe the multiple testing problem and employ basic remedies

## Nonparametric test: Wilcoxon

ILO: to contrast pros and cons of Wilcoxon vs t-test



# Power

The **power** of a test is the **chance** of obtaining a **significant result** when the null hypothesis is indeed false.



# Power

The **power** of a test is the **chance** of obtaining a **significant result** when the **null hypothesis is indeed false**.

- ▶ Power =  $1 - \beta$ , i.e. 1 minus the risk of a “false negative” result ( $\beta$ ), i.e. 1 minus risk of Type-II error.
- ▶ Although we can control the type-I error ( $\alpha = 5\%$ ) by appropriately computing the p-value and comparing it to 5%, the computation does not control the risk of type-II error,  $\beta$ .
- ▶ The power of a two-sample t-test depends on:
  - ▶ **sample sizes**  $n_1$  and  $n_2$  (the larger the better).
  - ▶ **standard deviations**  $\sigma_1$  and  $\sigma_2$  (i.e. variability, the smaller the better).
  - ▶ **difference in mean**  $\delta = |\mu_1 - \mu_2|$  (i.e. effect size, the larger the better).





# Textbook power formula (approximation for two-sample t-test)

$$\delta = (z_{1-\beta} - z_{\alpha/2}) \sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}}$$

- ▶  $z_{\alpha/2} = -1.96$  for  $\alpha = 5\%$ .<sup>12</sup>
- ▶  $z_{1-\beta} = 0.84$  and  $1.28$  for  $1 - \beta = 80\%$  and  $90\%$ .
- ▶ maximal power when  $n_1 = n_2$ , for a given total sample size  $n_1 + n_2$ , when  $\sigma_1 = \sigma_2$ .

## Useful for computing:

- ▶ **Sample size:**  $n_1 = n_2$  for given “guesses” of  $\sigma_1$ ,  $\sigma_2$  and  $\delta$  and desired  $1 - \beta$  and  $\alpha$ .
- ▶ **Power for a given budget/sample size:**  $1 - \beta$  for “guesses” of  $\sigma_1$ ,  $\sigma_2$  and  $\delta$  and desired  $n_1$ ,  $n_2$  and  $\alpha$ .
- ▶ **Least detectable difference:**  $\delta$  for given  $n_1$  and  $n_2$ , “guesses” of  $\sigma_1$  and  $\sigma_2$  and desired  $\alpha$  and minimal power  $1 - \beta$ .



# Use a software ! (e.g. R)

Often it is “good enough” to assume  $\sigma_1 = \sigma_2$  and then sensible to choose  $n_1 = n_2$ . Then standard software can be used, e.g. with R<sup>13</sup>:

```
power.t.test(power = .80, delta = 0.5)
```

```
Two-sample t test power calculation
```

```
      n = 63.76576
delta = 0.5
    sd = 1
sig.level = 0.05
  power = 0.8
alternative = two.sided
```

NOTE: n is number in *each* group

►  $n_1 = n_2 = 64$  subjects needed to detect 1/2 sd difference<sup>14</sup>.

---

<sup>13</sup>slightly more precise calculation performed than using the textbook formula.

<sup>14</sup>Note: it holds for whatever  $\sigma_1 = \sigma_2$  and  $\delta$ , as long as  $\delta/\sigma_1$ , the “signal-to-noise ratio”, is 1/2.



# Sample size calculation: which difference $\delta$ to use?

## Principled choices:

- ▶ expected/hypothesized difference.
- ▶ minimum (clinically) relevant difference.

But **small difference are difficult to detect** and may require a large sample size, with consequences on the budget, study length, etc.

Pragmatic choice: smallest difference “disappointing” to overlook.

If this still indicates a too large sample size, then discuss with your supervisor (try to avoid wasting time/money).



# Which guesses for the standard deviations?

For calculations, we need a “guess” of outcome variability, i.e.  $\sigma_1$ ,  $\sigma_2$ .

- ▶ **Estimate from previous studies** from your research group or published in the literature, but be aware of statistical uncertainty!
- ▶ **Expert guess** (supervisor/senior collaborators); asking them about the expected normal range<sup>15</sup> (and divide its width by 4) can help can be better to guess  $\sigma$  than directly asking about  $\sigma$ .

## Recommended practice:

- ▶ **use several likely values to do several calculations.**
- ▶ see how changes affect the results and discuss with your collaborators.
- ▶ be conservative (when appropriate).
- ▶ consider ethical issues (whenever relevant).

---

<sup>15</sup> Eg., ask “In which interval do you expect that approximately 95% of the outcome values will fall?” Personal experience suggests that this provides substantially more accurate guesses than asking the expected value of  $\sigma$  directly. Rationale is that the interval “mean  $\pm 2\sigma$ ” includes 95% of the observations, if the data are normally distributed, and more than 75% in any case



# Least detectable difference: sensitivity to $\sigma$

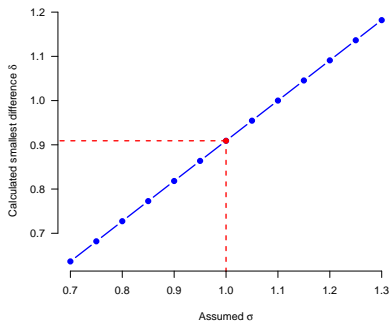
**Example:** my grant (money/time) can finance a sample size of  $n = 40$  (i.e. 20 per group). What is the smallest difference I can hope to show different from zero, with a decent power (e.g. 80%)?

```
power.t.test(n=20,sd=1,power=0.80)
```

Two-sample t test power calculation

```
n = 20  
delta = 0.9091306  
sd = 1  
sig.level = 0.05  
power = 0.8  
alternative = two.sided
```

NOTE: n is number in *each* group



**Note:** textbook formula gives  $\delta = 2.8 \cdot \sigma \cdot \sqrt{2/20}$ .



# Power: sensitivity to $\sigma$

**Example:** an initial calculation suggests  $n = 74$  (i.e. 37 per group), for the Minimal Clinically Important Difference (MCID)  $\delta = 2$ , with our best expert guess  $\sigma = 3$  (with 80% power). But what does the power become if we over or underestimate  $\sigma$  by up to 50%?

```
power.t.test(sd=4,delta=2,n=37)
```

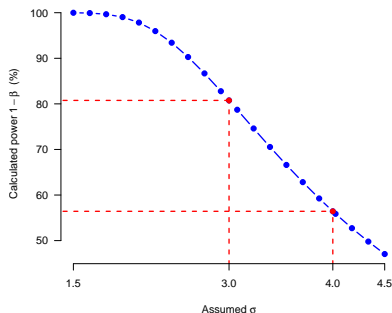
Two-sample t test power calculation

```

      n = 37
    delta = 2
      sd = 4
sig.level = 0.05
  power = 0.5642987
alternative = two.sided

```

NOTE: n is number in *each* group



**Note:** textbook formula gives  $z_{1-\beta} = (2/\sigma) \cdot (\sqrt{37}/\sqrt{2}) - 1.96$  and tables and software give  $z_{1-\beta} = 1.64, 1.28, 0.84,$

$0.25, -0.52$  for  $1 - \beta = 95, 90, 80, 60$  and  $30\%$ , respectively.



# Outline/Intended Learning Outcome (ILOs)

## Hypothesis testing

ILO: to describe the principles and logic of hypothesis testing

## One and two sample tests for continuous responses: t-test

ILO: to identify when, how and why to use a t-test

ILO: to define a p-value and contrast its use with that of a confidence interval

## Power and Sample size calculation

ILO: to identify why and how to make power and sample size calculations

ILO: to analyse their strengths and limitations

## Multiple testing

ILO: to describe the multiple testing problem and employ basic remedies

## Nonparametric test: Wilcoxon

ILO: to contrast pros and cons of Wilcoxon vs t-test



# A multiple testing example

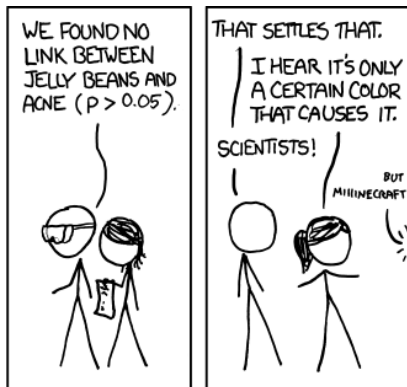


Are jelly beans associated with acne?

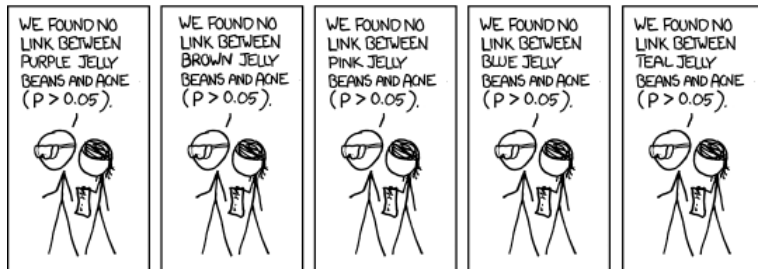


(cartoon from: <https://xkcd.com/882/>)

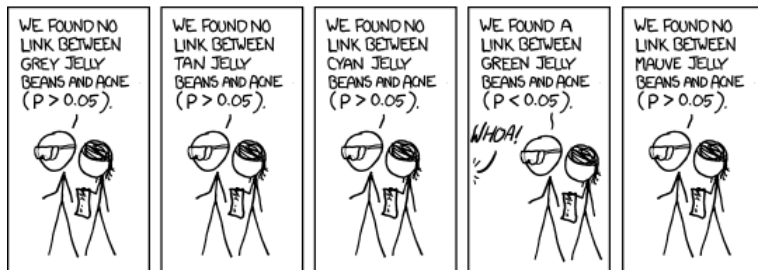




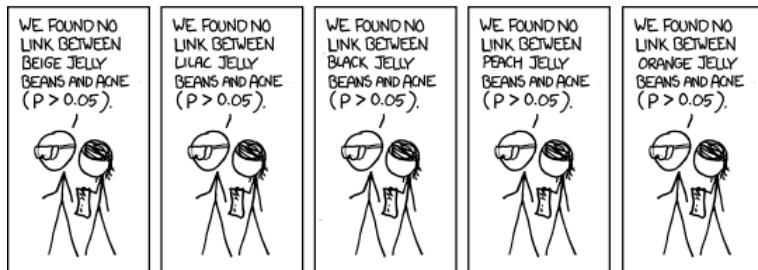
- ▶ First test is not significant.
- ▶ Move on to other tests.



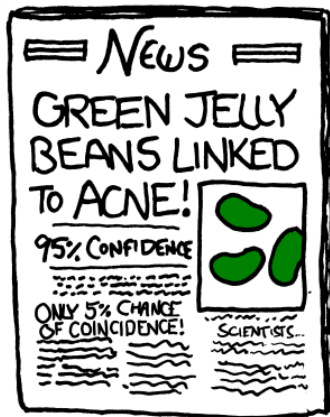
- ▶ Five more tests are not significant.
- ▶ Move on to other tests.



- ▶ Four more tests are not significant, but one is significant (Green!).
- ▶ Move on to other tests.



- ▶ Five more tests are not significant.
- ▶ Stop testing.



► Conclude.

Is the conclusion correct? Why?

# Multiple testing issue

- ▶ The risk of type-I error of **each** test is controlled (usually at 5%).
- ▶ i.e. thinking of each hypothesis test separately, each corresponding to a specific research question and specific study, the risk of false positive finding is controlled for each of them.
- ▶ But, if we consider them part of the same study and consider that we have a finding if at least one test is significant, then we do not control the risk of false positive finding.
- ▶ i.e. the risk of having **at least one** significant p-value although there is no association is not controlled.

**Family-wise error rate (FWER):** probability of making one or more false discoveries when performing multiple hypotheses tests



## FWER in the example

We have computed  $K = 16$  different p-values. For simplicity, we assume that the data to compute each of them are different (independent).

$$\begin{aligned}
 \text{FWER} &= P(\text{at least one of the } K \text{ p-values are significant}) \\
 &= 1 - P(\text{none of the } K \text{ p-values are significant}) \\
 &= 1 - P(1\text{st is not significant}) \times \cdots \times P(K\text{-th is not significant}) \\
 &= 1 - (1 - 0.05) \times \cdots \times (1 - 0.05) \quad (\text{as no association exists}) \\
 &= 1 - (1 - 0.05)^K
 \end{aligned}$$

K	1	2	3	4	5	10	16	20	50
FWER (%)	5	10	14	18	23	40	56	64	92

**Cartoon:** 56% chance of at least one significant false finding if no association exists.



# FWER control

When **we plan** to compute  $K \geq 1$  p-values, we can **adjust** their computation **to control the FWER**.

## Bonferroni adjustment:

- ▶ adjusted p-value =  $K \times$  original p-value
- ▶ adjusted significance level =  $\alpha/K$ .<sup>16</sup>

---

<sup>16</sup>

Can be used to compute adjusted confidence intervals.





# FWER control

When **we plan** to compute  $K \geq 1$  p-values, we can **adjust** their computation **to control the FWER**.

## Bonferroni adjustment:

- ▶ adjusted p-value =  $K \times$  original p-value
- ▶ adjusted significance level =  $\alpha/K$ .<sup>16</sup>

## Intuition:

- ▶ equally share/split the original significance level  $\alpha$  between the tests.
- ▶ the “total” risk of error (FWER) cannot exceed the sum of the errors of each test.

## Remarks:

- ▶ always works: no specific assumption.
- ▶ but only works if we **prespecify** the analysis with  $K$  tests.<sup>17</sup>

---

<sup>16</sup>

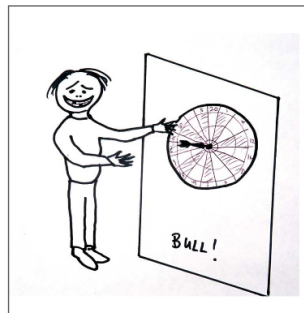
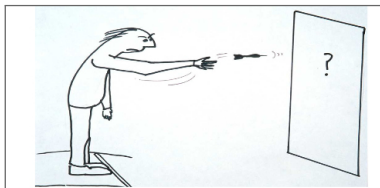
Can be used to compute adjusted confidence intervals.

<sup>52/64</sup>  
<sup>17</sup>

Not allowed to keep testing until one significant result pops up and then multiply all p-values by the number of tests performed.



# Prespecification matters



- ▶ Concluding significance without prespecification is like drawing a dart-board around where the dart lands.
- ▶ “If you torture the data long enough, it will confess to anything.”<sup>18</sup>

# Bonferroni-Holm adjusted p-values

1. sort the p-values:  $p_{(1)} \leq p_{(2)} \leq \dots \leq p_{(K)}$
2. adjust the first as with Bonferroni, i.e.  $\tilde{p}_{(1)} = K \cdot p_{(1)}$  and others as

$$\tilde{p}_{(i)} = \max \left\{ \tilde{p}_{(i-1)}, (K - i + 1) \cdot p_{(i)} \right\}$$

( $\approx$  multiply the 1st by  $K$ , the 2nd by  $K - 1$ , the 3rd by  $K - 2$ , . . .)

## Remarks:

- ▶ same as for Bonferroni (no specific assumption).
- ▶ we **cannot compute** corresponding adjusted significance levels and adjusted **confidence intervals**.
- ▶ **less conservative than Bonferroni**, i.e. adjusted p-values are always smaller.



# Example

We compare 6 doses of treatments (10-60 mg) to placebo (0 mg).

Comparison	60 mg	50 mg	40mg	30mg	20mg	10mg
Original p-value	<b>0.005</b>	<b>0.009</b>	0.1	0.15	0.3	0.6
Bonferroni	<b>0.03</b>	0.054	0.6	0.9	1	1
Bonferroni-Holm	<b>0.03</b>	<b>0.045</b>	0.4	0.45	0.6	0.6

Note: we “truncate” the p-value to 1.



# FWER vs FDR (1/2)

Controlling the **FWER** is important in “**confirmatory**” studies.

- ▶ When there is a clear **prespecified** scientific hypothesis and the aim is to “prove” it. E.g. **clinical trial**.

Controlling the **FDR** is often better suited in “**exploratory**” studies.

- ▶ When nice data are available, but **no specific research questions** / scientific hypotheses. You want to look at many associations and report findings which are “likely enough” true findings. E.g. **Genomics**.

**False discovery rate (FDR):** expected proportion of falsely rejected hypotheses among the rejected hypotheses.



# Digression: confirmatory versus exploratory research

Exploratory research	Confirmatory research
No hypothesis required/hypothesis can be vague	Clear hypothesis required
Generate new hypothesis from data	Test <i>a priori</i> hypothesis with new data
High sensitivity desired, i.e. minimising the risk of false negatives	High specificity desired, i.e. minimising the risk of false positives
Suitable for making new discoveries and finding the unexpected	Suitable for establishing strong evidence and confirming the expected
<b>For example:</b> Testing of new compounds in mice	<b>For example:</b> Assessing the efficacy of a drug in humans

*"It is essential to distinguish between exploratory and confirmatory research, and they are equally important to the scientific enterprise. Finding the questions to ask is at least as crucial as answering them, if not more so. But when the two concepts get confused, and the two worlds collide, the fallout can be disastrous"*<sup>19</sup>



# FWER vs FDR (2/2)

Hypotheses	Not rejected	Rejected	Total
True	U	V	$K_0$
False	T	S	$K - K_0$
Total	W	R	$K$

- ▶  $FWER = P(V > 0)$
- ▶  $FDR = E(V/R)$  (where here we set  $V/R = 0$  if  $R = 0$ ).
- ▶ **controlling the FDR is less conservative than controlling the FWER:**  
p-values adjusted to control the FDR are smaller than those adjusted to control the FWER.
- ▶ See **Benjamini-Hochberg** (1995) method to control FDR at e.g. 5%.



# Multiple testing corrections: none can be fine/best !

It is not always needed, or even possible, to adjust for multiple testing.

*"In exploratory studies without prespecified hypotheses there is typically no clear structure in the multiple tests, so an appropriate multiple test adjustment is difficult or even impossible. Hence, we prefer that data of exploratory studies are analysed without multiplicity adjustment. However, 'significant' results based on exploratory analyses should be clearly labelled as exploratory results. To confirm these results, the corresponding hypotheses have to be tested in confirmatory studies."*

Bender & Lange. (1999). Multiple test procedures other than Bonferroni's deserve wider use. BMJ, 1999, 318:600.





# Outline/Intended Learning Outcome (ILOs)

## Hypothesis testing

ILO: to describe the principles and logic of hypothesis testing

## One and two sample tests for continuous responses: t-test

ILO: to identify when, how and why to use a t-test

ILO: to define a p-value and contrast its use with that of a confidence interval

## Power and Sample size calculation

ILO: to identify why and how to make power and sample size calculations

ILO: to analyse their strengths and limitations

## Multiple testing

ILO: to describe the multiple testing problem and employ basic remedies

## Nonparametric test: Wilcoxon

ILO: to contrast pros and cons of Wilcoxon vs t-test



# Wilcoxon-Mann-Whitney Test: motivation

## Limitation of the two-sample t-test:

- ▶ Data should be normally distributed in each group
- ▶ **OR** the sample size of each group should be large.

## Challenge:

What if we want a reliable computation of a p-value to compare two groups, with small sample data not necessarily normally distributed?

## A solution:

We can use a rank-based test<sup>20</sup>: the Wilcoxon-Mann-Whitney test<sup>21</sup>. It provides “exact” p-values.<sup>22</sup>

Another advantage of Wilcoxon is its “robustness” to outliers, which might be convenient.

---

<sup>20</sup> also often called “non-parametric” test

<sup>21</sup> sometimes just called “Wilcoxon” or “Mann-Whitney” test.

<sup>22</sup> exact means that p-values are always valid (i.e. no “large  $n$ ” approximation.)



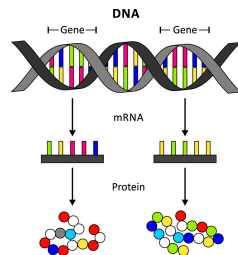
# Case: gene expression

## ► Research question:

Is the length of the candidate gene NACP associated with the level of expressed alpha synuclein mRNA, which has been shown to be associated with alcoholism?

## ► Outcome: level of expressed alpha synuclein mRNA.

## ► Compared groups: “short” vs “long” allele length (sum score built from additive dinucleotide repeat length categorized into groups).

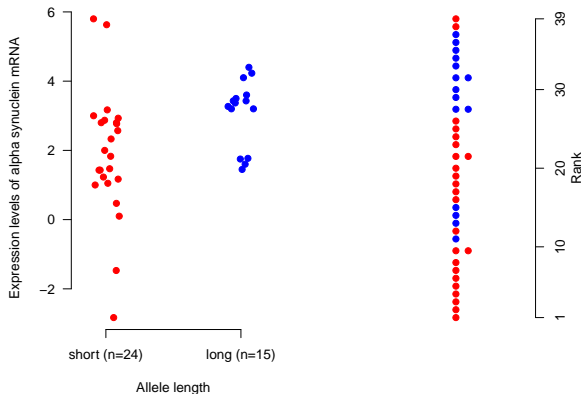


## Challenges:

- small sample size  $n = 24$  (short) + 15 (long)
- outcome not known to be normally distributed.
- aim to **confirm** that this gene is linked to alcohol dependence.

# Wilcoxon test: example

p-value=0.002



## Why using the ranks:

If the two groups are similar, then the ranks should be equally distributed between the two groups. **Whatever the distribution of the observations** in each group, a randomly drawn **blue observation** should be larger than a randomly drawn **red observation** in about 50% of the draws. (Here  $P(X > Y) = 79.2\%$ )



# Wilcoxon test: practical limitation

When a significant difference is shown **we can conclude that the distribution in the two groups are different, but nothing else...** which can be **frustrating**.

**Common error**/overinterpretation: conclude to a difference in median.

We cannot estimate a nice matching 95% CI to quantify the “effect size”.  
By contrast, to complement the p-value of a t-test we can provide a matching 95% CI of the difference in mean.

Hence unless an “exact” p-value computation is really needed, using a t-test, possibly after having transformed the data, can often be preferred<sup>23</sup>.

---

<sup>23</sup>See e.g. le Cessie, Goeman, and Dekkers. "Who is afraid of non-normal data? Choosing between parametric and non-parametric tests." *European Journal of Endocrinology* (2020).

