Faculty of Health Sciences

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

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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 confidence intervals

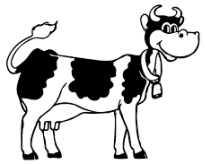
Power and Sample size calculation
- ILO: to identify why and how to make power and sample size calculations
- ILO: to analyse their strengths and limitations

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.[1]

[1] **Note:** important complementary information is given by the confidence interval of the effect size.

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 conclusion

Case:

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

Hypothesis testing and risk control

We want to ensure that risk of wrongly rejecting the null hypothesis (α) 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 our 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 should be 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.

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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
```

etc...

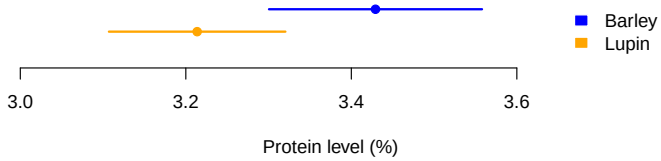
	Barley	Lupin
Mean (SD):	3.43 (0.31)	3.21 (0.27)

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

First approach (not optimal for testing)

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 with the null hypothesis $\boxed{\mathcal{H}_0 : \mu_1 = \mu_2}$, 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 big 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 it is used to compute a p-value.

Two-sample t-test (2/2)

The key idea to use the t-statistics is that under the model assumption, it follows the specific distribution[2] whatever the value of the (population) means (μ_1, μ_2) and standard deviations (σ_1, σ_2) in each group.

Hence we can assume $\mu_1 = \mu_2$ and calculate how unlikely it should be 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.

[2] the t-distribution or Student's distribution, which depends on the two sample sizes n_1 and n_2; already encountered in Lecture 1.

The p-value

Interpretation:
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.

- If the **p-value is small** the data are at odds with the null hypothesis and the finding is said to be statistically significant.
- If the **p-value is large**, the finding is said to be not statistically significant.

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.

p-value and strength of evidence

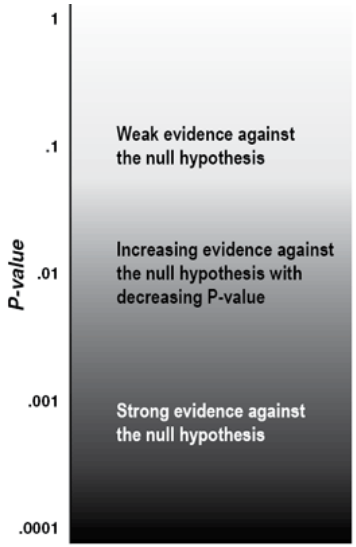


Figure 8.2 in Kirkwood & Sterne (2003), *Essential medical statistics, 2nd edition.*

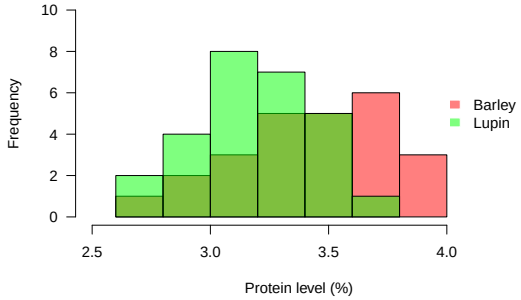
Case: Two-sample t-test

- $\bar{x}_1 = 3.43$, $\bar{x}_2 = 3.21$
- $\bar{x}_1 - \bar{x}_2 = 0.22$
- $n_1 = 25$, $n_2 = 27$
- $s_1 = 0.31$, $s_2 = 0.27$
- $s.e.(\bar{x}_1 - \bar{x}_2) = 0.081$
- $t = 2.66$
- p-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).

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**[3].

However, **skewed data can be transformed** to facilitate the interpretation and reduce the influence of outliers.

[3] due to the central limit theorem.

Confidence interval of the difference

Good practice: report an estimate of the mean difference and a 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 ≥ 15).
- software will take care.

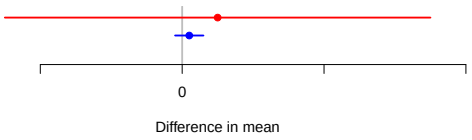
Case: mean difference of -0.22 (CI-95% = [0.05;0.38]; p-value = 0.011).

Confidence interval vs p-value

- if 0 is { **in** / **not in** } the CI, then the difference { **is not** / **is** } 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 { **in** / **not in** } the CI, then the difference { **is not** / **is** } 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.

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 of equal standard deviations $\sigma_1 = \sigma_2$.
- Different formula for s.e. and degrees of freedom ($df = n_1 + n_2 - 2$).

Welch' 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.

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 μ 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.

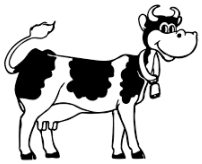
$$\text{p-value} = \text{P}(|T| > |t|)$$

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.
- p-value $\leq 5\%$ $\iff$ μ not in 95% CI.

One-sample t-test: example results

- $\bar{x} = 0.46$
- $n = 25$
- $s = 0.31$
- $t = 7.43$
- p-value= $\text{P}(|T| > |t|) < 0.001$.



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

Note: this one-sample t-test corresponds to a **paired t-test**[4].

[4] two samples of observations (two times) paired by cow. More on Lecture 8.

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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 (β), 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, β.

- The power of a two-sample t-test depends on:
  - sample sizes n_1 and n_2 (the larger the better).
  - standard deviations σ_1 and σ_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 $\alpha = 5\%$.[5]
- $z_{1-\beta} = 0.84$ 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 σ_1, σ_2 and δ and desired $1 - \beta$ and α.
- Power for a given budget/sample size: $1 - \beta$ for "guesses" of σ_1, σ_2 and δ and desired n_1, n_2 and α.
- Least detectable difference: δ for given n_1 and n_2, "guesses" of σ_1 and σ_2 and desired α and minimal power $1 - \beta$.

[5] where z_γ is the γ-quantile of a standard normal distribution.

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[6]:

```
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[7].

[6] slightly more precise calculation performed than using the textbook formula.

[7] Note: it holds whatever $\sigma_1 = \sigma_2$ and δ, as long as δ/σ_1=1/2.

Sample size calculation: which difference δ 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 the calculations, we need a "guess" for the variability in the outcome[8], i.e. σ_1, σ_2.

- Estimate from previous studies from your research group or published in the literature (be aware of statistical uncertainty).
- Expert guess (supervisor/senior collaborators).

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 (when appropriate).

[8] Thinking about the normal range width ($=4\sigma$) can help to guess σ.

Least detectable difference: sensitivity to σ

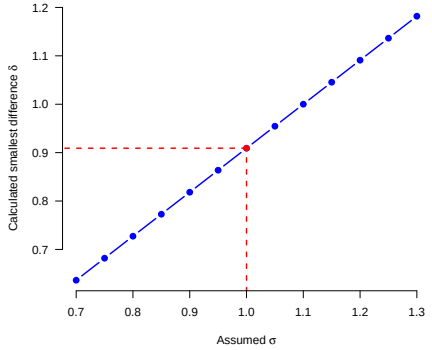
Example: my grant (money/time) can finance a sample size of $n = 40$ (i.e. 20 per group), what is the smallest difference I can hope to show with a decent power (e.g. 80%)?

```
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 σ

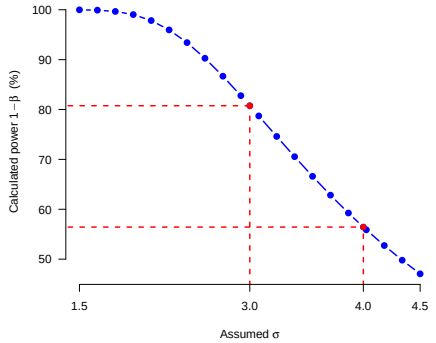
Example: an initial calculation suggests $n = 74$ (i.e. 37 per group), for the minimum difference $\delta = 2$ that we aim to show, with our best expert guess $\sigma = 3$ (with 80% power). But what does the power become if we over or underestimate σ by up to 50%?

```
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.

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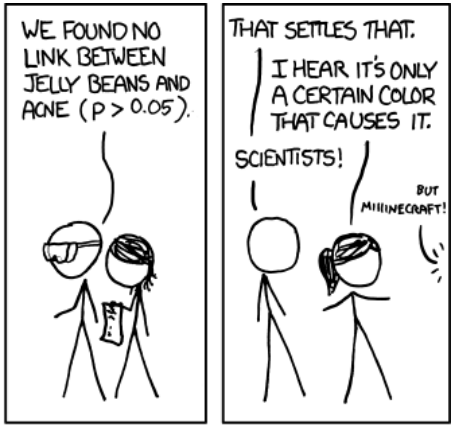
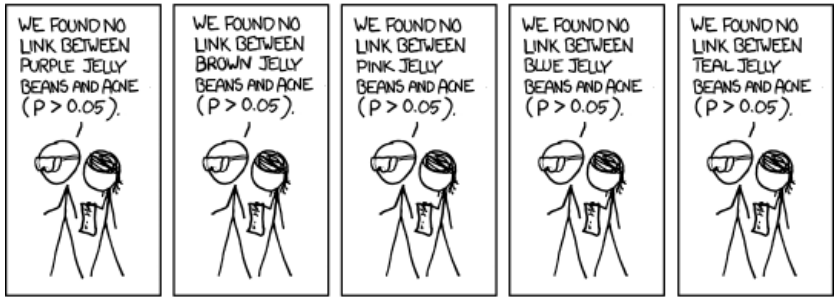
A multiple testing example

Are jelly beans associated with acne?

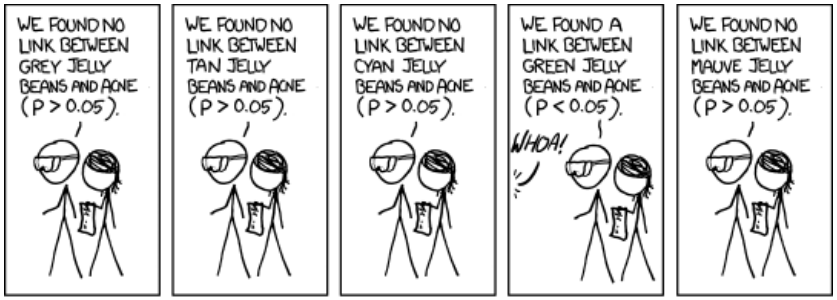
(cartoon from: https://xkcd.com/882/)



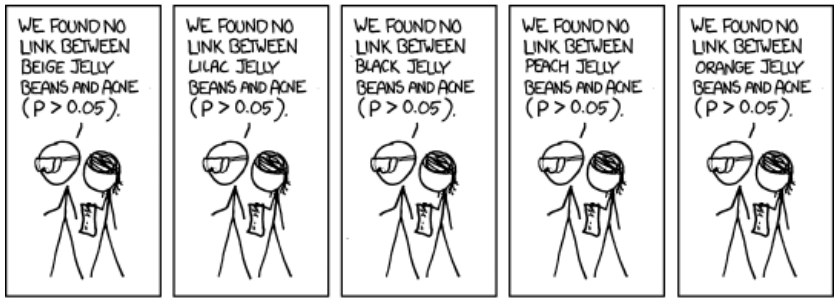
- First test is not significant.
- Move on to other tests.



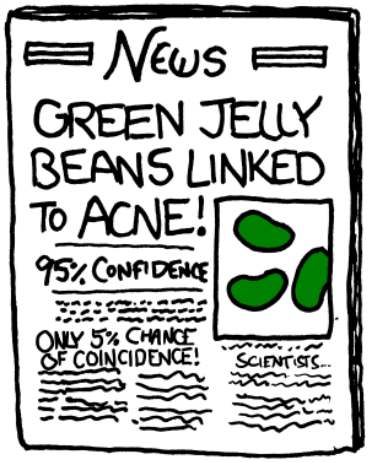
- Five more tests are not significant.
- Move on to other tests.



- 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).

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

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 $= \alpha/K$.[9]

[9] Can be used to compute adjusted confidence intervals.

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

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- adjusted significance level $= \alpha/K$.[9]

Intuition:

- equally share/split the original significance level α between the tests.
- the "total" risk of error (FWER) cannot exceed the sum of the errors of each test.

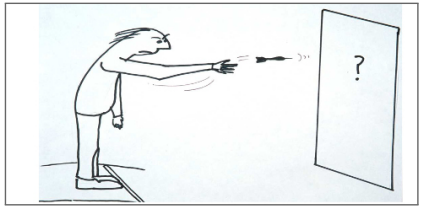
Remarks:

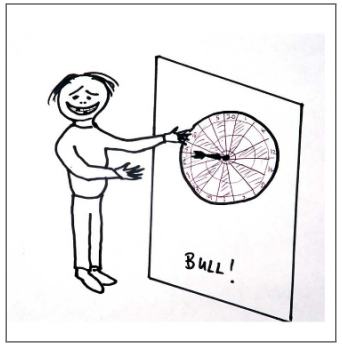
- always works: no specific assumption.
- but only works if we prespecify the analysis with K tests.[10]

[9] Can be used to compute adjusted confidence intervals.

[10] Not allowed to keep testing until one significant result pops up and then divide 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.

Bonferroni-Holm adjusted p-values

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

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

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

Remarks:

- same as for Bonferroni.
- 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	10 mg	20 mg	30mg	40mg	50mg	60mg
Original p-value	**0.005**	**0.009**	0.1	0.15	0.3	0.6
Bonferroni	**0.03**	0.054	0.6	0.9	1	1
Bonferroni-Holm	**0.03**	**0.045**	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.

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

- $FWER = \mathrm{P}(V > 0)$
- $FDR = \mathrm{E}(V/R)$ (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%.

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 confidence intervals

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 (say >15).

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

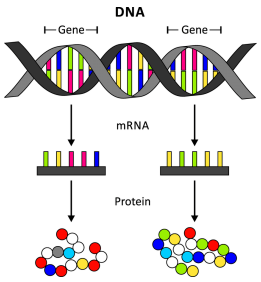
A solution:
We can use a rank-based test[11]: the Wilcoxon-Mann-Whitney test[12]. It provides "exact" p-values.[13]

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

[11] also often called "non-parametric" test
[12] sometimes just called "Wilcoxon" or "Mann-Whitney" test.
[13] 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).

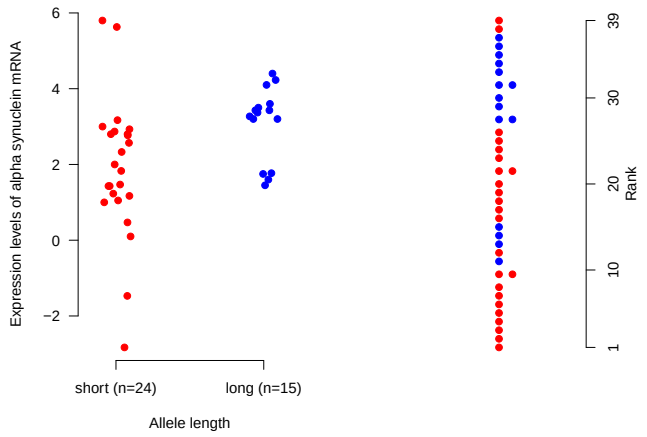


Challenges:

- 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[14].

[14] 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).