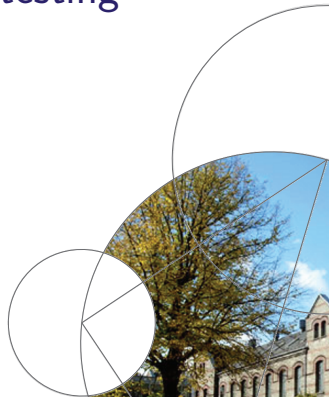Faculty of Health Sciences

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

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Outline

Hypothesis testing

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

Power and Sample size calculation

Multiple testing
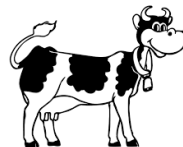
Nonparametric test: Wilcoxon

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]

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[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.

Outline

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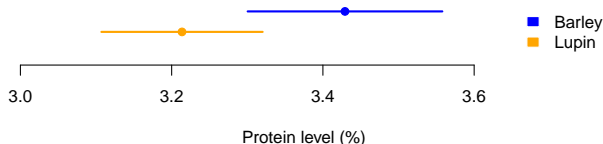
▶ Is the difference observed in the data sample large enough to conclude to a difference in the population?

First approach (not optimal for testing)

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.

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[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
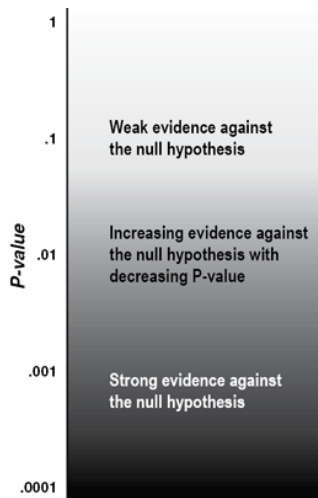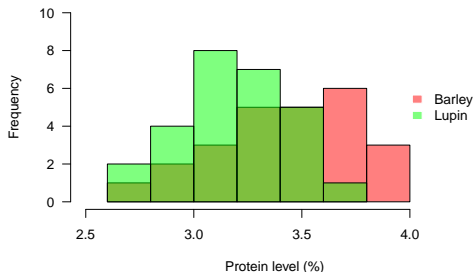
Case: Two-sample t-test

- $\bar{x}_1 = 3.43$, $\bar{x}_2 = 3.21$
- $\bar{x}_1 - \bar{x}_2 = 0.22$
- $n_1 = 25$, $n_2 = 27$
- $s_1 = 0.31$, $s_2 = 0.27$
- $s.e.(\bar{x}_1 - \bar{x}_2) = 0.081$
- $t = 2.66$
- 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 $\left\{ \begin{array}{c} \textbf{in} \\ \textbf{not in} \end{array} \right\}$ the CI, then the difference $\left\{ \begin{array}{c} \textbf{is not} \\ \textbf{is} \end{array} \right\}$ 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 $\left\{ \begin{array}{c} \textbf{in} \\ \textbf{not in} \end{array} \right\}$ the CI, then the difference $\left\{ \begin{array}{c} \textbf{is not} \\ \textbf{is} \end{array} \right\}$ 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).

Difference in mean
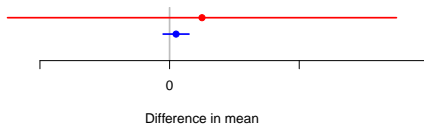
Two versions of the two-sample t-test

"Classical" Student's t-test (not recommended):

- ▶ Original t-test, described in many basic text books.
- ▶ 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.

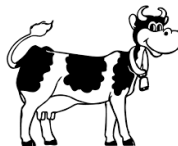$$\text{p-value} = P(|T| > |t|)$$

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= $P(|T| > |t|) < 0.001$.

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.

Outline

Hypothesis testing

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

Power and Sample size calculation

Multiple testing

Nonparametric test: Wilcoxon

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]a slightly more precise calculation is performed.

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

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).

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

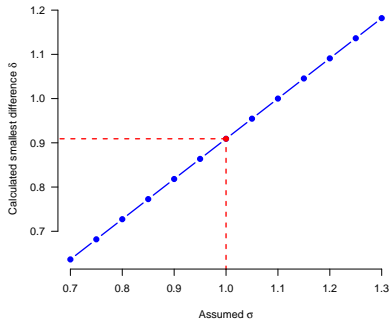## Least detectable difference: sensitivity to σ

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
```
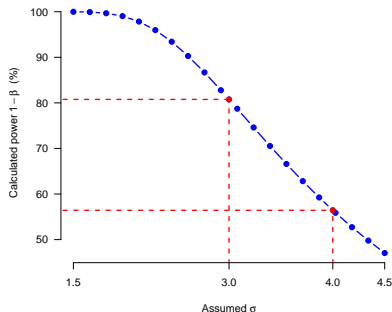

Note: textbook formula gives $z_{1-\beta} = (2/\sigma) \cdot (\sqrt{37}/\sqrt{2}) - 1.96$ and tables and software give $z_{1-\beta} = 1.64, 1.28, 0.84$,

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

Outline

Hypothesis testing

One and two sample tests for continuous responses: t-test
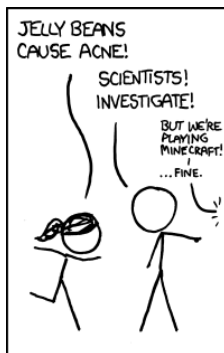
Power and Sample size calculation
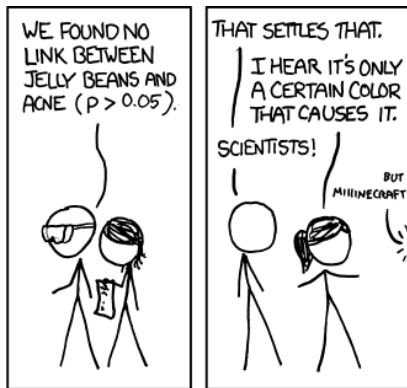
Multiple testing

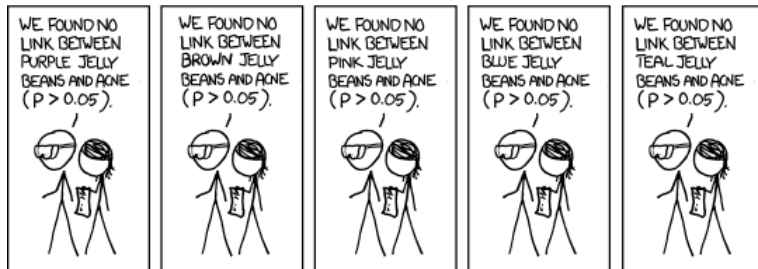Nonparametric test: Wilcoxon

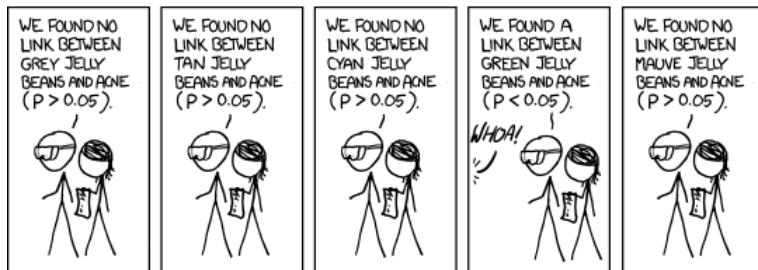# A multiple testing example

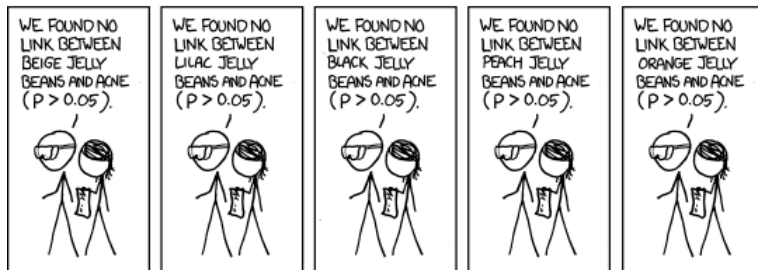Are jelly beans associated
with acne?

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

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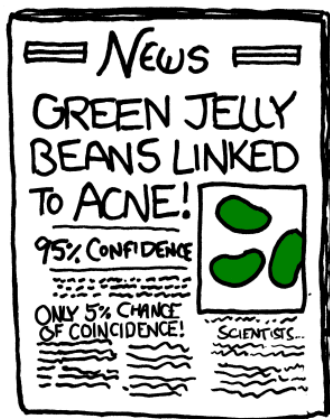
- Five more tests are not significant.
- Move on to other tests.

▶ 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).

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

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.

FWER control

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Bonferroni adjustment:

▶ adjusted p-value $= K \times$ original p-value

▶ 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]

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[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
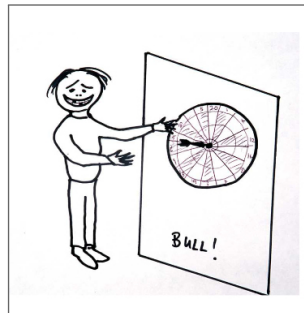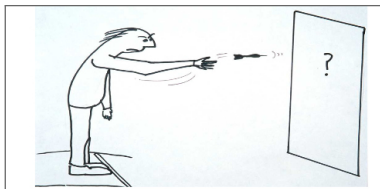
Concluding significance without prespecification is like drawing a dart-board around where the dart lands.

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

▶ $FWER = \mathsf{P}(V > 0)$

▶ $FDR = \mathsf{E}(V/R)$ (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

Hypothesis testing

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

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Nonparametric test: Wilcoxon

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.)
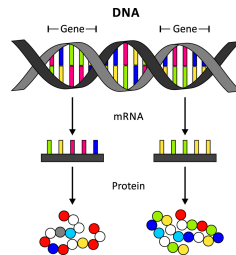
Case: gene expression

▶ **Research question:**
Is the candidate gene NACP, coding for alpha synuclein, associated with alcohol dependence?

▶ **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).
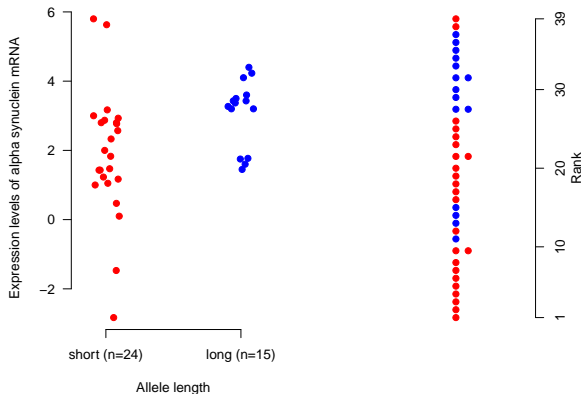

Challenges:

▶ 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

Idea of using ranks:
If the two groups are similar, then we know what to expect whatever the distribution of the observations in each group (approx. same rank distribution in the two groups).

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].

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[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).