Analysis of Quantitative data
One-Way + Two-Way ANOVA

Anne Segonds-Pichon
v2020-08

Babraham Institute

Babraham Bioinformatics

Comparison between more than 2 groups

One factor = One predictor

One-Way ANOVA

Signal-to-noise ratio

$$\frac{\text{Difference}}{\text{Variability}} = \frac{\text{Signal}}{\text{Noise}}$$

$$\frac{\text{Signal}}{\text{Noise}}$$ = statistical significance

$$\frac{\text{Signal}}{\text{Noise}}$$ = no statistical significance

Analysis of variance: how does it work?

$$\frac{\text{Signal}}{\text{Noise}} = \frac{\text{Difference between the means}}{\text{Variability in the groups}}$$

$$= \text{F ratio}$$

One-Way Analysis of variance

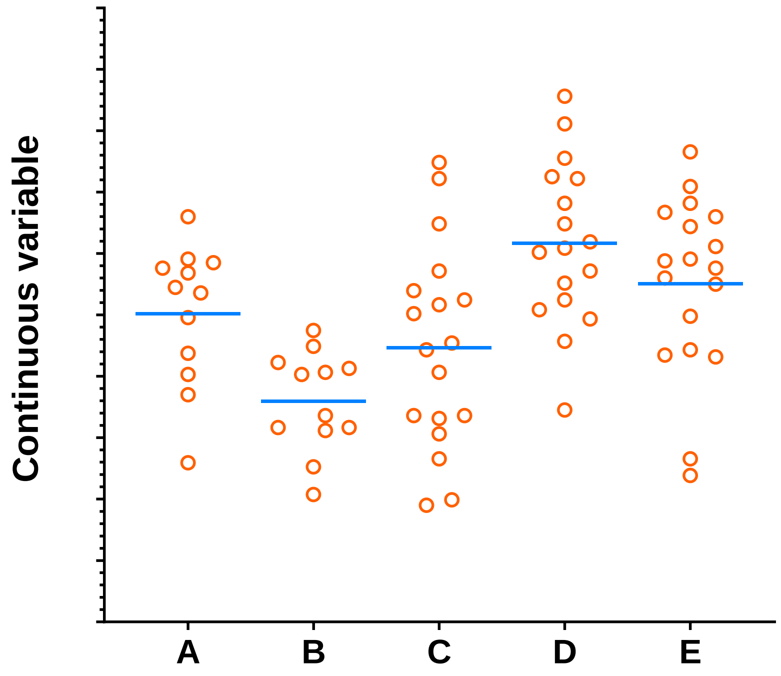
<u>Step 1</u>: Omnibus test

- It tells us if there is a difference between the means but not which means are significantly different from which other ones.

<u>Step 2</u>: Post-hoc tests

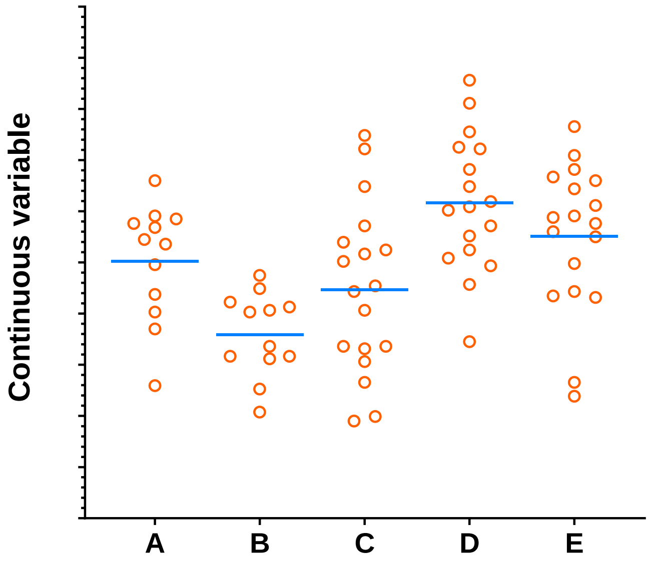
- They tell us if there are differences between the means pairwise.

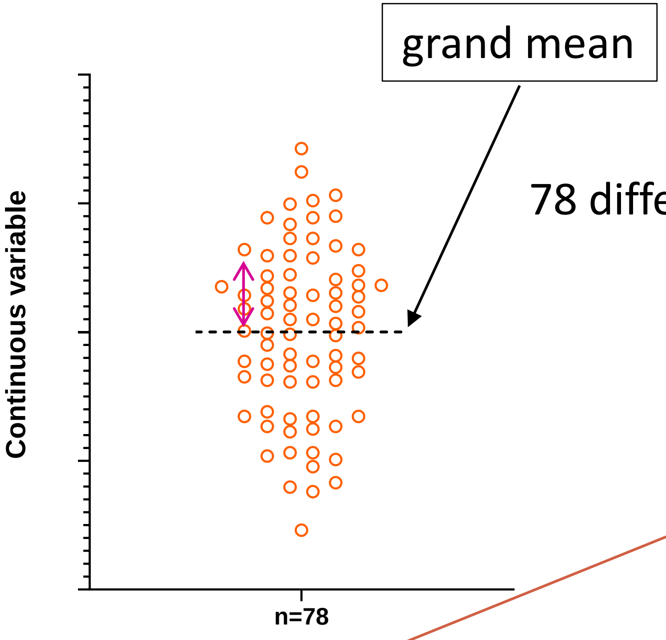
Analysis of variance: how does it work?



Source of variation	Sum of Squares	df	Mean Square	F	p-value
Between Groups	18.1	4	4.5	6.32	0.0002
Within Groups	51.8	73	0.71		
Total	69.9				

Analysis of variance: how does it work?





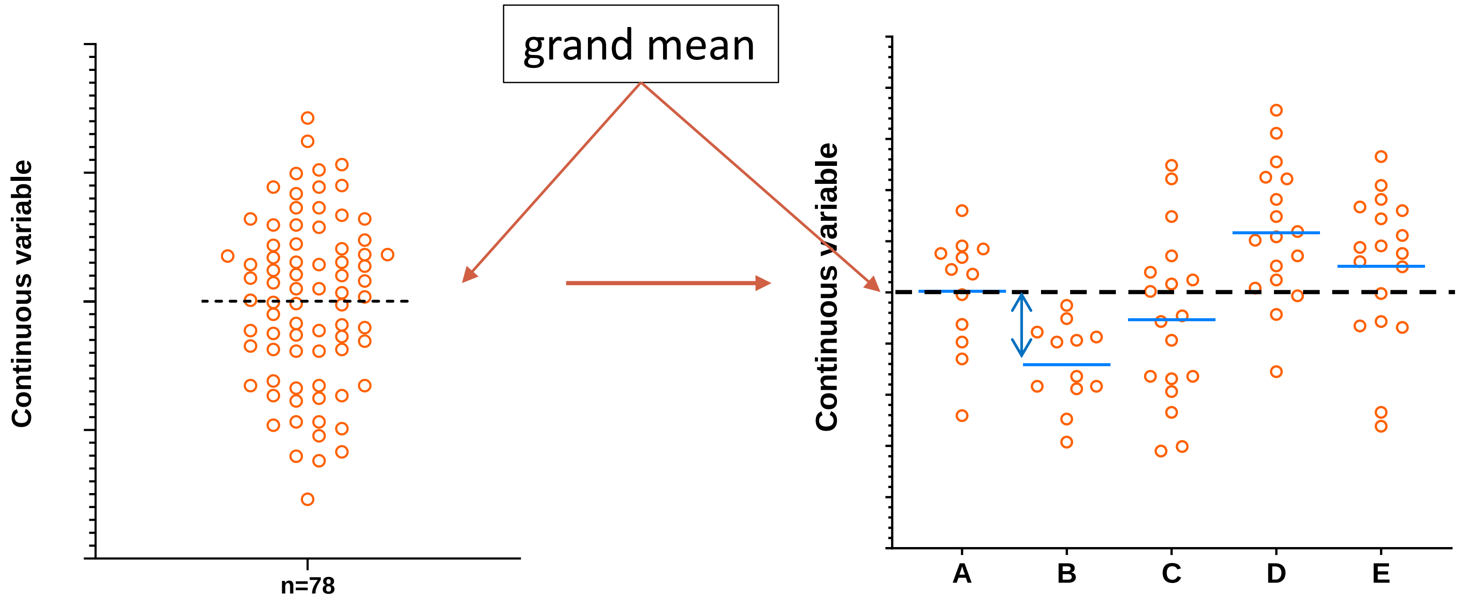
78 differences: $\sum_{1}^{78} (\mathrm{value}_n - \text{grand mean})^2$

=

Sum of squared errors

Source of variation	Sum of Squares	df	Mean Square	F	p-value
Between Groups					
Within Groups					
Total	69.9				

Analysis of variance: how does it work?



5 differences: $\sum_1^5 (\text{mean}_n - \text{grand mean})^2$

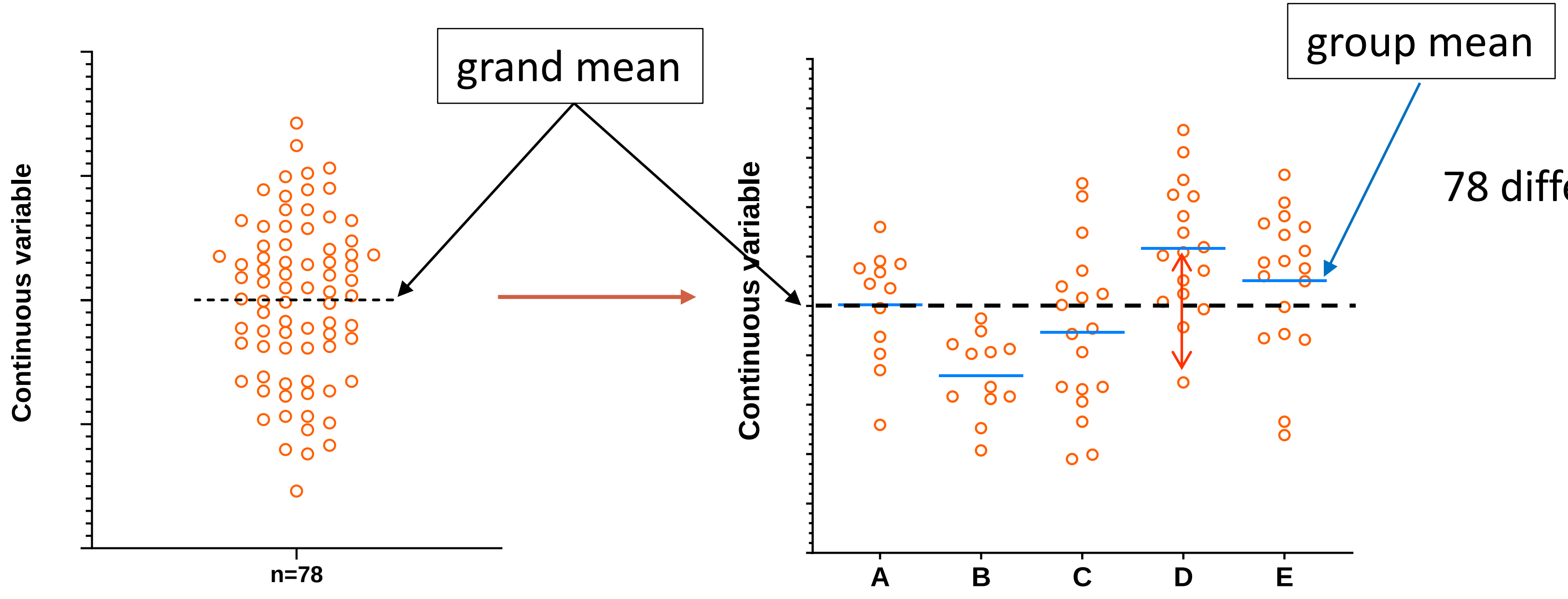
=

Sum of squared errors

Between the groups

Source of variation	Sum of Squares	df	Mean Square	F	p-value
Between Groups	18.1				
Within Groups					
Total	69.9				

Analysis of variance: how does it work?



78 differences: $\sum_1^{78}(\text{value}_n - \text{group mean})^2$

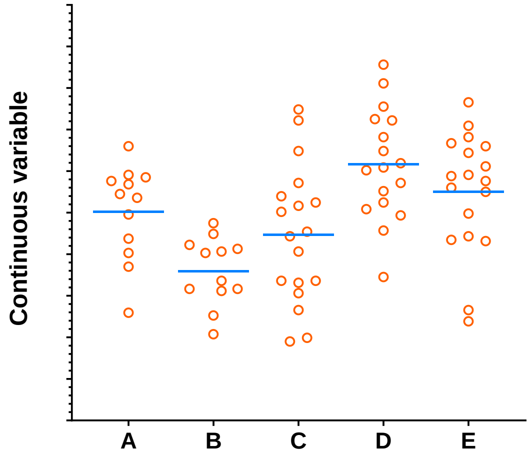
=

Sum of squared errors

Within the Groups

Source of variation	Sum of Squares	df	Mean Squares	F	p-value
Between Groups	18.1				
Within Groups	51.8				
Total	69.9				

Analysis of variance: how does it work?



	Source of variation	Sum of Squares	df	Mean Squares	F ratio	p-value
Signal	Between Groups	18.1	k-1			
Noise	Within Groups	51.8	n-k			
	Total	69.9				

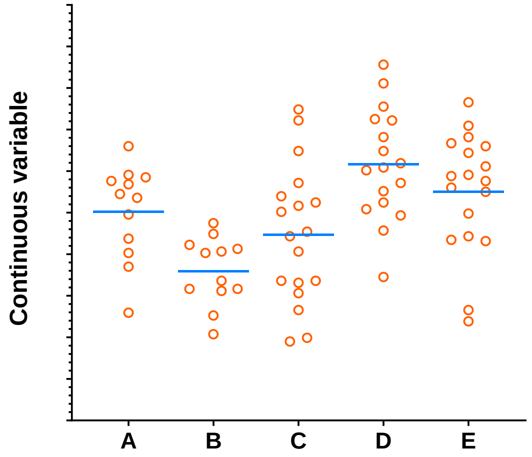
df: degree of freedom with df = n-1

n = number of values, k=number of groups

Between groups: df = 4 (k-1)

Within groups: df = 73 ($n-k = n_1-1 + \dots + n_5-1$)

Analysis of variance: how does it work?



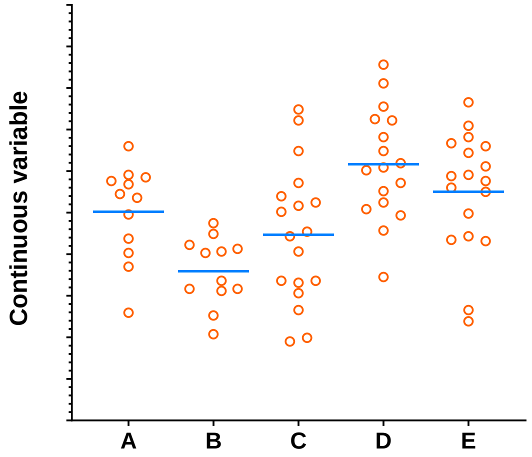
	Source of variation	Sum of Squares	df	Mean Squares	F ratio	p-value
Signal	Between Groups	18.1	4	4.5		
Noise	Within Groups	51.8	73	0.71		
	Total	69.9				

df: degree of freedom with df = n-1

18.2/4 = 4.5 **51.8/73 = 0.71**

Mean squares = **Sum of Squares / n-1 = Variance!**

Analysis of variance: how does it work?



Source of variation	Sum of Squares	df	Mean Squares	F ratio	p-value
Between Groups	18.1	4	4.5	6.34	0.0002
Within Groups	51.8	73	0.71		
Total	69.9				

Mean squares = **Sum of Squares / n-1 = Variance**

$$\text{F ratio} = \frac{\text{Variance between the groups}}{\text{Variance within the groups (individual variability)}} = \frac{4.5}{0.71} = \mathbf{6.34}$$

Comparison of more than 2 means

- Running multiple tests on the same data increases the **familywise error rate**.
- What is the familywise error rate?
  - The error rate across tests conducted on the same experimental data.
- One of the basic rules ('laws') of probability:
  - The Multiplicative Rule: The probability of the joint occurrence of 2 or more independent events is the product of the individual probabilities.

$$P(A,B) = P(A) \times P(B)$$

For example:

$$P(2\ Heads) = P(head) \times P(head) = 0.5 \times 0.5 = 0.25$$

Familywise error rate

- **<u>Example</u>**: All pairwise comparisons between 3 groups A, B and C:
  - A-B, A-C and B-C
- Probability of making the Type I Error: **5%**
  - The probability of <u>not making the Type I Error</u> is 95% (=1 – 0.05)
- Multiplicative Rule:
  - Overall probability of <u>no Type I errors</u> is: 0.95 * 0.95 * 0.95 = 0.857
- So the probability of making <u>at least one Type I Error</u> is 1-0.857 = 0.143 or **14.3%**
  - The probability has increased from 5% to 14.3%
- Comparisons between 5 groups instead of 3, the familywise error rate is **40%** (=1-$(0.95)^n$)

Familywise error rate

- Solution to the increase of familywise error rate: correction for multiple comparisons
  - **Post-hoc tests**

- Many different ways to correct for multiple comparisons:
  - Different statisticians have designed corrections addressing different issues
    - e.g. unbalanced design, heterogeneity of variance, liberal vs conservative

- However, they all have **one thing in common**:
  - the more tests, the higher the familywise error rate: the more stringent the correction

- Tukey, Bonferroni, Sidak, Benjamini-Hochberg …
  - Two ways to address the multiple testing problem
    - **Familywise Error Rate** (FWER) vs. **False Discovery Rate** (FDR)

Multiple testing problem

- **FWER: Bonferroni**: α_{adjust} = 0.05/n comparisons e.g. 3 comparisons: 0.05/3=0.016
  - Problem: very conservative leading to loss of power (lots of false negative)
  - 10 comparisons: threshold for significance: 0.05/10: 0.005
  - Pairwise comparisons across 20.000 genes ☹

- **FDR: Benjamini-Hochberg**: the procedure controls the expected proportion of "discoveries" (significant tests) that are false (false positive).
  - Less stringent control of Type I Error than FWER procedures which control the probability of at least one Type I Error
  - More power at the cost of increased numbers of Type I Errors.

- **Difference between FWER and FDR**:
  - a p-value of 0.05 implies that 5% of all tests will result in false positives.
  - a FDR adjusted p-value (or **q-value**) of 0.05 implies that 5% of significant tests will result in false positives.

One-Way Analysis of variance

Step 1: Omnibus test

- It tells us if there is (or not) a difference between the means but not which means are significantly different from which other ones.

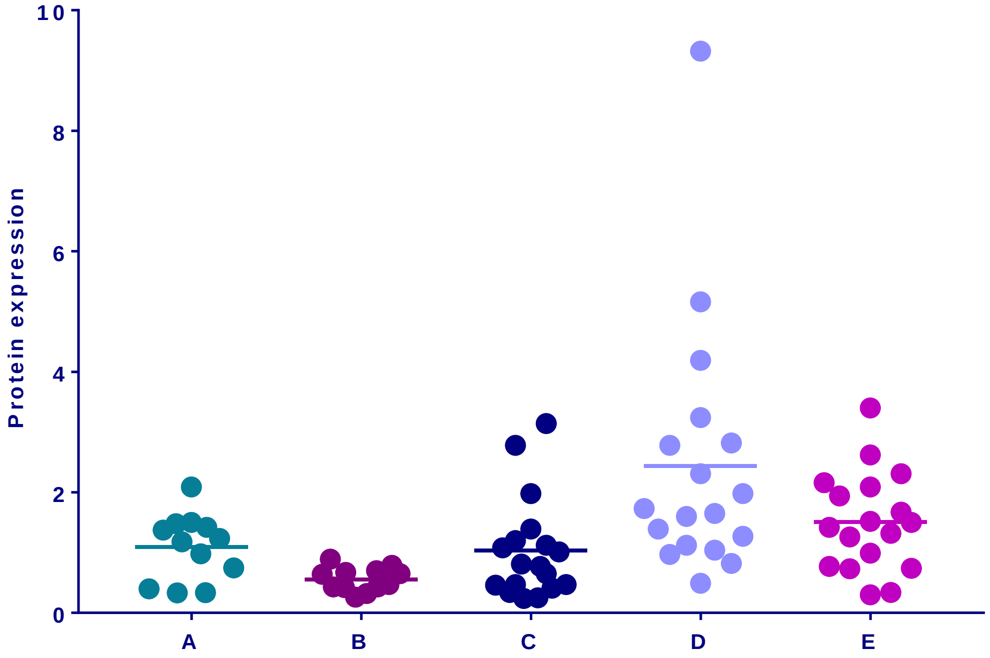
Step 2: Post-hoc tests

- They tell us if there are (or not) differences between the means pairwise.
- A correction for multiple comparisons will be applied on the p-values.
- These post hoc tests should only be used when the ANOVA finds a significant effect.

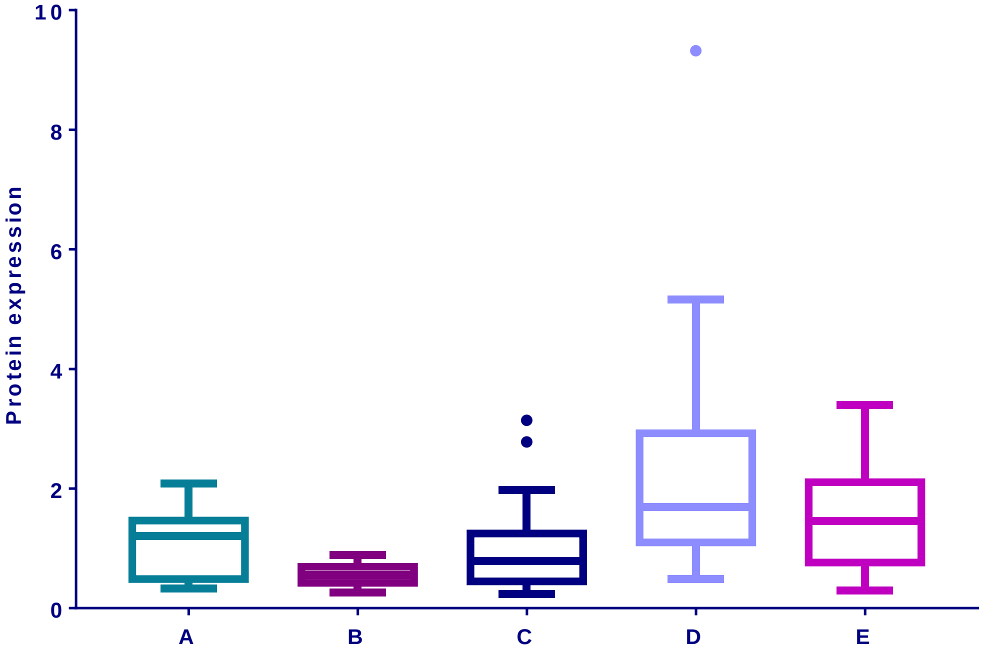
Exercise: One-way ANOVA
protein expression.xlsx

- **Question**: is there a difference in protein expression between the 5 cell lines?

- **1 Plot the data**
- **2 Check the assumptions for parametric test**



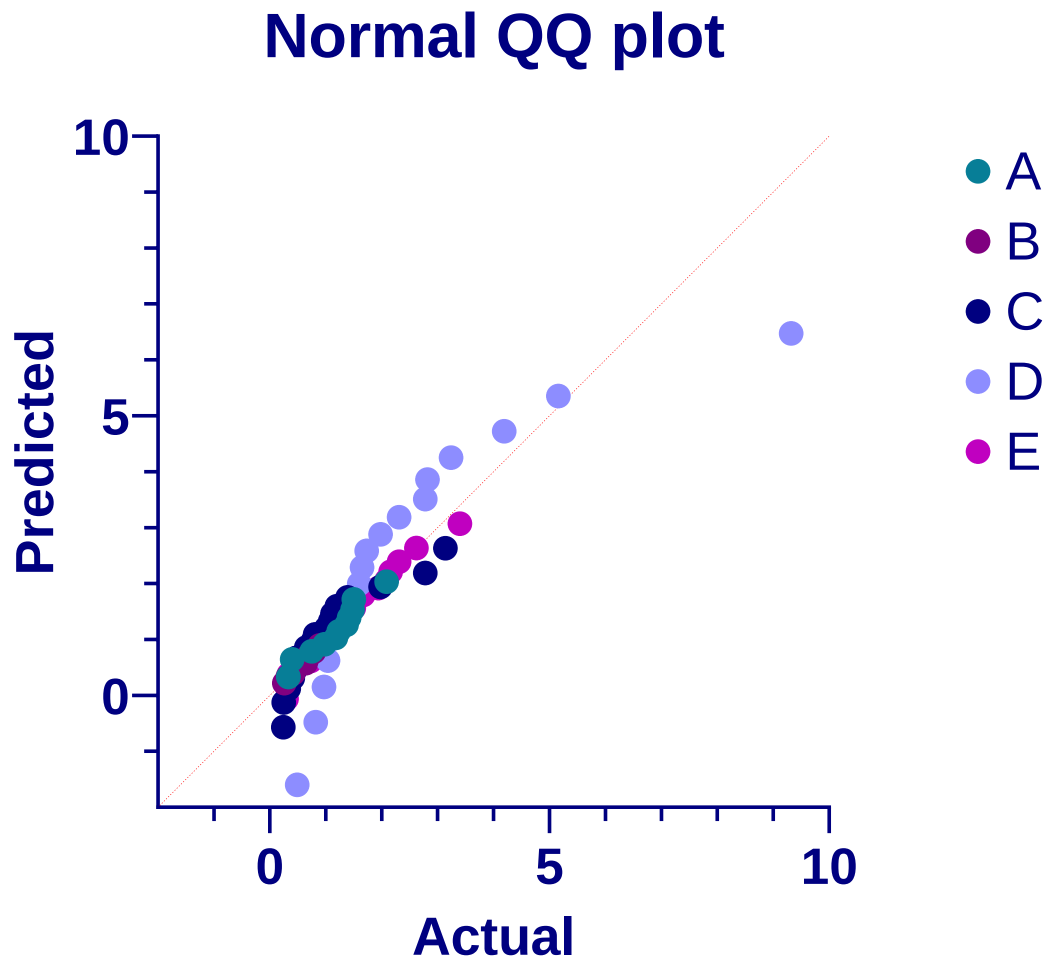
Protein expression
10
8
6
4
2
0
A
B
C
D
E

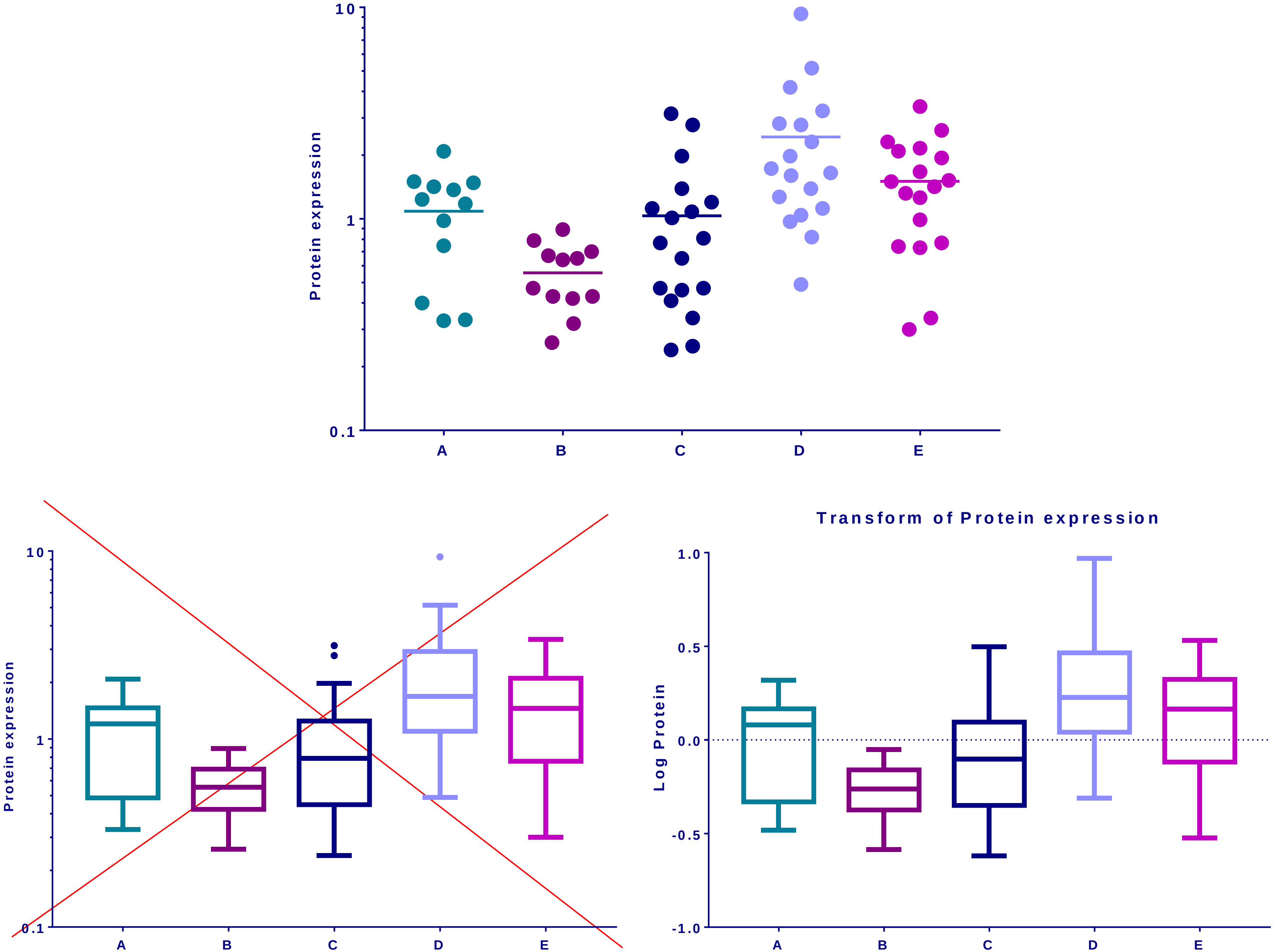


Protein expression
10
8
6
4
2
0
A
B
C
D
E

Parametric tests assumptions

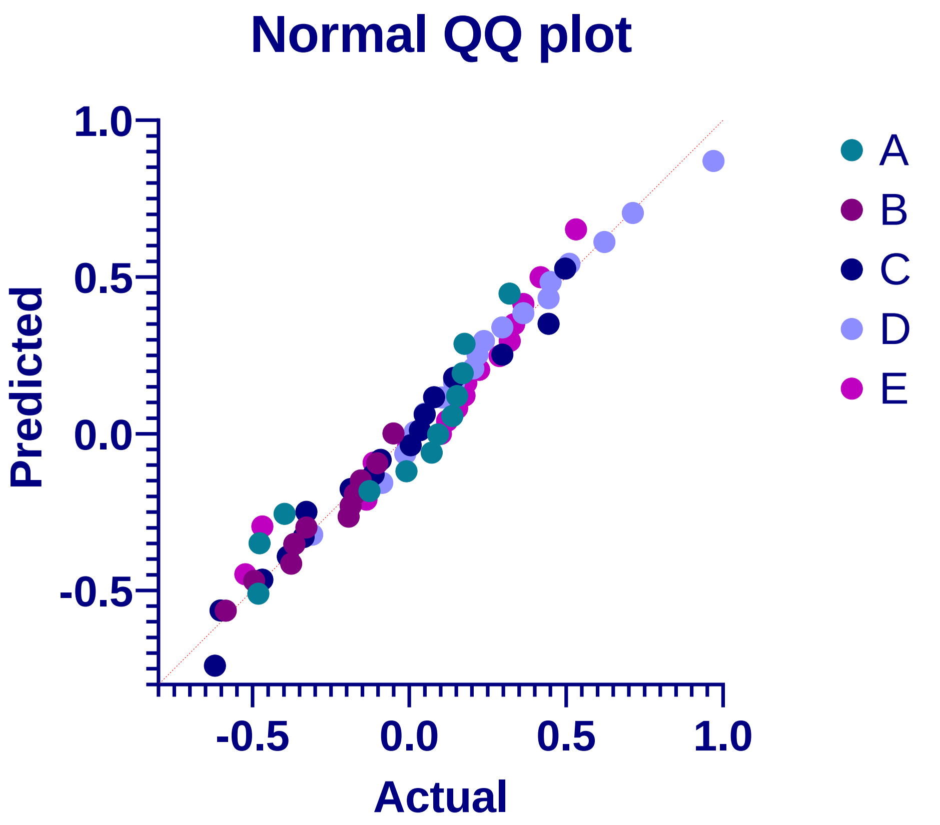
1	Test for normal distribution					
2	Anderson-Darling test					
3	A2*	0.3797	0.3141	1.166	1.439	0.2011
4	P value	0.3446	0.5029	0.0035	0.0007	0.8590
5	Passed normality test (alpha=0.05)?	Yes	Yes	No	No	Yes
6	P value summary	ns	ns	**	***	ns
7						
8	D'Agostino & Pearson test					
9	K2	0.1236	0.7508	9.375	22.59	1.280
10	P value	0.9401	0.6870	0.0092	<0.0001	0.5274
11	Passed normality test (alpha=0.05)?	Yes	Yes	No	No	Yes
12	P value summary	ns	ns	**	****	ns
13						
14	Shapiro-Wilk test					
15	W	0.9295	0.9535	0.8197	0.7531	0.9671
16	P value	0.3752	0.6888	0.0029	0.0004	0.7411
17	Passed normality test (alpha=0.05)?	Yes	Yes	No	No	Yes
18	P value summary	ns	ns	**	***	ns
19						
20	Kolmogorov-Smirnov test					
21	KS distance	0.1485	0.1704	0.1980	0.2058	0.1035
22	P value	>0.1000	>0.1000	0.0603	0.0424	>0.1000
23	Passed normality test (alpha=0.05)?	Yes	Yes	Yes	No	Yes
24	P value summary	ns	ns	ns	*	ns
25						
26	Number of values	12	12	18	18	18



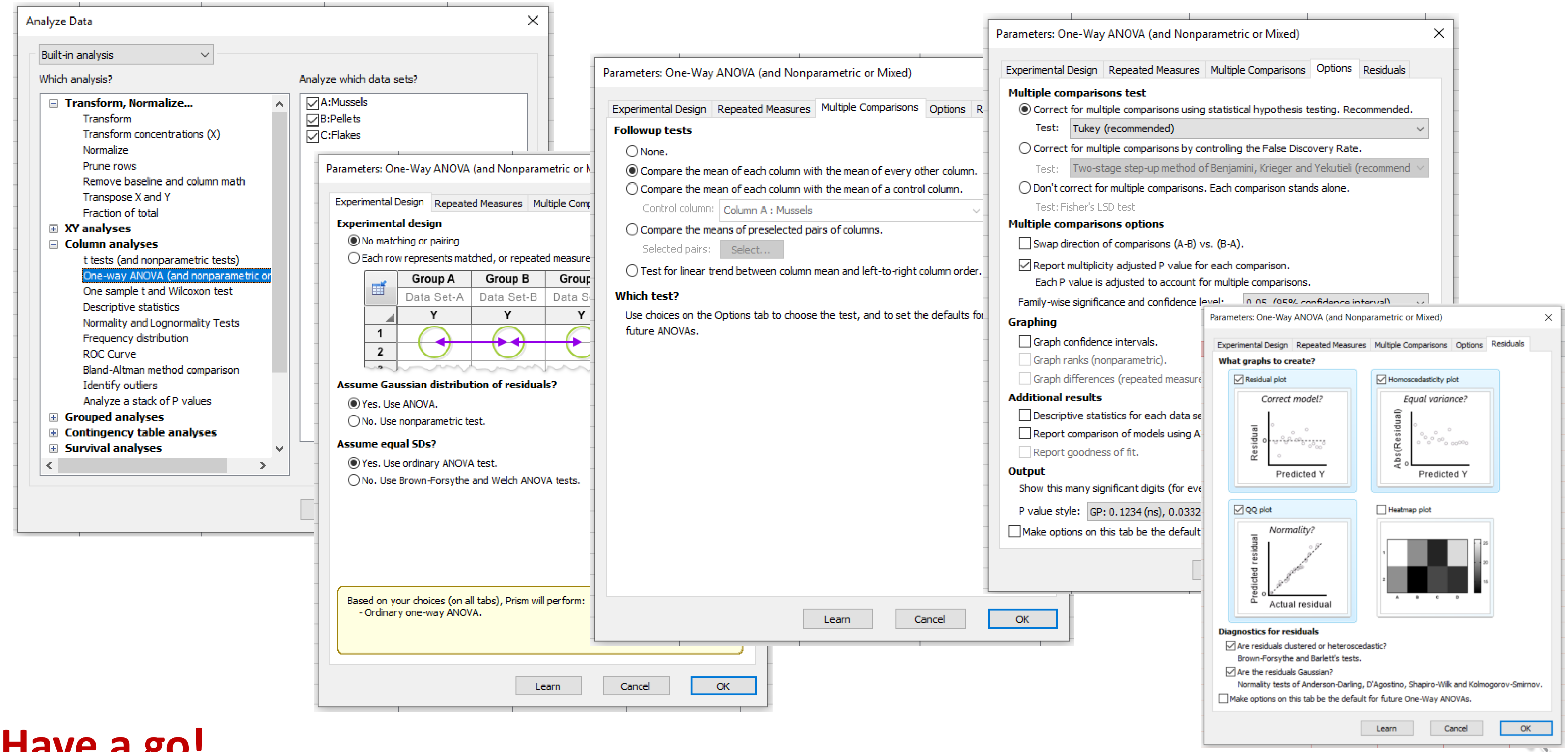

10
1
0.1
Protein expression
A
B
C
D
E
10
1
0.1
Protein expression
A
B
C
D
E
Transform of Protein expression
1.0
0.5
0.0
-0.5
-1.0
Log Protein
A
B
C
D
E

Parametric tests assumptions

1	Test for normal distribution					
2	Anderson-Darling test					
3	A2*	0.7849	0.3412	0.2086	0.1524	0.4727
4	P value	0.0295	0.4303	0.8386	0.9495	0.2138
5	Passed normality test (alpha=0.05)?	No	Yes	Yes	Yes	Yes
6	P value summary	*	ns	ns	ns	ns
7						
8	D'Agostino & Pearson test					
9	K2	2.037	0.6827	0.5884	0.8869	2.902
10	P value	0.3611	0.7108	0.7451	0.6418	0.2344
11	Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes	Yes
12	P value summary	ns	ns	ns	ns	ns
13						
14	Shapiro-Wilk test					
15	W	0.8553	0.9458	0.9657	0.9868	0.9313
16	P value	0.0427	0.5773	0.7142	0.9935	0.2050
17	Passed normality test (alpha=0.05)?	No	Yes	Yes	Yes	Yes
18	P value summary	*	ns	ns	ns	ns
19						
20	Kolmogorov-Smirnov test					
21	KS distance	0.2278	0.2049	0.1373	0.1016	0.1646
22	P value	0.0857	>0.1000	>0.1000	>0.1000	>0.1000
23	Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes	Yes
24	P value summary	ns	ns	ns	ns	ns
25						
26	Number of values	12	12	18	18	18



One-Way ANOVA in Prism 8



Have a go!

Analysis of variance: results

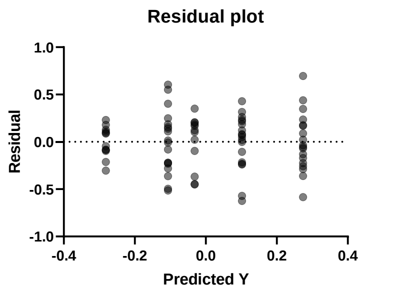
Ordinary one-way ANOVA
ANOVA results

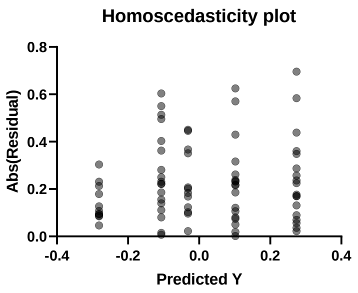
1	Table Analyzed	Transform of Protein expression			
2	Data sets analyzed	A-E			
3					
4	**ANOVA summary**				
5	F	8.127			
6	P value	<0.0001			
7	P value summary	****			
8	Significant diff. among means (P < 0.05)?	Yes			
9	R square	0.3081			
10					
11	**Brown-Forsythe test**				
12	F (DFn, DFd)	0.9831 (4, 73)			
13	P value	0.4222			
14	P value summary	ns			
15	Are SDs significantly different (P < 0.05)?	No			
16					
17	**Bartlett's test**				
18	Bartlett's statistic (corrected)	5.829			
19	P value	0.2123			
20	P value summary	ns			
21	Are SDs significantly different (P < 0.05)?	No			
22					
23	**ANOVA table**	**SS**	**DF**	**MS**	
24	Treatment (between columns)	2.691	4	0.6727	
25	Residual (within columns)	6.043	73	0.08278	
26	Total	8.734	77		
27					
28	**Normality of Residuals**				
29	**Test name**	**Statistics**	**P value**	**Passed normality test (al**	
30	Anderson-Darling (A2*)	0.4188	0.3201	Yes	
31	D'Agostino-Pearson omnibus (K2)	0.1697	0.9187	Yes	
32	Shapiro-Wilk (W)	0.9863	0.5717	Yes	
33	Kolmogorov-Smirnov (distance)	0.07889	0.1000	Yes	
34					

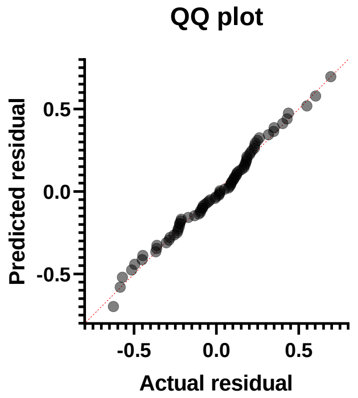
Homogeneity of variance ☑

F=0.6727/0.08278=8.13

Normality ☑



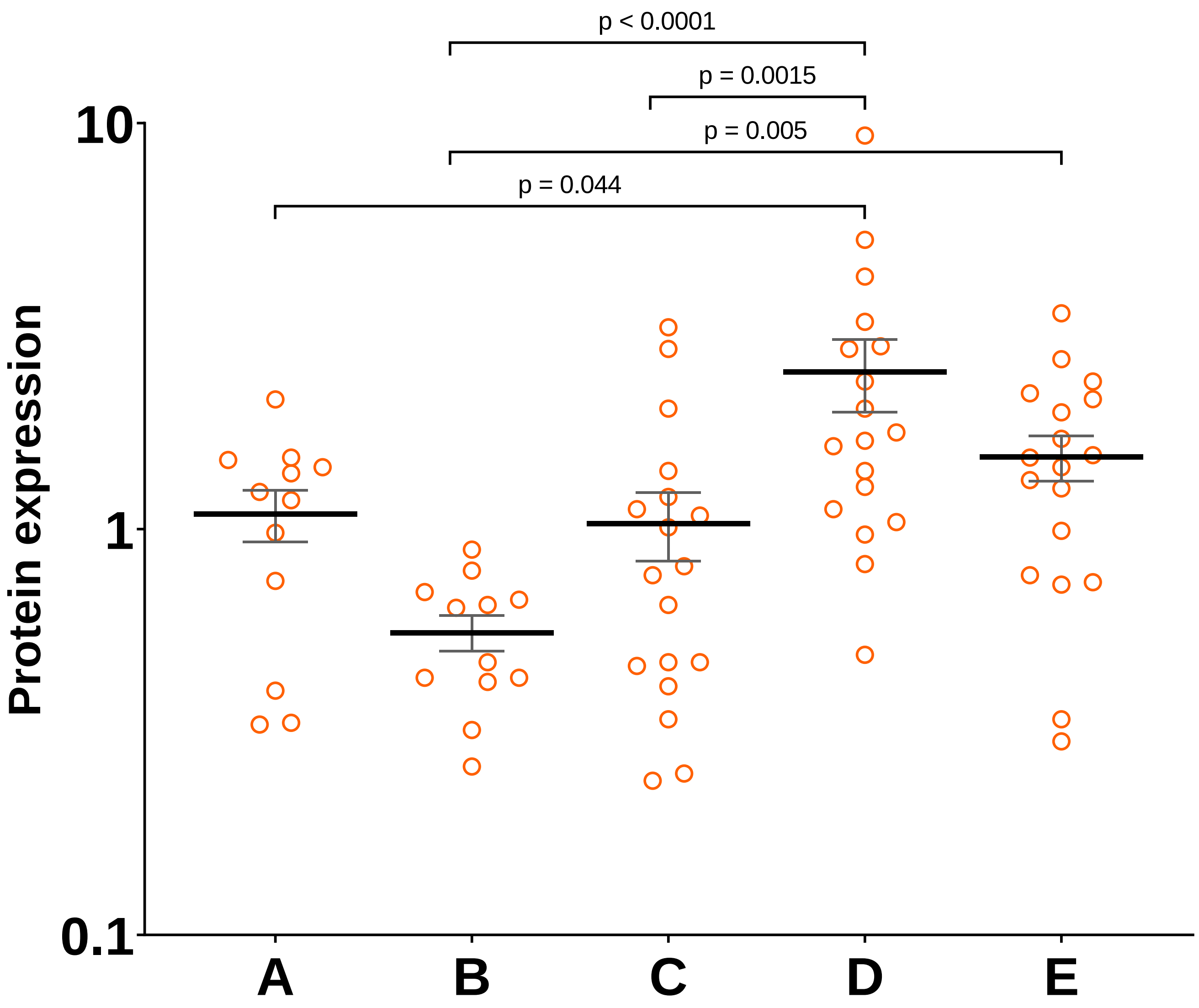




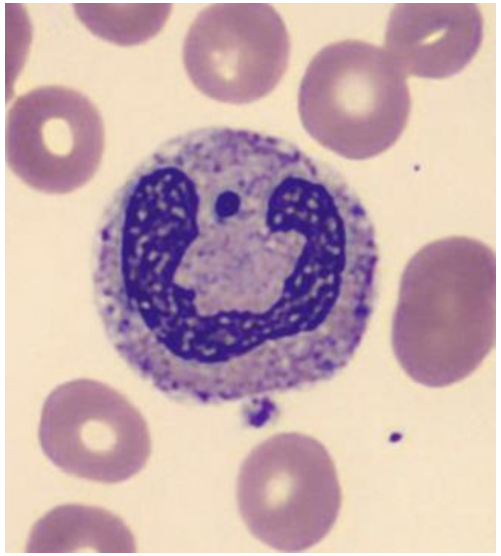
Ordinary one-way ANOVA
Multiple comparisons

1	Number of families	1							
2	Number of comparisons per family	10							
3	Alpha	0.05							
4									
5	**Tukey's multiple comparisons test**	**Mean Diff.**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**			
6	A vs. B	0.2505	-0.07808 to 0.5790	No	ns	0.2177	A-B		
7	A vs. C	0.07521	-0.2247 to 0.3751	No	ns	0.9555	A-C		
8	A vs. D	-0.3053	-0.6052 to -0.005359	Yes	*	0.0440	A-D		
9	A vs. E	-0.1331	-0.4330 to 0.1669	No	ns	0.7275	A-E		
10	B vs. C	-0.1753	-0.4752 to 0.1247	No	ns	0.4807	B-C		
11	B vs. D	-0.5557	-0.8557 to -0.2558	Yes	****	<0.0001	B-D		
12	B vs. E	-0.3835	-0.6834 to -0.08360	Yes	**	0.0055	B-E		
13	C vs. D	-0.3805	-0.6487 to -0.1122	Yes	**	0.0015	C-D		
14	C vs. E	-0.2083	-0.4765 to 0.05998	No	ns	0.2021	C-E		
15	D vs. E	0.1722	-0.09604 to 0.4405	No	ns	0.3839	D-E		
16									
17	**Test details**	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**	**n2**	**q**	**DF**
18	A vs. B	-0.03123	-0.2817	0.2505	0.1175	12	12	3.016	73
19	A vs. C	-0.03123	-0.1064	0.07521	0.1072	12	18	0.9920	73
20	A vs. D	-0.03123	0.2740	-0.3053	0.1072	12	18	4.026	73
21	A vs. E	-0.03123	0.1018	-0.1331	0.1072	12	18	1.755	73
22	B vs. C	-0.2817	-0.1064	-0.1753	0.1072	12	18	2.311	73
23	B vs. D	-0.2817	0.2740	-0.5557	0.1072	12	18	7.330	73
24	B vs. E	-0.2817	0.1018	-0.3835	0.1072	12	18	5.058	73
25	C vs. D	-0.1064	0.2740	-0.3805	0.09590	18	18	5.611	73
26	C vs. E	-0.1064	0.1018	-0.2083	0.09590	18	18	3.071	73
27	D vs. E	0.2740	0.1018	0.1722	0.09590	18	18	2.540	73
28									

Analysis of variance: results



Exercise: Repeated measures ANOVA
neutrophils.xlsx

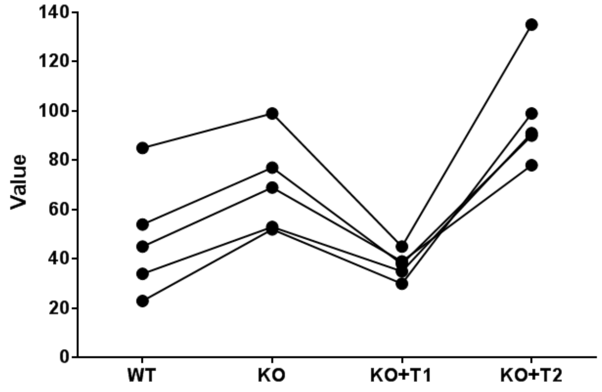


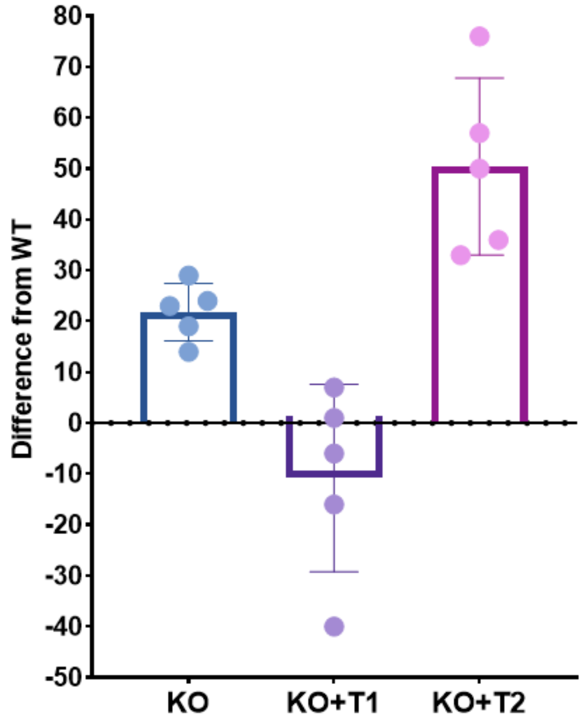
- A researcher is looking at the difference between 4 cell groups. He has run the experiment 5 times. Within each experiment, he has neutrophils from a WT (control), a KO, a KO+Treatment 1 and a KO+Treatment2.

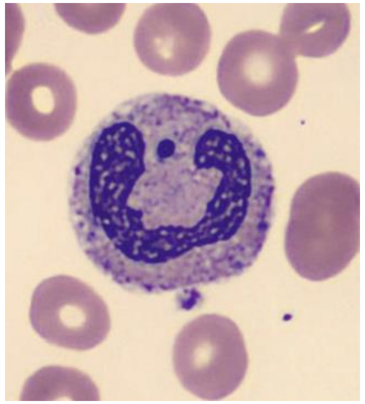
- **Question**: Is there a difference between KO with/without treatment and WT?

Exercise: Repeated measures ANOVA

neutrophils.xlsx







	ANOVA					
1	Table Analyzed	Repeated measures one-way ANOVA data2				
2						
3	Repeated measures ANOVA summary					
4	Assume sphericity?	No				
5	F	28.57				
6	P value	0.0002				
7	P value summary	***				
8	Statistically significant (P < 0.05)?	Yes				
9	Geisser-Greenhouse's epsilon	0.6916				
10	R square	0.8772				
11						
12	Was the matching effective?					
13	F	8.239				
14	P value	0.0020				
15	P value summary	**				
16	Is there significant matching (P < 0.05)?	Yes				
17	R square	0.2522				
18						
19	ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
20	Treatment (between columns)	10948	3	3649	F (2.075, 8.299) = 28.57	P = 0.0002
21	Individual (between rows)	4209	4	1052	F (4, 12) = 8.239	P = 0.0020
22	Residual (random)	1533	12	127.7		
23	Total	16689	19			
24						

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value	A-?	
WT vs. KO	-21.80	-30.91 to -12.69	Yes	**	0.0022	B	KO
WT vs. KO+T1	10.80	-19.02 to 40.62	No	ns	0.4941	C	KO+T1
WT vs. KO+T2	-50.40	-78.53 to -22.27	Yes	**	0.0067	D	KO+T2

Answer: There is a significant difference from WT for the first and third groups.

Comparison between more than 2 groups

Two factors = Two predictors

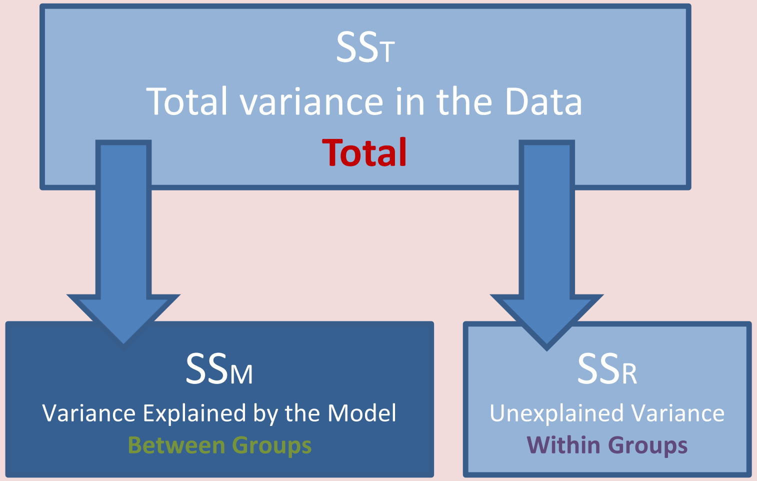
Two-Way ANOVA

Two-way Analysis of Variance (Factorial ANOVA)

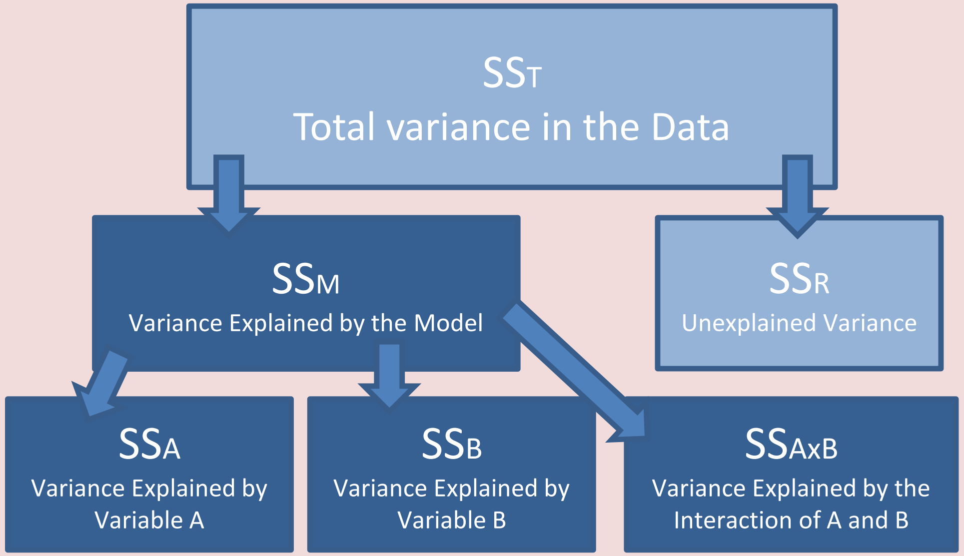
Source of variation	Sum of Squares	Df	Mean Square	F	p-value
Variable A (Between Groups)	2.665	4	0.6663	8.42	<0.0001
Within Groups (Residual)	5.775	73	0.0791		
Total	8.44	77			

Source of variation	Sum of Squares	Df	Mean Square	F	p-value
Variable A * Variable B	1978	2	989.1	F (2, 42) = 11.91	P < 0.0001
Variable B (Between groups)	3332	2	1666	F (2, 42) = 20.07	P < 0.0001
Variable A (Between groups)	168.8	1	168.8	F (1, 42) = 2.032	P = 0.1614
Residuals	3488	42	83.04		

One-way ANOVA= 1 predictor variable



2-way ANOVA= 2 predictor variables: A and B



Two-way Analysis of Variance

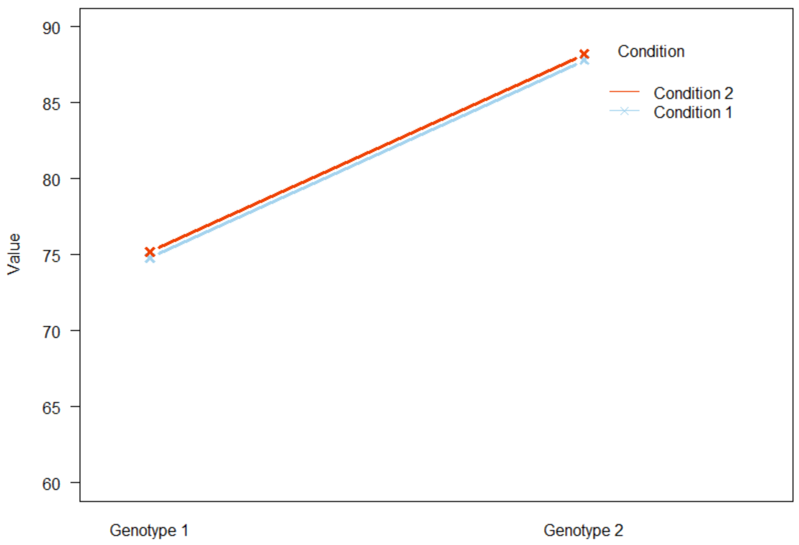
- **Interaction plots: Examples**
  - Fake dataset:
    - 2 factors: **Genotype** (2 levels) and **Condition** (2 levels)

Genotype	Condition	Value
Genotype 1	Condition 1	74.8
Genotype 1	Condition 1	65
Genotype 1	Condition 1	74.8
Genotype 1	Condition 2	75.2
Genotype 1	Condition 2	75
Genotype 1	Condition 2	75.2
Genotype 2	Condition 1	87.8
Genotype 2	Condition 1	65
Genotype 2	Condition 1	74.8
Genotype 2	Condition 2	88.2
Genotype 2	Condition 2	75
Genotype 2	Condition 2	75.2

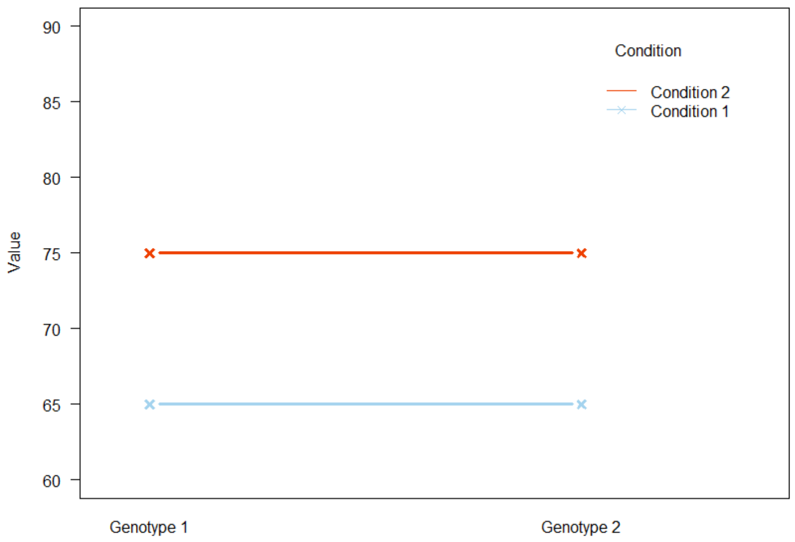
Two-way Analysis of Variance

- **<u>Interaction plots</u>: Examples**
  - <u>2 factors</u>: **Genotype** (2 levels) and **Condition** (2 levels)

<u>Single Effect</u>



Genotype Effect

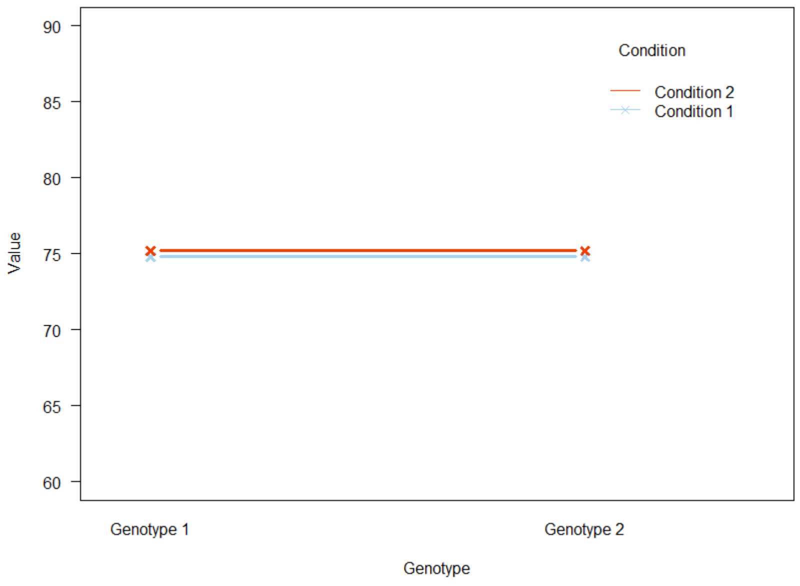


Condition Effect

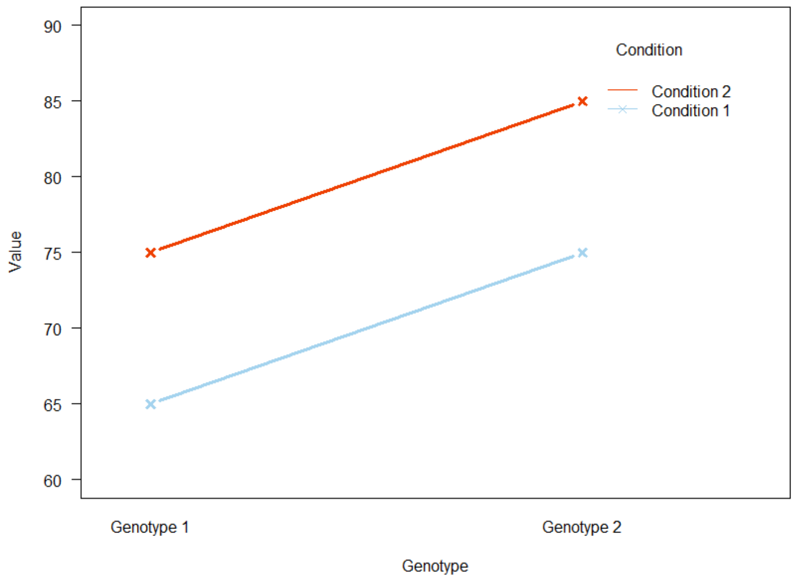
Two-way Analysis of Variance

- **Interaction plots: Examples**
  - 2 factors: **Genotype** (2 levels) and **Condition** (2 levels)

Zero or Both Effect



Zero Effect

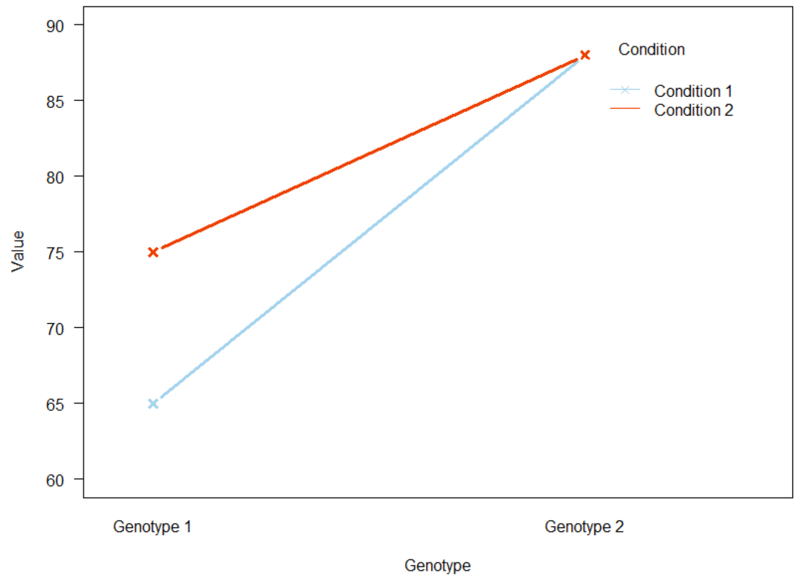


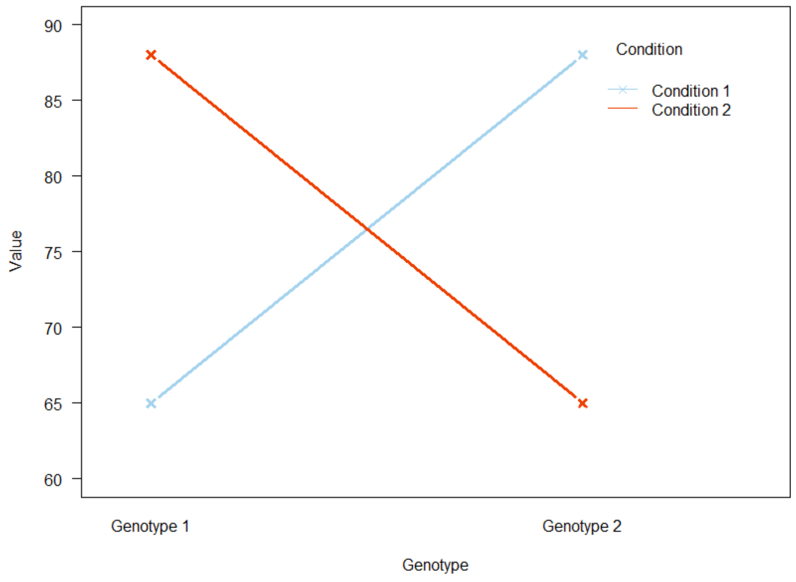
Both Effect

Two-way Analysis of Variance

- **<u>Interaction plots</u>: Examples**
  - <u>2 factors</u>: **Genotype** (2 levels) and **Condition** (2 levels)

<u>Interaction</u>





Two-way Analysis of Variance

Alcohol	None		2 Pints		4 Pints	
Gender	Female	Male	Female	Male	Female	Male
	65	50	70	55	45	30
	70	55	65	65	60	30
	60	80	60	70	85	30
	60	65	70	55	65	55
	60	70	65	55	70	35
	55	75	60	60	70	20
	60	75	60	50	80	45
	55	65	50	50	60	40

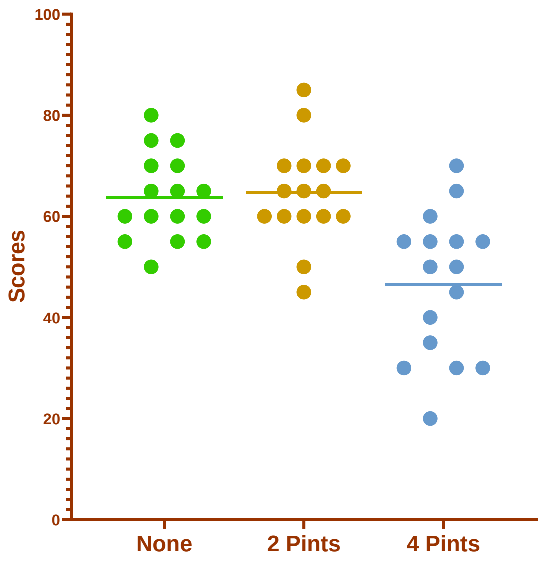
Example: goggles.xlsx

- The ‘beer-goggle’ effect
  - The term refers to finding people more attractive after you’ve had a few beers. Drinking beer provides a warm, friendly sensation, lowers your inhibitions, and helps you relax.

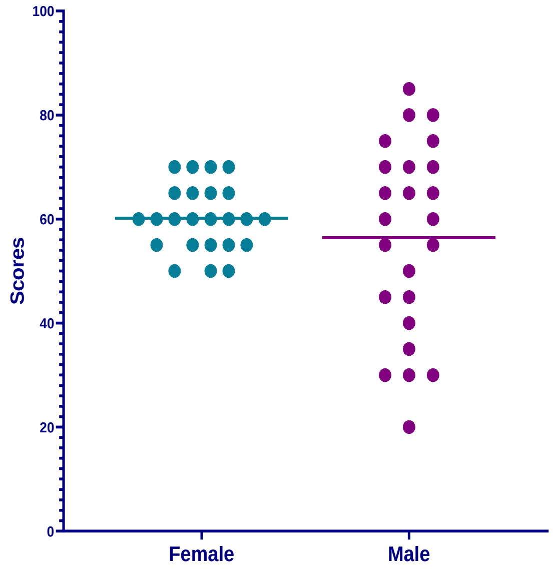
- Study: effects of alcohol on mate selection in night-clubs.
- Pool of independent judges scored the levels of attractiveness of the person that the participant was chatting up at the end of the evening.
- **Question**: is subjective perception of physical attractiveness affected by alcohol consumption?
  - Attractiveness on a scale from 0 to 100

Two-way Analysis of Variance

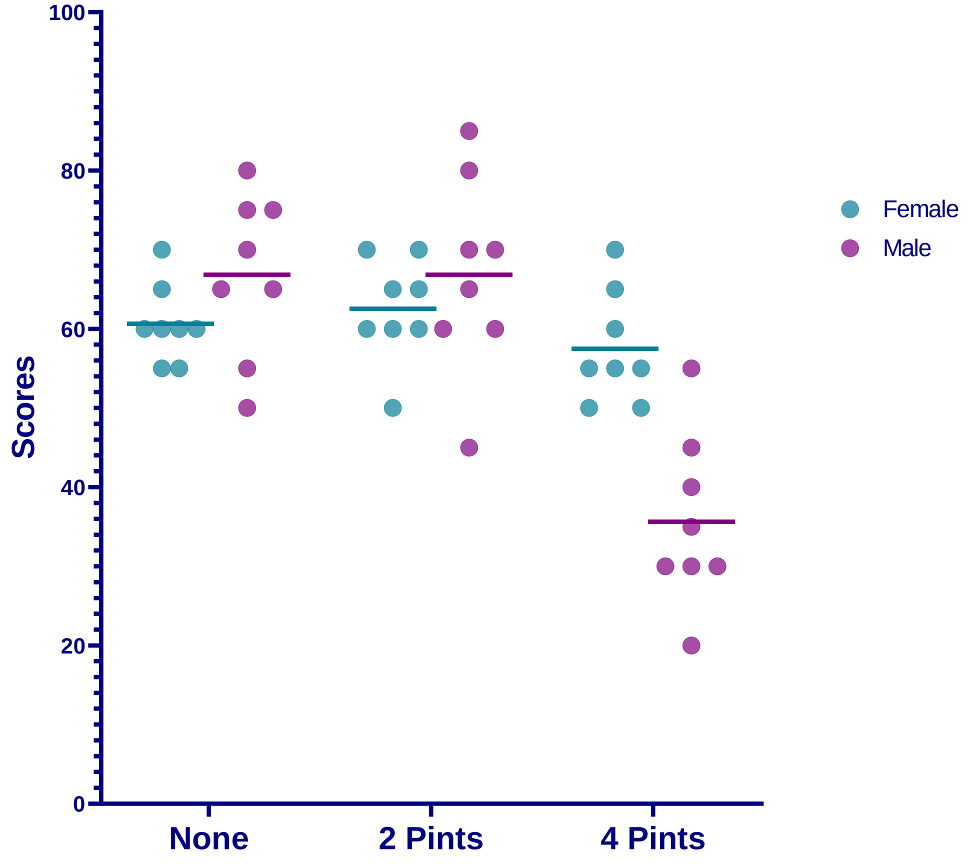
Main effect of Alcohol



Main effect of Gender



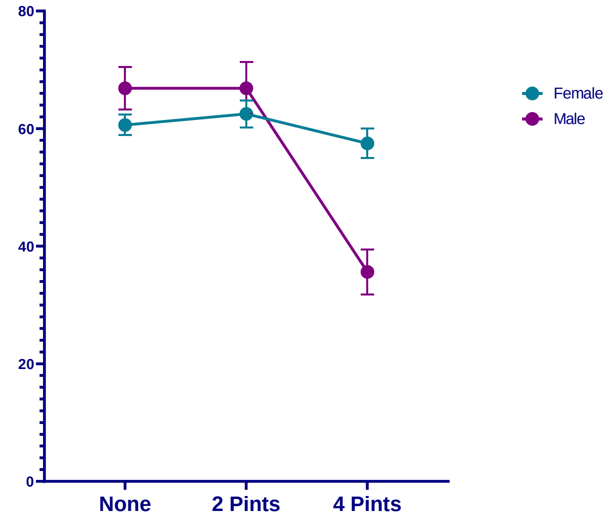
Interaction
between Alcohol and Gender



Two-way Analysis of Variance

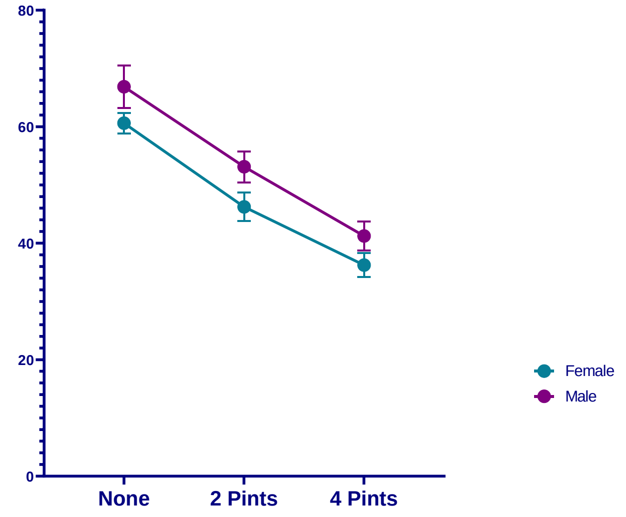
With significant interaction (real data)

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1978	2	989.1	F (2, 42) = 11.91	< 0.0001
Alcohol Consumption	3332	2	1666	F (2, 42) = 20.07	< 0.0001
Gender	168.8	1	168.8	F (1, 42) = 2.032	0.1614
Residual	3488	42	83.04		

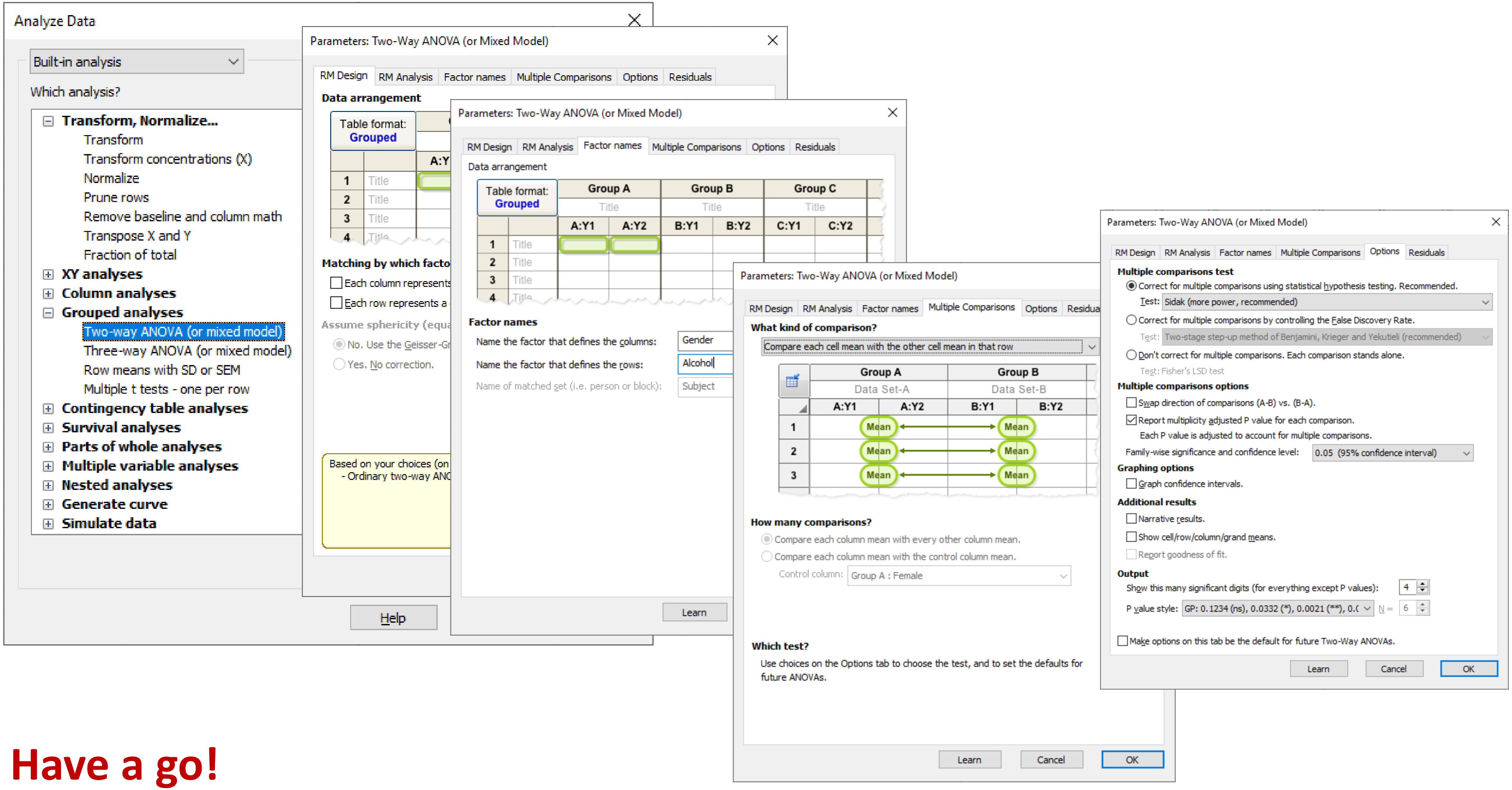


Without significant interaction (fake data)

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	7.292	2	3.646	F (2, 42) = 0.06872	0.9337
Alcohol Consumption	5026	2	2513	F (2, 42) = 47.37	< 0.0001
Gender	438.0	1	438.0	F (1, 42) = 8.257	0.0063
Residual	2228	42	53.05		



Two-way Analysis of Variance

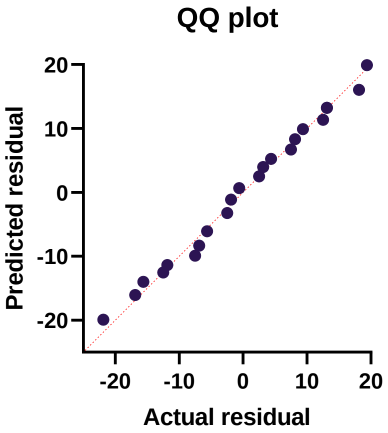


Have a go!

Two-way Analysis of Variance

2way ANOVA
ANOVA results

1	Table Analyzed	data for 2-way				
2						
3	**Two-way ANOVA**	Ordinary				
4	Alpha	0.05				
5						
6	**Source of Variation**	**% of total variation**	**P value**	**P value summary**	**Significant?**	
7	Interaction	22.06	<0.0001	****	Yes	
8	Alcohol Consumption	37.16	<0.0001	****	Yes	
9	Gender	1.882	0.1614	ns	No	
10						
11	**ANOVA table**	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
12	Interaction	1978	2	989.1	F (2, 42) = 11.91	P<0.0001
13	Alcohol Consumption	3332	2	1666	F (2, 42) = 20.07	P<0.0001
14	Gender	168.8	1	168.8	F (1, 42) = 2.032	P=0.1614
15	Residual	3488	42	83.04		



Sidak's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Female - Male					
None	-6.250	-17.58 to 5.080	No	ns	0.4434
2 Pints	-4.375	-15.70 to 6.955	No	ns	0.7157
4 Pints	21.88	10.55 to 33.20	Yes	****	<0.0001