# Biostatistics Workshop 1 – Repeated Measures Analysis

## …with GraphPad Prism

Musculoskeletal Research Center Educational Series

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# Acknowledgements

Deb Veis
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# Repeated Measures Data

- same quantity is measured repeatedly over time in the same experimental unit (e.g., subject, patient, mouse, a foot) = serial data
- hypotheses concern the change across the repeated measurements
  - $H_0$: Mean response is the same at all time points
  - $H_0$: Change in the mean response over time does not depend on the level of some between-subjects factor
- does not reduce the serial data into a single summary value
  - e.g., mean response, difference between 1$^{st}$ and last measurement, AUC, slope, etc.
  - must be based on biological considerations

# Fixed vs. Random Effect Factors

| Fixed | Random |
| --- | --- |
| levels are purposefully selected by a nonrandom process, or, the levels consist of the entire population of possible levels | levels consist of a random sample of levels from a population of possible levels |
| levels comprise the universe about which conclusions will be drawn | levels are a sample from the universe of interest |
| levels are fixed by design | may have different values if observed more than once at a single time |
| is observed without error | is observed with error |

# Why do I care?

ANOVA partitions variation among values into different components

- fixed effects include one component of variance
- random effects include two components of variance
- **To enter your data properly in Prism, you need to be able to identify your effects as fixed or random!**

# Repeated Measures Analysis

1. **Standard repeated measures ANOVA:**
   - treats all factors (subject, group or treatment, time) as fixed effects
   - thus, will exclude a subject with a missing value at any time point

# Repeated Measures Analysis

## 2. Mixed effects repeated measures analysis:

- allows both random & fixed effects
  - ❖subject is random effect
  - ❖group (or treatment), time, and interaction are fixed effects
- thus, allows for missing values
  - ❖specify the correlation of measurements across time (i.e., covariance structure)
  - ❖assumes missing at random

# Example Data Summary

- female mice from two genotypes:
  - wild-type, controls  (CON), N=10
  - experimental (CKO), N=9
- mice were measured at baseline (time zero), then all fed the high fat diet and re-measured weekly for 8 weeks
  - measurement time points are the same for all mice and evenly spaced
- outcome is weight (g)
  - there are some missing values
- hypothesis: “CKO mice will respond less to the high fat diet as compared to CON mice”
  - expect less weight gain in the CKO as compared to the controls
  - concerned with mean change, not rate of change
  - no hypotheses about specific time points

# Example Data Design

- Outcome variable:
  - change in weight from time zero
  - there are missing data
- Design:
  - Genotype → between subjects effect, fixed effect
  - Time → within subjects, fixed effect
  - Subject=Mouse → random effect
- Analysis: mixed effects repeated measures ANOVA

# Mixed Model Assumptions

1) Within-subjects factor consists of ≥ two categorical, 'related groups' (same subjects) or 'matched pairs'
   - ✓Time = same mice at each of 9 time points
2) Between-subjects factor consists of ≥ two categorical, independent groups
   - ✓Genotype = different mice for each of 2 groups
3) Outcome variable is continuously distributed
   - ✓Weight = theoretically continuous
   - ✓Plots of the *actual* data

# Mixed Model Assumptions

4) Outcome variable has no 'significant' outliers in any level of the within- or between-subjects factors
   - ✓Plots of the *actual* data
     - at each time point, regardless of genotype
     - for each genotype, regardless of time point
   - ✓Plots of residuals from the data *fitted* in the mixed model

5) Outcome variable is approximately normally distributed for each combination of the two factors
   - ✓Plots of the *actual* data and statistical tests
     - at each combination of genotype WITH time point
   - ✓Q-Q plots of residuals from the *fitted* data

# Mixed Model Assumptions

6) Homogeneity of variances for each combination of the two factors
   - ✓SDs of the *actual* data and statistical tests
     - at each combination of genotype WITH time point
7) Sphericity (or circularity) = Homogeneity of variances of the differences between the related groups of the within-subject factor for all groups of the between-subjects factor
   - ✓SDs of the difference between each pair of time points (regardless of genotype) in *actual* data and statistical indicators

# Mixed Model Assumptions

8) Properly specified covariance structure of repeated data
   - ✓Correlations between all pairs of time points in *actual* data
     Prism assumes compound symmetry: equal correlation between measurements from all pairs of time points - often unrealistic in repeated measures data
   - ✓Fit statistics from *fitted* model
     Not available in Prism

9) Data are missing at random: No association between the reason for missingness and the within- or between-subjects effects
   - ✓ No association between time or genotype with missingness, theoretically
   - ✓Evaluate the pattern of missingness in *actual* data

Assumptions are about the population(s) from which the data are sampled, not just the actual dataset.

Mixed models are robust to minor deviations.

# Analysis Steps

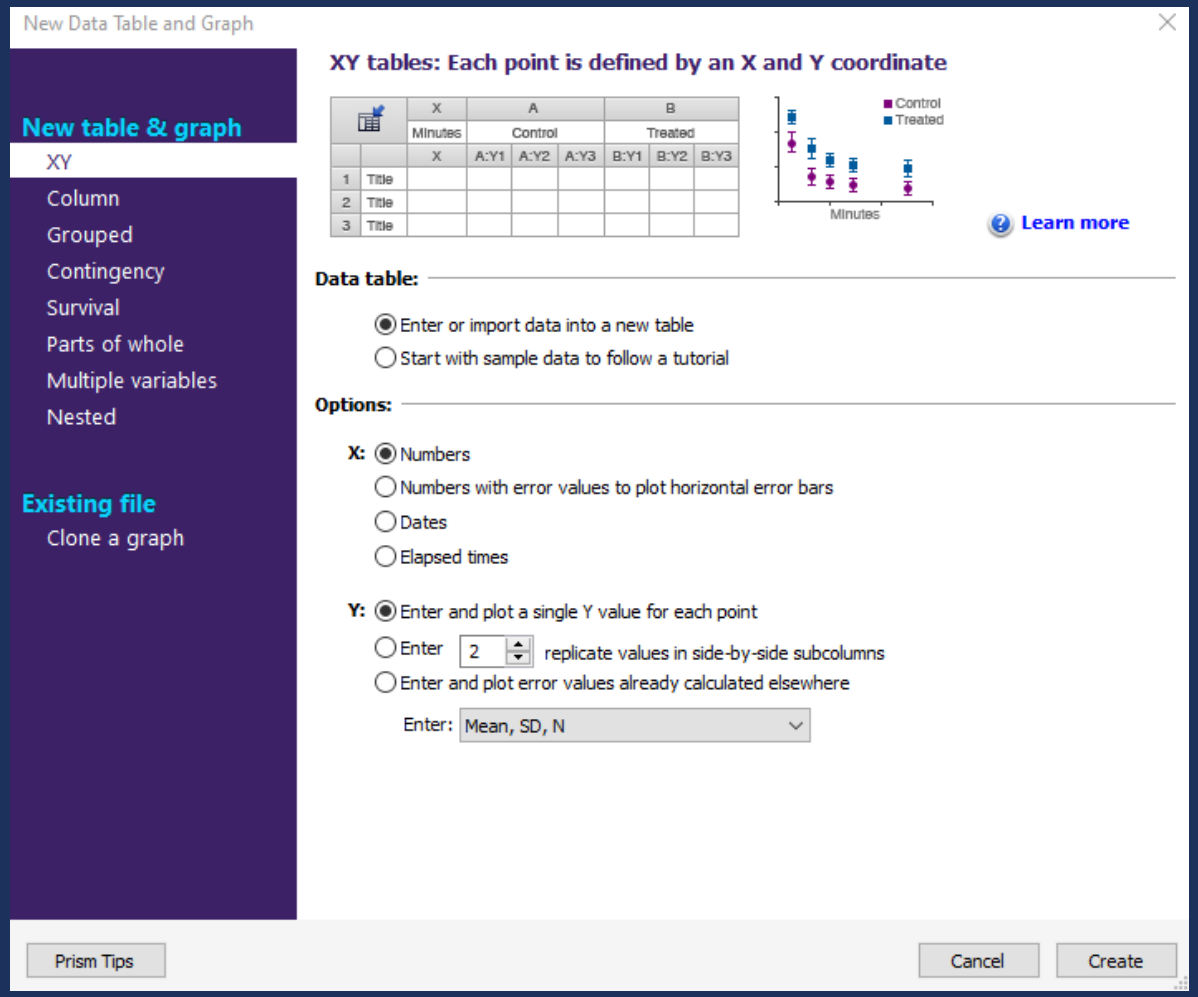
1) Assumptions: Determine if can adjust analytic model options to account for violations or determine if data need transformation prior to mixed model
2) Run the mixed model to determine model fit
3) Adjust model specifications until have the best model fit

   Not available in Prism
4) Run the final mixed model and examine p-values

# Setting-Up the Data in Prism

- the data arrangement in Prism depends upon the type of analysis
- you WILL need more than one arrangement to fully assess and analyze the data
- TIP: Immediately label the data and all output clearly in Prism so apparent what you did – Prism doesn't easily attach your modeling specification with the output

# Data for Assumptions




File > New Data Table and Graph > XY

Data table: Enter or import data into a new table

Options:

X Numbers

Y Enter and plot a single Y value for each point

# Data for Assumptions (subset)

Table Format

**Each column represents the level of one factor = Time (0-8)**

| Table format: XY | X | Group A | Group B | Group C | Group D | Group E | Group F | Group G | Group H | Group I |
|---|---|---|---|---|---|---|---|---|---|---|
| | ID | Time 0 | Time 1 | Time 2 | Time 3 | Time 4 | Time 5 | Time 6 | Time 7 | Time 8 |
| | X | Y | Y | Y | Y | Y | Y | Y | Y | Y |
| 1 | Title | 1 | 19.4 | 20.0 | 20.7 | 21.4 | 24.3 | 28.3 | 26.4 | 28.3 | 28.6 |
| 2 | Title | 2 | 20.2 | 21.0 | 21.6 | 22.0 | 23.1 | 24.6 | 24.8 | 25.0 | 25.7 |
| 3 | Title | 3 | 21.1 | 22.0 | 22.7 | 22.3 | 26.3 | 25.2 | 26.4 | 26.6 | 26.8 |
| 4 | Title | 4 | 18.2 | 19.5 | 20.1 | 21.5 | 22.9 | 24.2 | 28.0 | 25.7 | 26.3 |
| 5 | Title | 5 | 17.8 | 18.9 | 20.8 | 21.8 | 22.4 | 23.5 | 24.8 | 26.2 | 28.3 |
| 6 | Title | 6 | 19.4 | 20.9 | 20.2 | 21.6 | 23.0 | 24.7 | 25.8 | 28.0 | 29.2 |
| 7 | Title | 7 | 17.7 | 18.4 | 18.5 | 19.4 | 20.9 | 21.9 | 23.2 | | |
| 8 | Title | 8 | 18.4 | 19.0 | 19.7 | | | | | | |
| 9 | Title | 9 | 22.8 | 26.8 | 27.8 | | | | | | |

**Each row represents a different level of the other factor = Mouse**

# Summary Data (subset)

Analyze Data > Column Analysis > Descriptive statistics > *Select columns to analyze*

| | | A | B | C | D |
|---|---|---|---|---|---|
| | | Time 0 | Time 1 | Time 2 | Time 3 |
| 1 | Number of values | 9 | 9 | 9 | 7 |
| 2 | | | | | |
| 3 | Minimum | 17.70 | 18.40 | 18.50 | 19.40 |
| 4 | 25% Percentile | 18.00 | 18.95 | 19.90 | 21.40 |
| 5 | Median | 19.40 | 20.00 | 20.70 | 21.60 |
| 6 | 75% Percentile | 20.65 | 21.50 | 22.15 | 22.00 |
| 7 | Maximum | 22.80 | 26.80 | 27.80 | 22.30 |
| 8 | Range | 5.100 | 8.400 | 9.300 | 2.900 |
| 9 | | | | | |
| 10 | Mean | 19.44 | 20.72 | 21.34 | 21.43 |
| 11 | Std. Deviation | 1.694 | 2.559 | 2.691 | 0.9464 |

# Normality – Tests (subset)

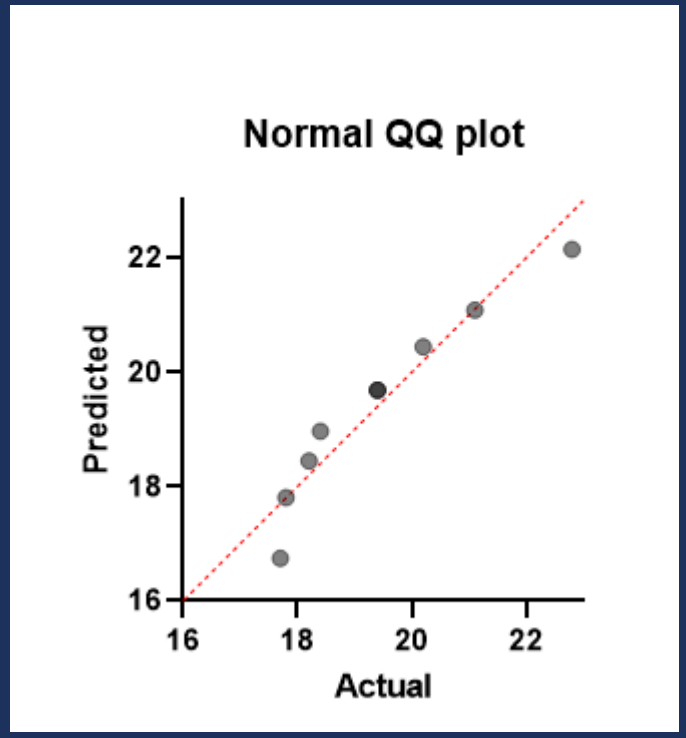
- Analyze Data > Column Analysis > Normality and Lognormality Tests *(see Appendix A for options)*
- non-significant p-values indicate that distribution does not deviate significantly from normal

| | Normality and Lognormality Tests | A<br>Time 0 | B<br>Time 1 | C<br>Time 2 | D<br>Time 3 |
|---|---|---|---|---|---|
| 1 | **Test for normal distribution** | | | | |
| 2 | **Anderson-Darling test** | | | | |
| 3 | A2* | 0.3572 | 0.7145 | 0.7981 | N too small |
| 4 | P value | 0.3687 | 0.0397 | 0.0234 | |
| 5 | Passed normality test (alpha=0.05)? | Yes | No | No | |
| 6 | P value summary | ns | * | * | |
| 7 | | | | | |
| 8 | **D'Agostino & Pearson test** | | | | |
| 9 | K2 | 2.207 | 11.87 | 12.82 | N too small |
| 10 | P value | 0.3317 | 0.0026 | 0.0016 | |
| 11 | Passed normality test (alpha=0.05)? | Yes | No | No | |
| 12 | P value summary | ns | ** | ** | |
| 13 | | | | | |
| 14 | **Shapiro-Wilk test** | | | | |
| 15 | W | 0.9071 | 0.8016 | 0.7984 | 0.7677 |
| 16 | P value | 0.2958 | 0.0213 | 0.0196 | 0.0193 |
| 17 | Passed normality test (alpha=0.05)? | Yes | No | No | No |
| 18 | P value summary | ns | * | * | * |
| 19 | | | | | |
| 20 | **Kolmogorov-Smirnov test** | | | | |
| 21 | KS distance | 0.1771 | 0.2346 | 0.2468 | 0.3451 |
| 22 | P value | >0.1000 | >0.1000 | >0.1000 | 0.0117 |
| 23 | Passed normality test (alpha=0.05)? | Yes | Yes | Yes | No |
| 24 | P value summary | ns | ns | ns | * |
| 25 | | | | | |
| 26 | **Number of values** | 9 | 9 | 9 | 7 |

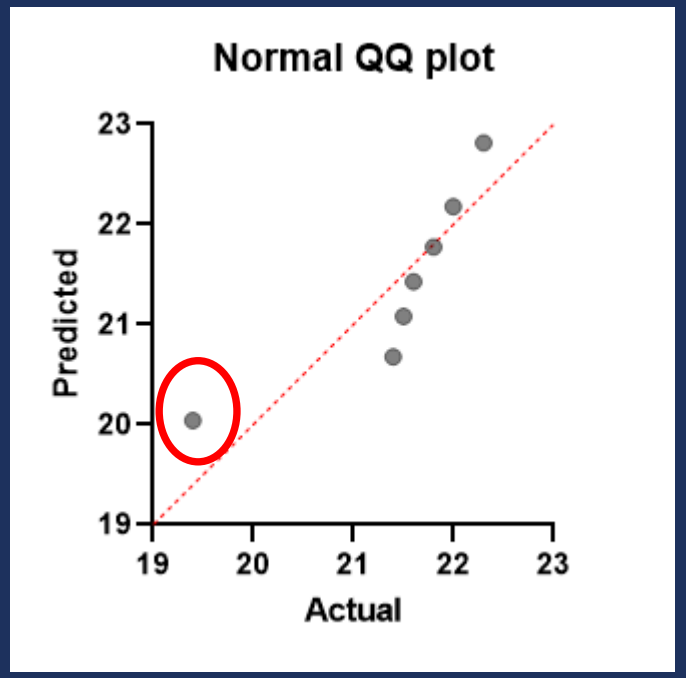
# Normality & Outliers - Plots

- Analyze Data > Column Analysis > Normality and Lognormality Tests *(see Appendix A for options)* > Create a QQ plot > *Select each column separately to get Q-Q plot of each time point*
- the dots will be linear, on the line of identify, if the data are truly sampled from a normal distribution

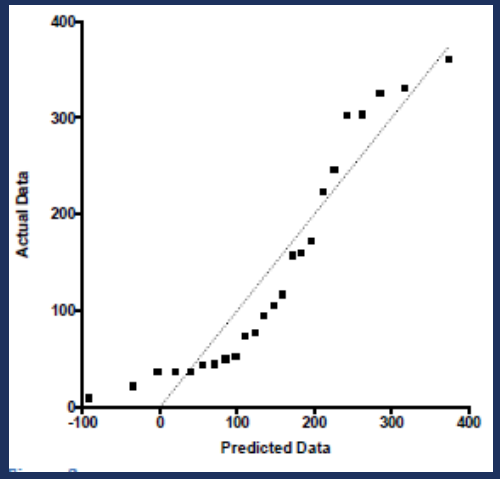
**CKO Time 0**




**CKO Time 3**

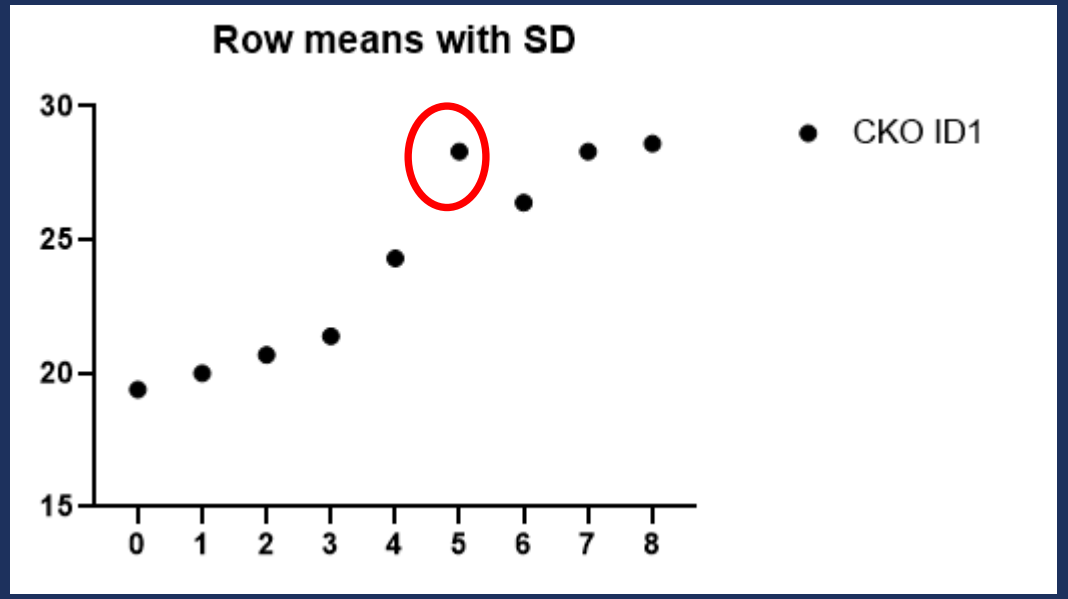


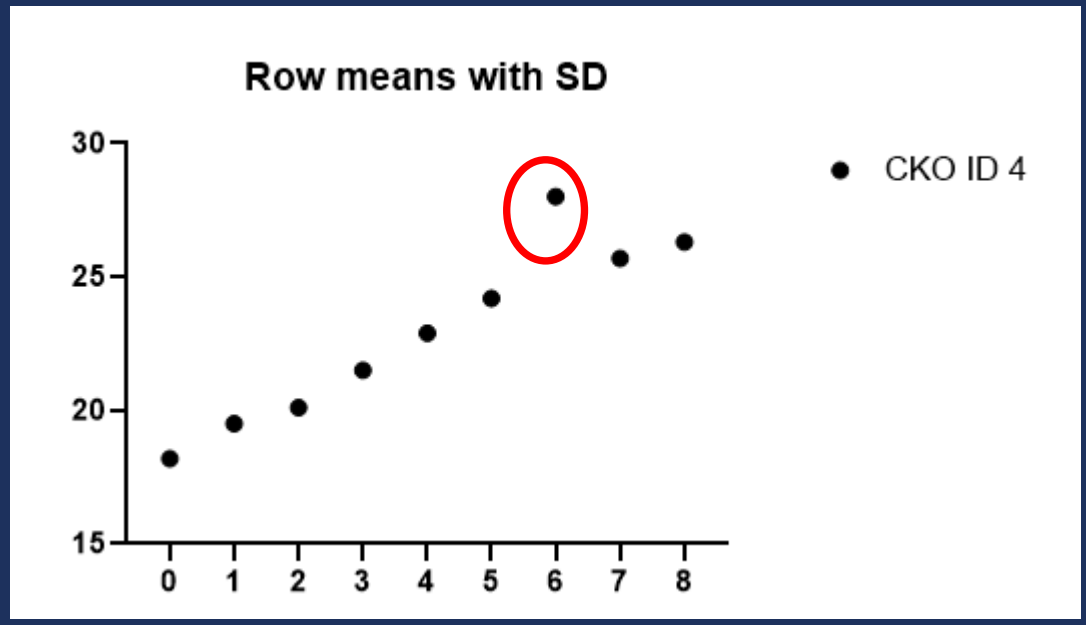

**example of log normal distribution**



# Outliers – Plots Within Mouse

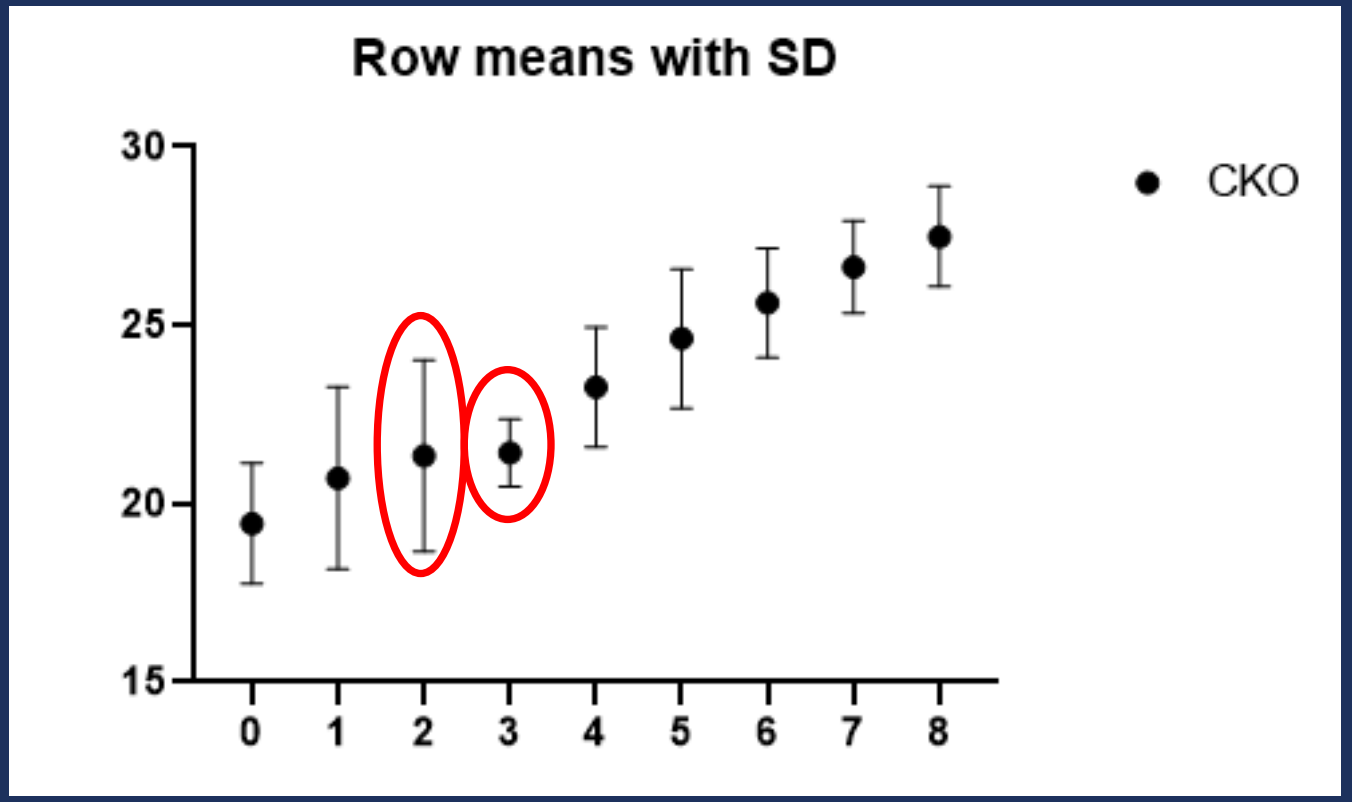
- Analyze Data > XY Analysis > Row statistics > Create a graph of the results
- *data needs to be in format used for the mixed model*



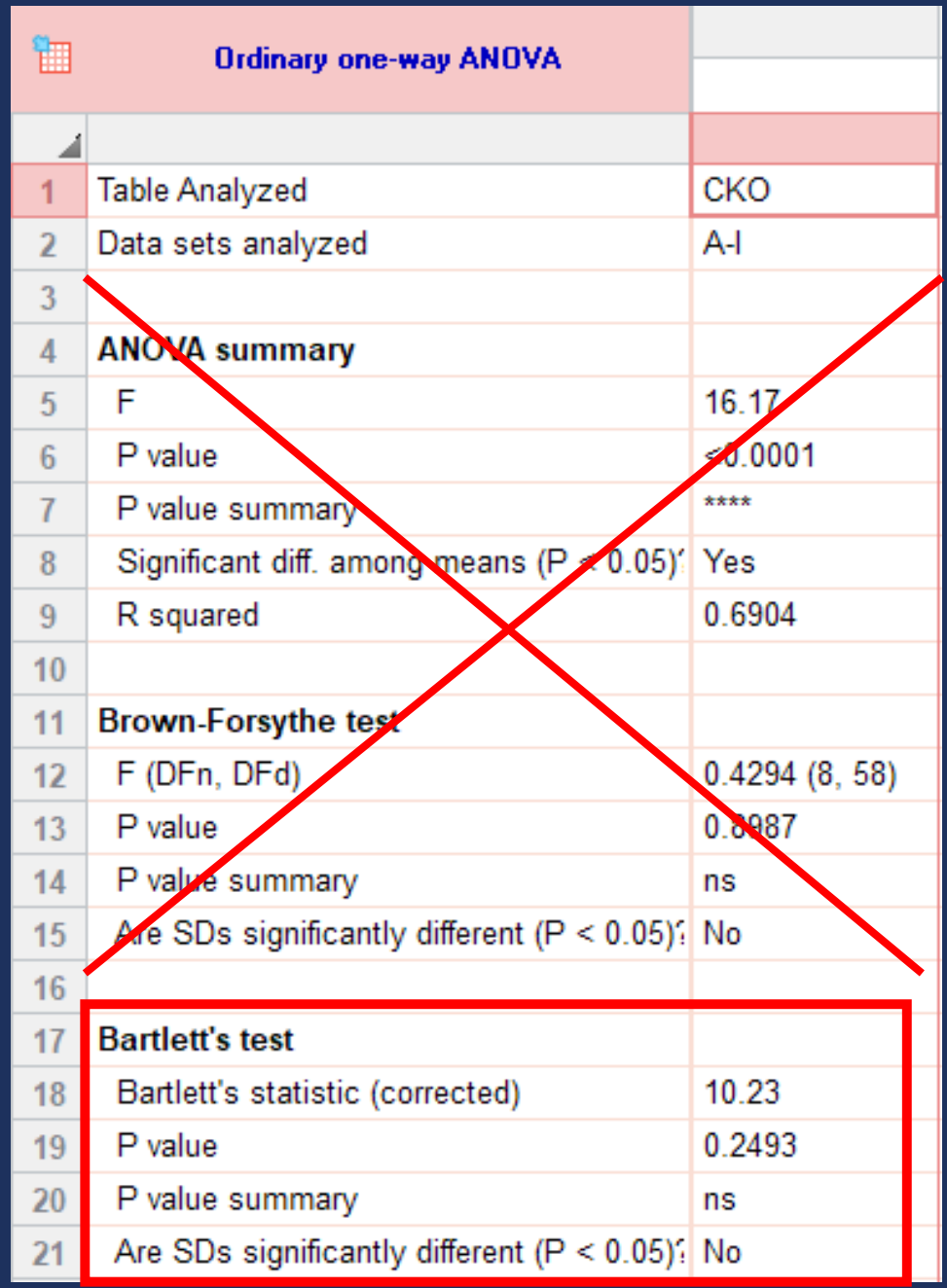
# Variances – Plots

- Analyze Data > XY Analysis > Row statistics > Create a graph of the results
- *data needs to be in format used for the mixed model*



# Variances – Tests

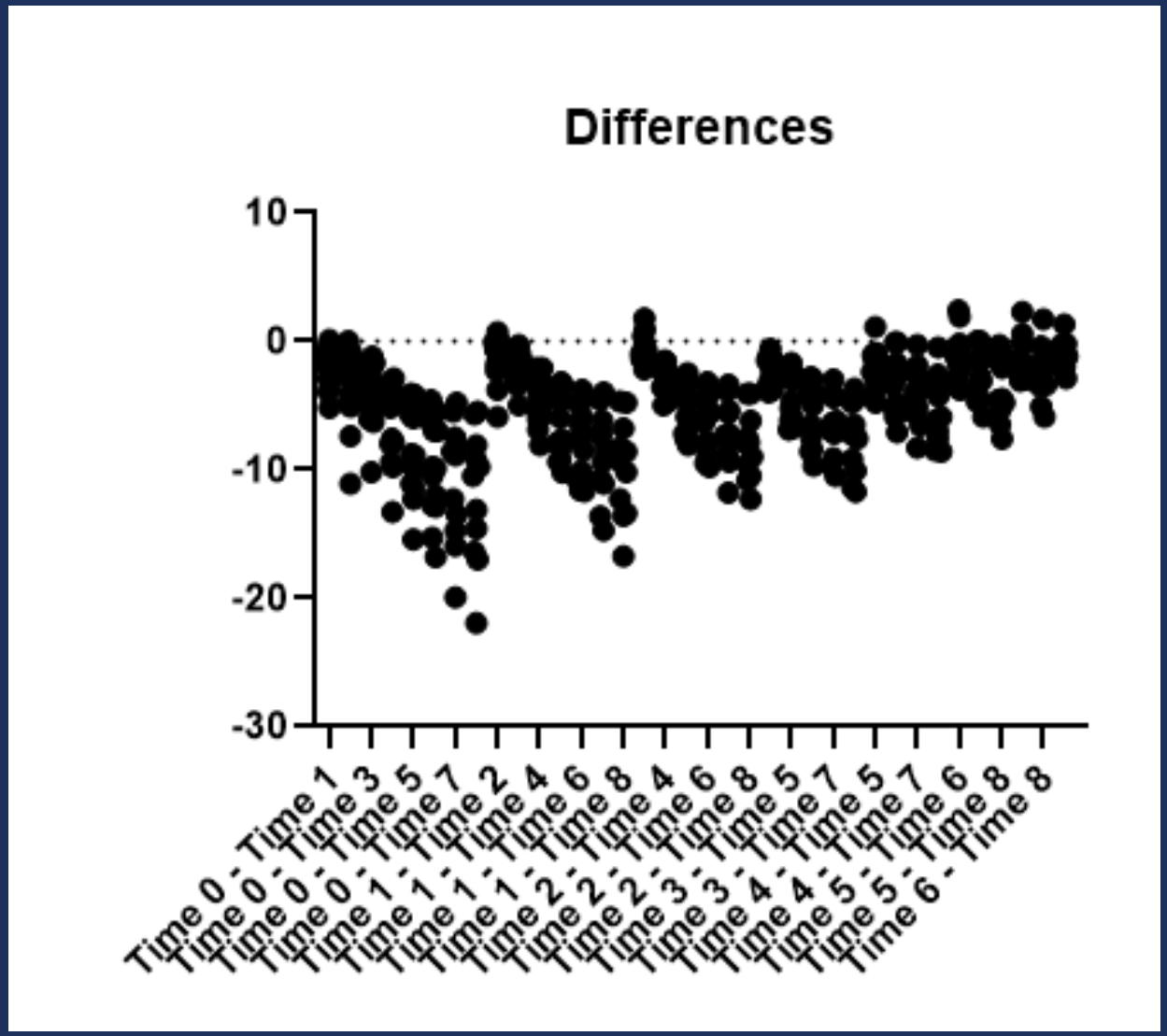
- Analyze Data > Column Analysis > One-way ANOVA > *Select all time points*
- Experimental Design Tab: No matching or pairing; Yes. Use ANOVA; Yes. Use ordinary ANOVA test.
- **ANOVA within genotype to test for homogeneity of variance across time points**



| | Ordinary one-way ANOVA | |
|---|---|---|
| 1 | Table Analyzed | CKO |
| 2 | Data sets analyzed | A-I |
| 3 | | |
| 4 | **ANOVA summary** | |
| 5 | F | 16.17 |
| 6 | P value | <0.0001 |
| 7 | P value summary | **** |
| 8 | Significant diff. among means (P < 0.05)? | Yes |
| 9 | R squared | 0.6904 |
| 10 | | |
| 11 | **Brown-Forsythe test** | |
| 12 | F (DFn, DFd) | 0.4294 (8, 58) |
| 13 | P value | 0.8987 |
| 14 | P value summary | ns |
| 15 | Are SDs significantly different (P < 0.05)? | No |
| 16 | | |
| 17 | **Bartlett's test** | |
| 18 | Bartlett's statistic (corrected) | 10.23 |
| 19 | P value | 0.2493 |
| 20 | P value summary | ns |
| 21 | Are SDs significantly different (P < 0.05)? | No |

# Variances – Sphericity

- Analyze Data > Column Analysis > One-way ANOVA > *Select all time points*
- Experimental Design Tab: Each row represents matched, or repeated measures, data; Yes. Use ANOVA
- Multiple Comparisons Tab: Compare the mean of each column with the mean of every other column
- Options Tab: Graph differences.
- **ANOVA of all mice to get visual of differences between each pair of time points**

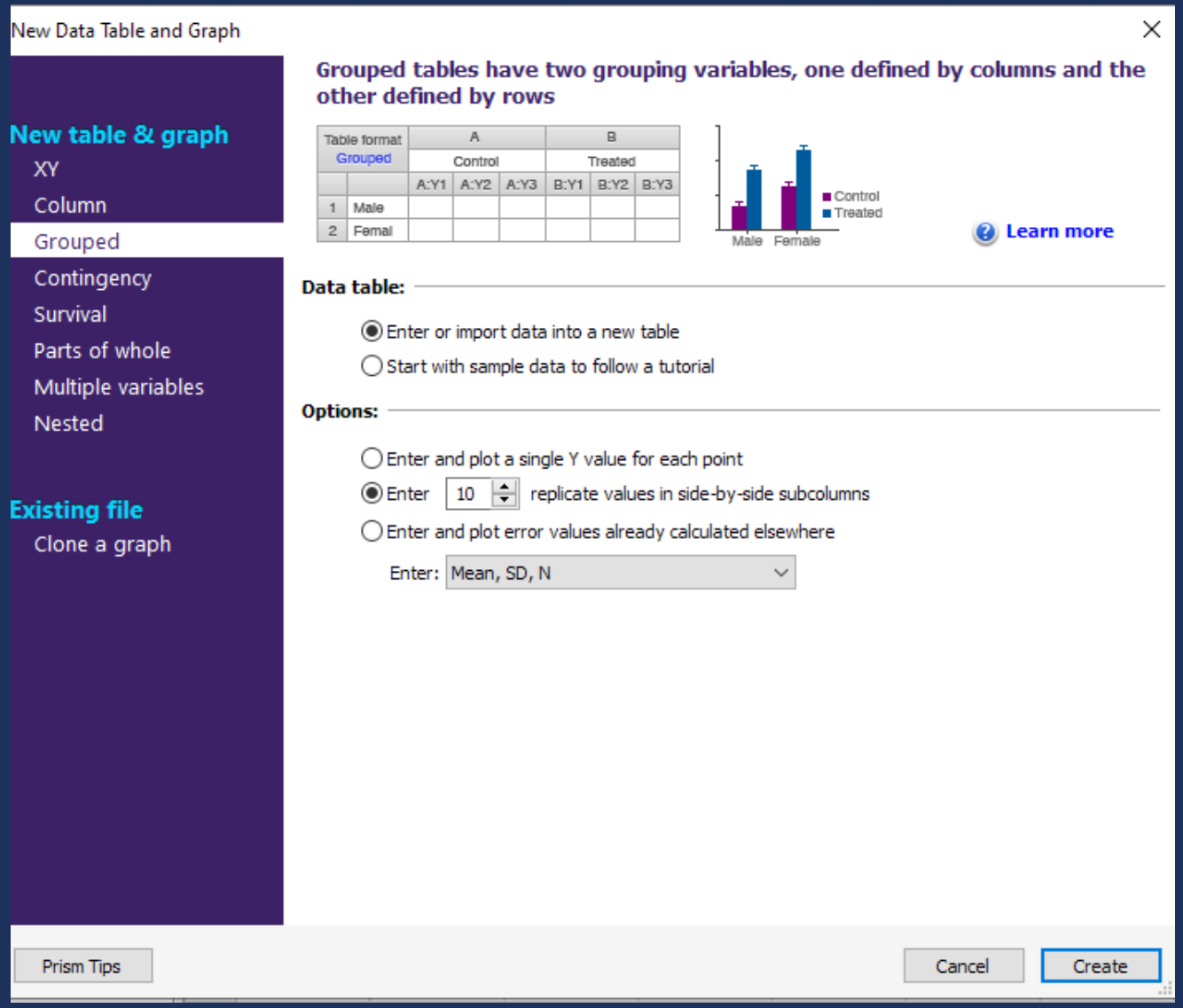


# Correlations Between Time Points (subset)

- Analyze Data > XY Analysis > Correlation *(see Appendix A for options)*
- compute Spearman correlation for every pair of time points
- Prism assumes equal correlations between all pairs (i.e., compound symmetry)

| | Time 0 | Time 1 | Time 2 | Time 3 | Time 4 | Time 5 | Time 6 | Time 7 | Time 8 |
|---|---|---|---|---|---|---|---|---|---|
| Time 0 | 1.000 | 0.979 | 0.812 | 0.721 | 0.937 | 0.793 | 0.413 | 0.058 | -0.232 |
| Time 1 | 0.979 | 1.000 | 0.817 | 0.750 | 0.893 | 0.750 | 0.382 | 0.029 | -0.200 |
| Time 2 | 0.812 | 0.817 | 1.000 | 0.893 | 0.714 | 0.500 | 0.109 | -0.143 | -0.314 |
| Time 3 | 0.721 | 0.750 | 0.893 | 1.000 | 0.536 | 0.286 | 0.109 | -0.429 | -0.429 |
| Time 4 | 0.937 | 0.893 | 0.714 | 0.536 | 1.000 | 0.929 | 0.546 | 0.371 | -0.029 |
| Time 5 | 0.793 | 0.750 | 0.500 | 0.286 | 0.929 | 1.000 | 0.600 | 0.714 | 0.371 |
| Time 6 | 0.413 | 0.382 | 0.109 | 0.109 | 0.546 | 0.600 | 1.000 | 0.265 | 0.000 |
| Time 7 | 0.058 | 0.029 | -0.143 | -0.429 | 0.371 | 0.714 | 0.265 | 1.000 | 0.886 |
| Time 8 | -0.232 | -0.200 | -0.314 | -0.429 | -0.029 | 0.371 | 0.000 | 0.886 | 1.000 |

# Data in Prism for Mixed




File > New Data Table and Graph > Grouped

Data table: Enter or import data into a new table

Options: Enter 10 replicate values in side-by-side subcolumns

# Data in Prism for Mixed (subset)

Grouped Data Table

"Group A": Each column represents the level of one factor = Genotype (CON, CKO)

| Table format: Grouped | | CON | | | |
|---|---|---|---|---|---|
| | | A:1 | A:2 | A:3 | A:4 |
| 1 | 0 | 20.3 | 21.0 | 18.4 | 19.7 |
| 2 | 1 | 25.5 | 24.6 | 21.3 | 22.0 |
| 3 | 2 | 31.4 | 28.4 | 23.5 | 23.8 |
| 4 | 3 | 30.5 | 26.6 | 24.5 | 25.1 |
| 5 | 4 | 33.6 | 30.4 | 28.2 | 27.3 |
| 6 | 5 | 35.7 | 32.6 | 30.7 | 29.7 |
| 7 | 6 | 37.1 | 36.3 | 28.3 | 32.6 |
| 8 | 7 | 40.2 | 35.7 | 30.7 | 35.6 |
| 9 | 8 | 42.2 | 38.0 | 31.5 | 34.3 |

"0, 1, …8": Each row represents a different level of the other factor = Time (0-8)

"A1": Matched values are stacked in a subcolumn = Mouse 1

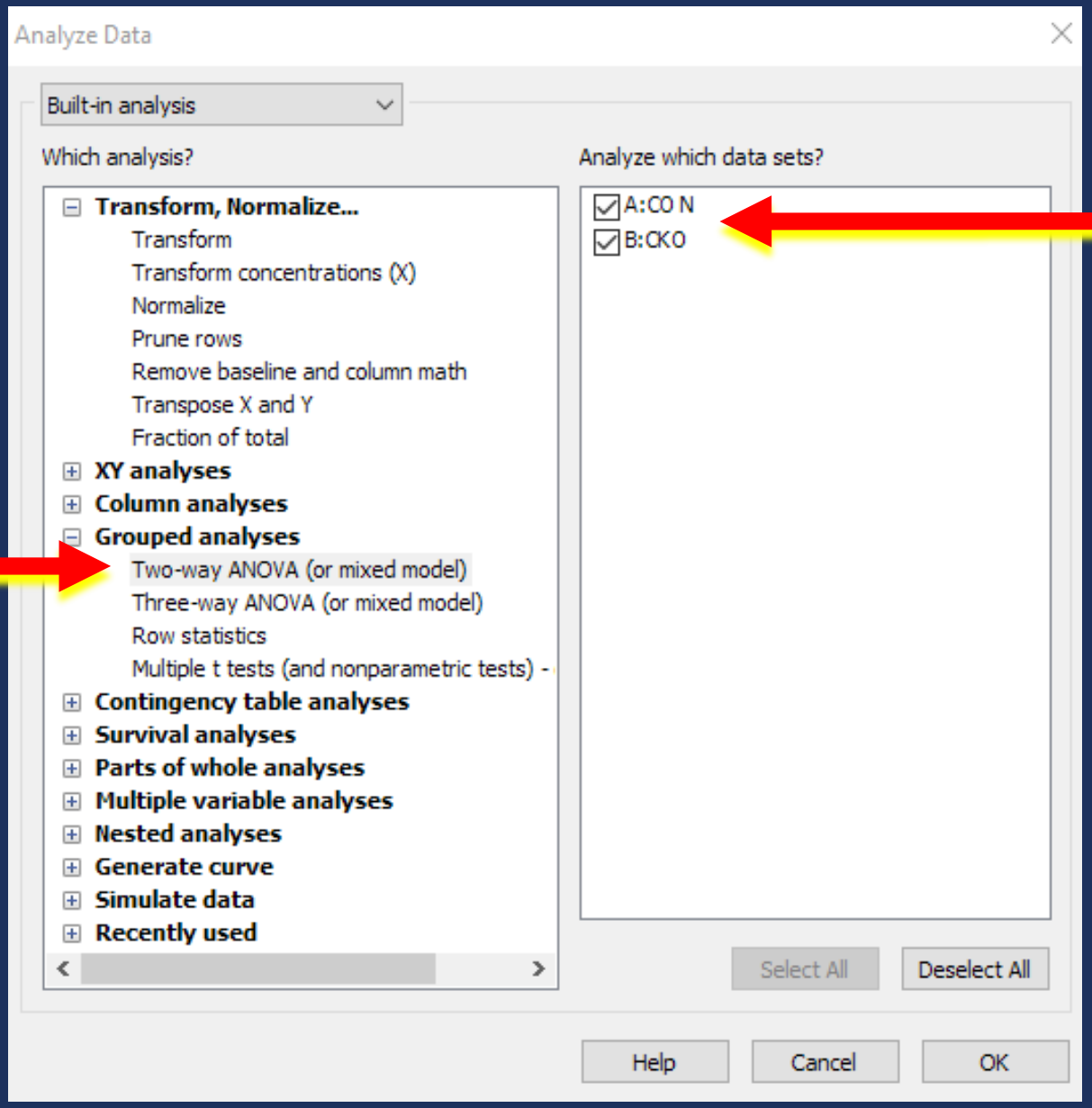
# Data in Prism for Mixed

| Table format: Grouped | Group A | | | | | | | | | | Group B | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CON | | | | | | | | | | CKO | | | | | | | | |
| | A:1 | A:2 | A:3 | A:4 | A:5 | A:6 | A:7 | A:8 | A:9 | A:10 | B:1 | B:2 | B:3 | B:4 | B:5 | B:6 | B:7 | B:8 | B:9 |
| 1 0 | 20.3 | 21.0 | 18.4 | 19.7 | 20.4 | 20.5 | 19.7 | 17.5 | 18.5 | 21.0 | 19.4 | 20.2 | 21.1 | 18.2 | 17.8 | 19.4 | 17.7 | 18.4 | 22.8 |
| 2 1 | 25.5 | 24.6 | 21.3 | 22.0 | 23.2 | 22.4 | 21.6 | 18.7 | 18.4 | 23.4 | 20.0 | 21.0 | 22.0 | 19.5 | 18.9 | 20.9 | 18.4 | 19.0 | 26.8 |
| 3 2 | 31.4 | 28.4 | 23.5 | 23.8 | 24.5 | 23.5 | 22.2 | 19.3 | 18.5 | 25.7 | 20.7 | 21.6 | 22.7 | 20.1 | 20.8 | 20.2 | 18.5 | 19.7 | 27.8 |
| 4 3 | 30.5 | 26.6 | 24.5 | 25.1 | 26.7 | 24.9 | 23.1 | | | | 21.4 | 22.0 | 22.3 | 21.5 | 21.8 | 21.6 | 19.4 | | |
| 5 4 | 33.6 | 30.4 | 28.2 | 27.3 | 28.3 | 28.5 | 24.6 | | | | 24.3 | 23.1 | 26.3 | 22.9 | 22.4 | 23.0 | 20.9 | | |
| 6 5 | 35.7 | 32.6 | 30.7 | 29.7 | 29.2 | 31.6 | 29.4 | | | | 28.3 | 24.6 | 25.2 | 24.2 | 23.5 | 24.7 | 21.9 | | |
| 7 6 | 37.1 | 36.3 | 28.3 | 32.6 | 30.9 | 33.3 | 31.7 | | | | 26.4 | 24.8 | 26.4 | 28.0 | 24.8 | 25.8 | 23.2 | | |
| 8 7 | 40.2 | 35.7 | 30.7 | 35.6 | 33.9 | | | | | | 28.3 | 25.0 | 26.6 | 25.7 | 26.2 | 28.0 | | | |
| 9 8 | 42.2 | 38.0 | 31.5 | 34.3 | 36.8 | | | | | | 28.6 | 25.7 | 26.8 | 26.3 | 28.3 | 29.2 | | | |

When Prism does mixed model analysis of repeated measures data, it assumes that the main factors (defined by the data set columns and rows in two-way) are fixed, but that subjects are random.

# Mixed Model in Prism

**Analyze >**
**Analyze Data >**
**Grouped analyses >**
**Two-way ANOVA (or mixed model)**

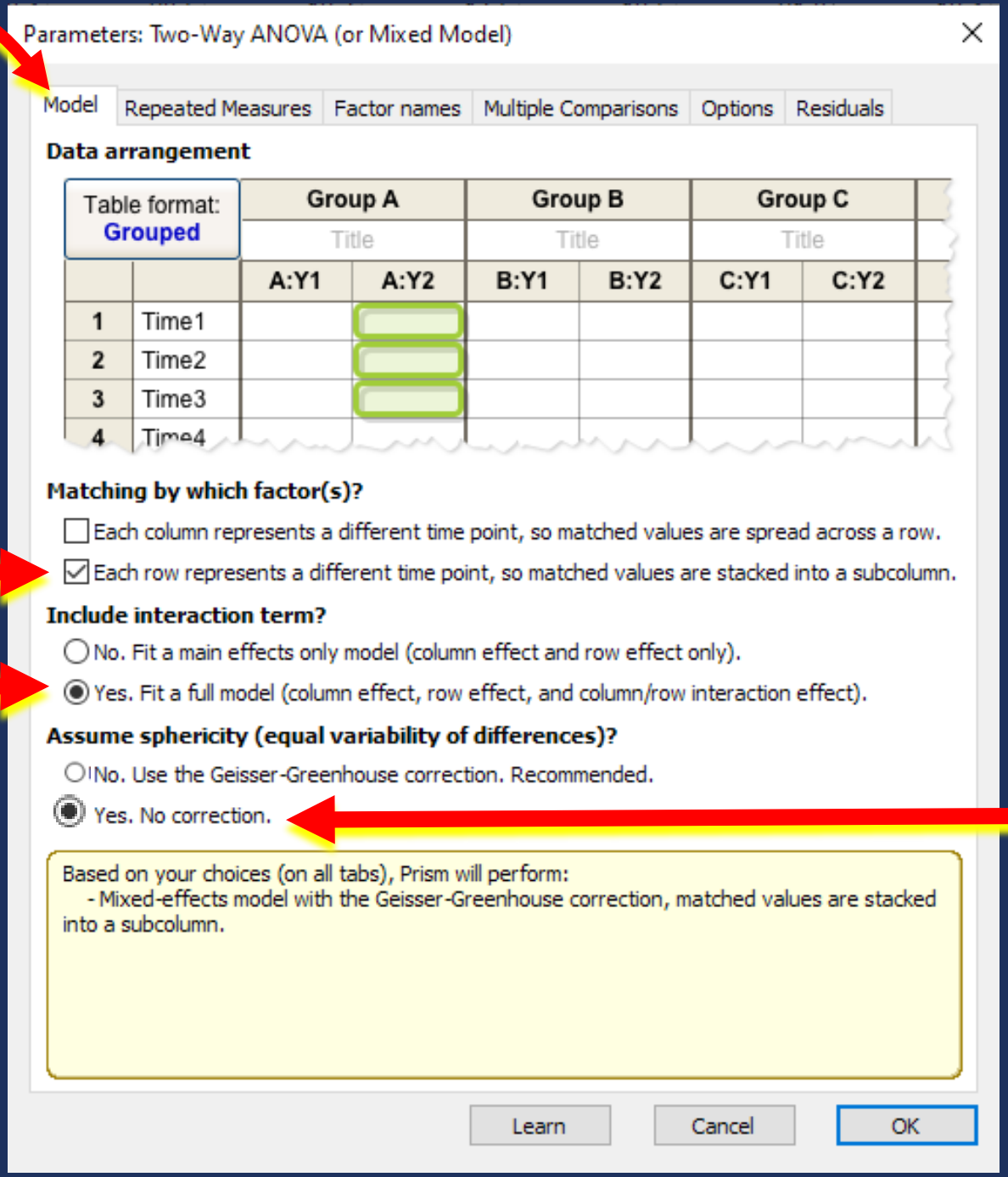



specify grouped data to include

**Model**

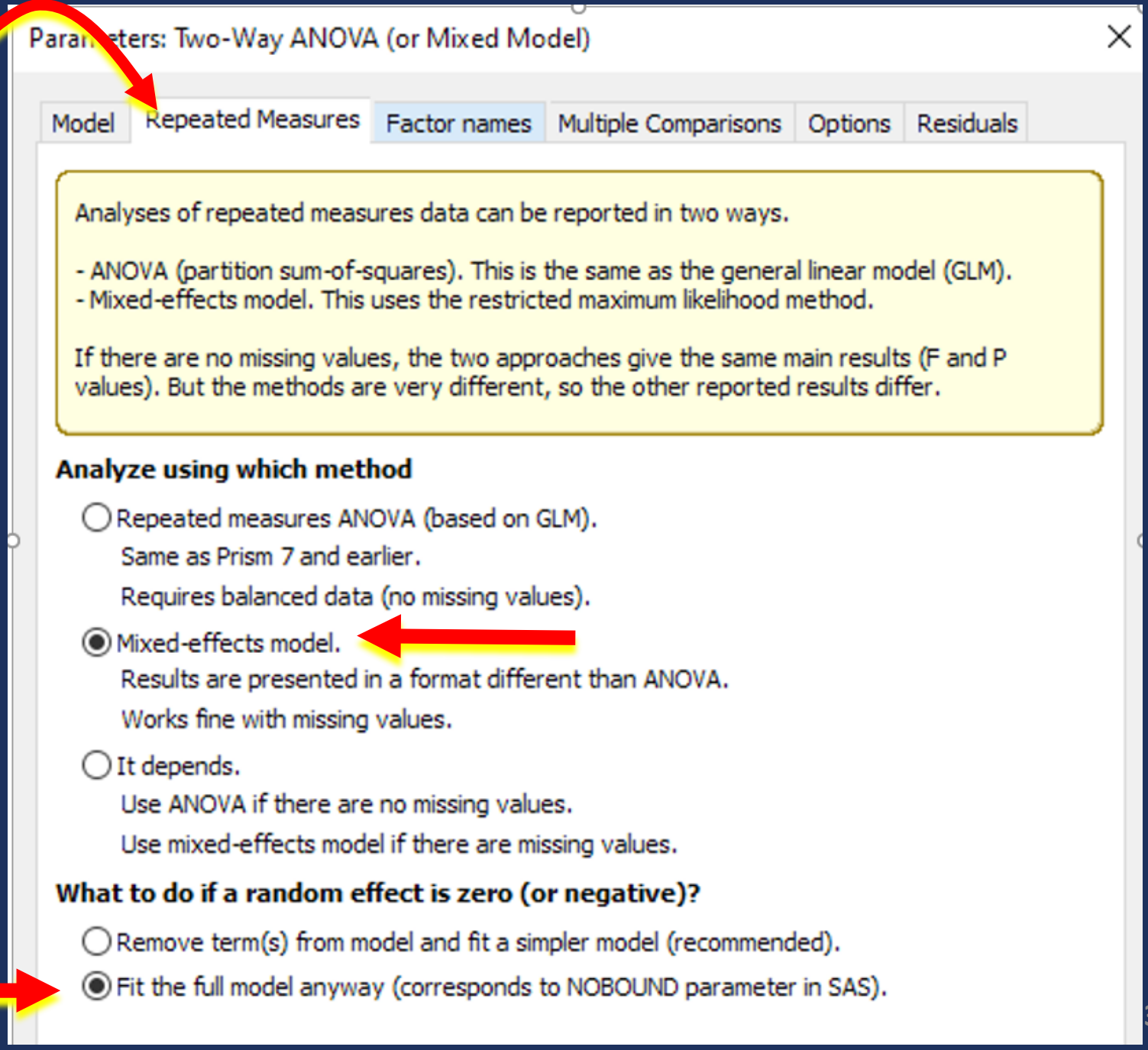
repeated measurements from the same animal

test effects for genotype, time, genotype*time




Sphericity: Caution, use only if serious violations. The correction affects how other results are calculated (particularly the multiple comparisons).

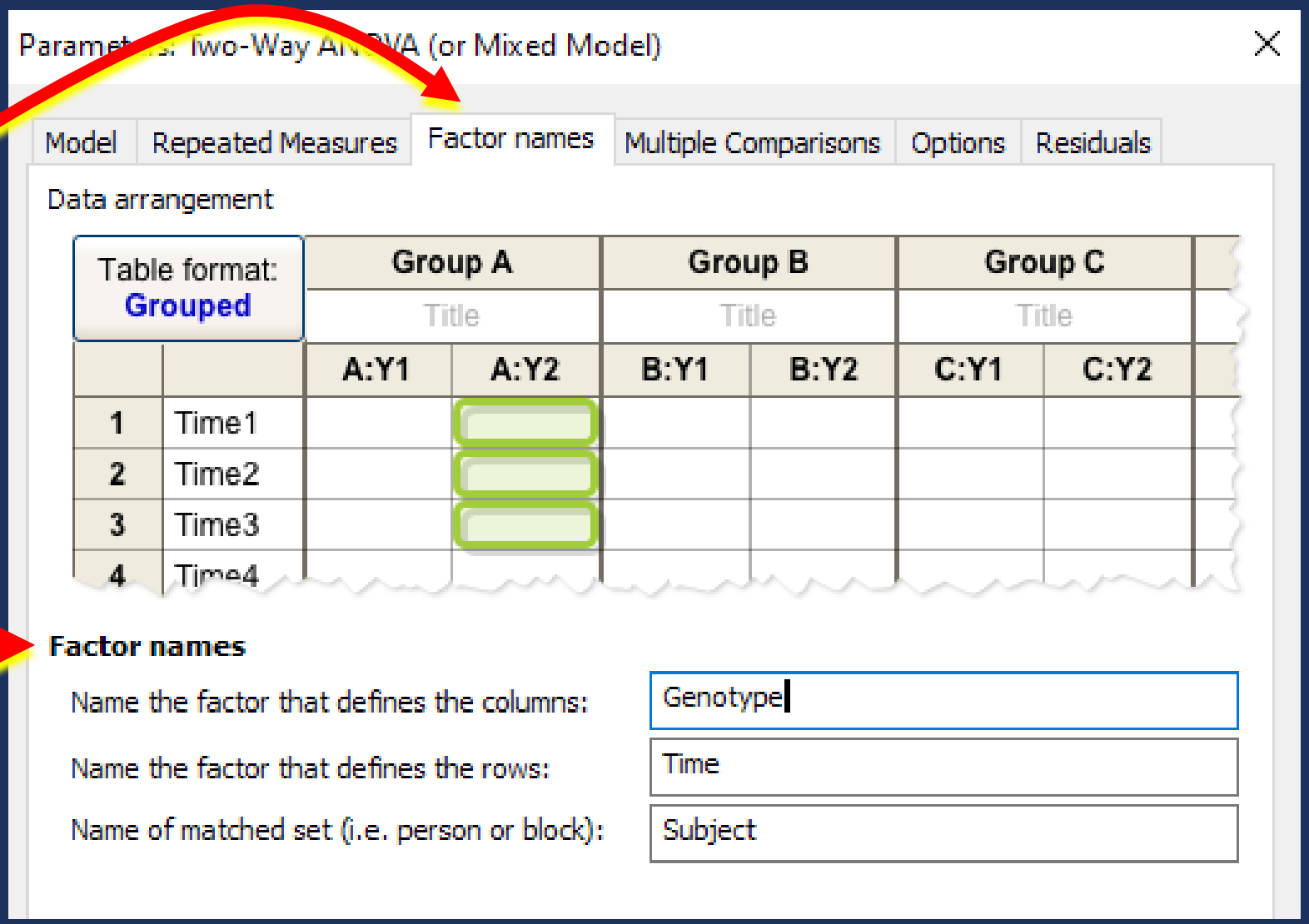
**Repeated Measures**




recommended when we need to respect the study design in the sense that sources of variation that are part of the design must be accounted for

**Factor Names**

customize the labels in the output

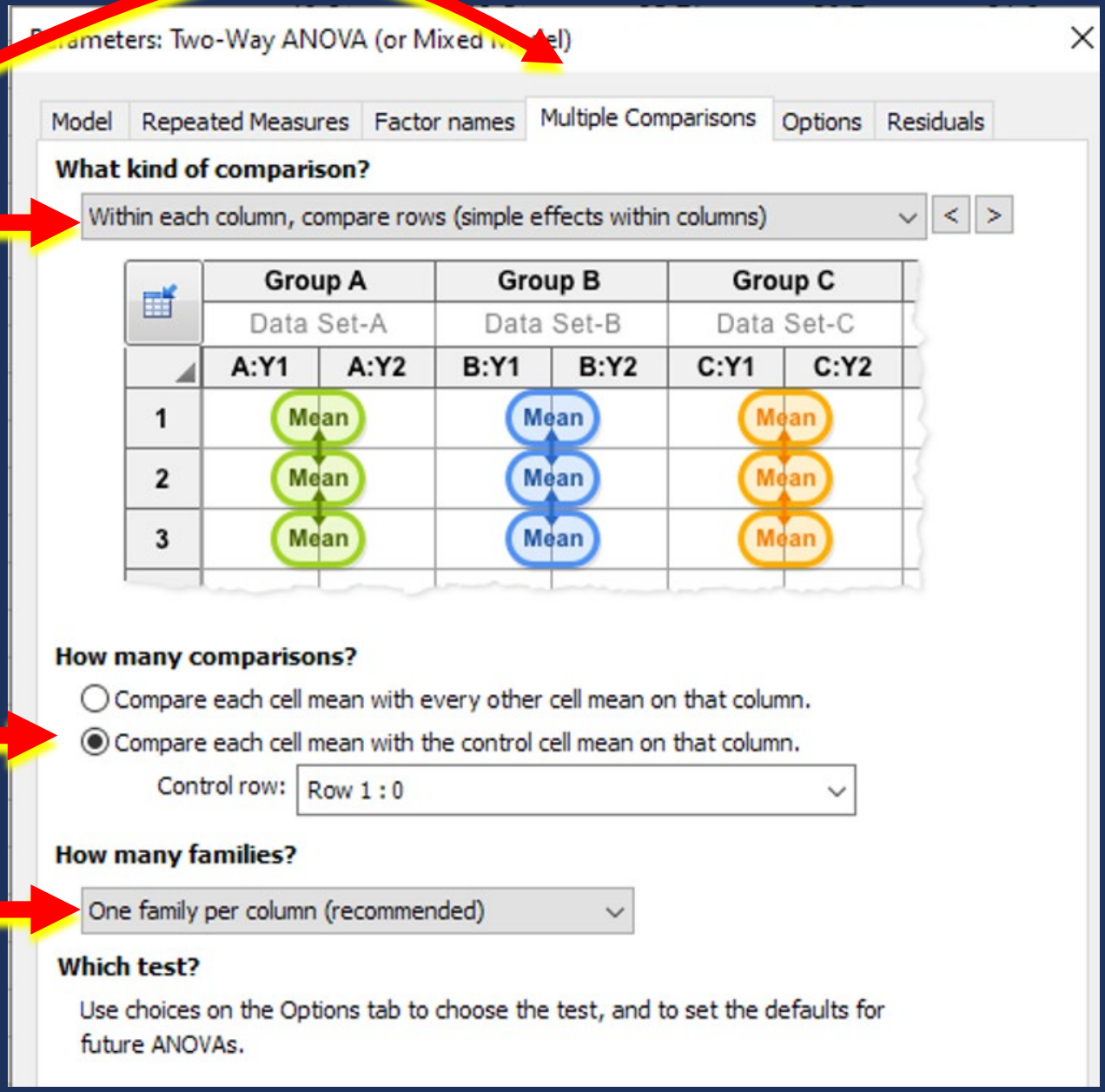


**Multiple Comparisons – choices depend upon study design and research question**

within each genotype, compare time points

compare each time point to time zero

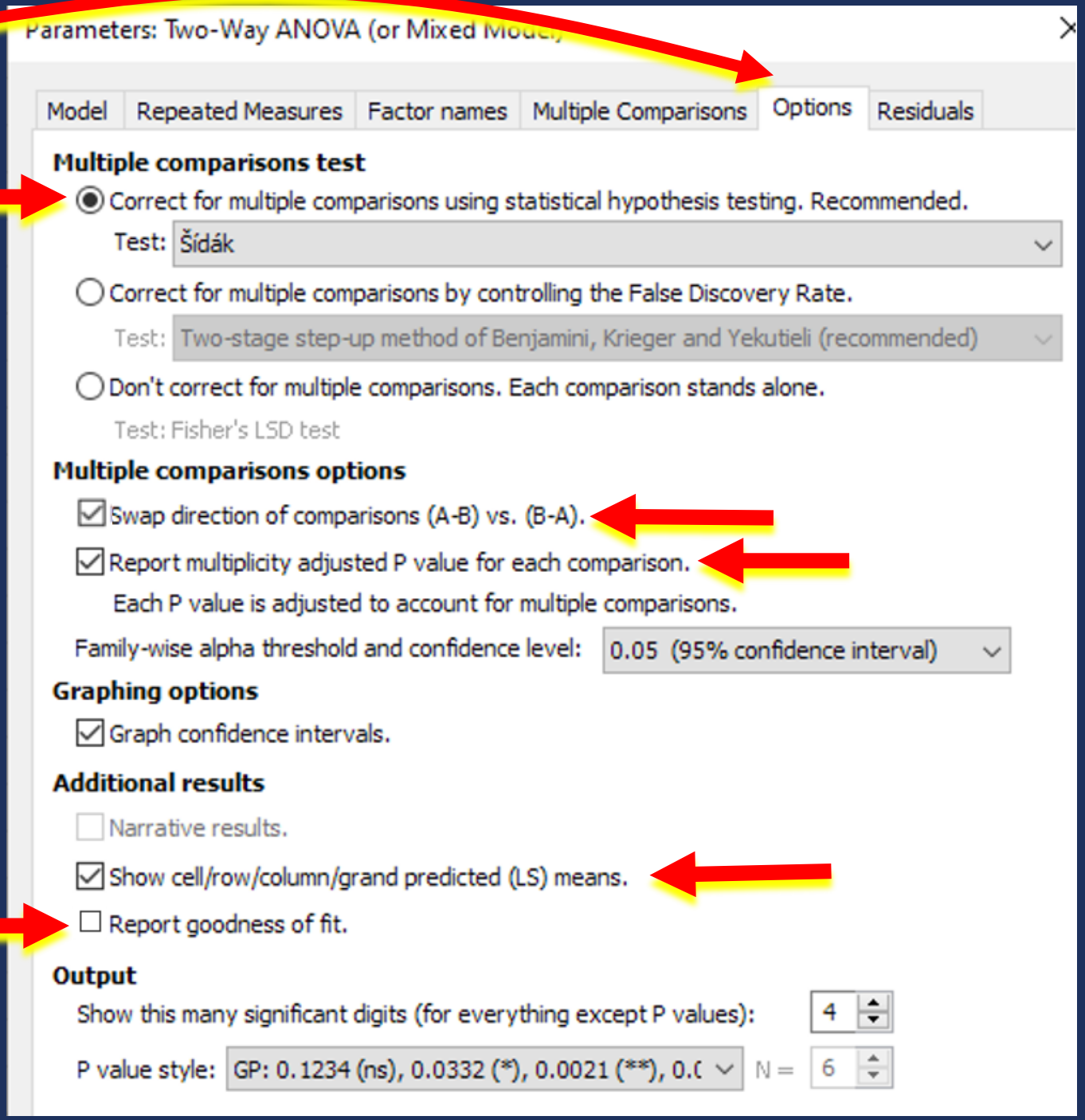
apply correction for the # of multiple comparisons performed within each genotype



# Options

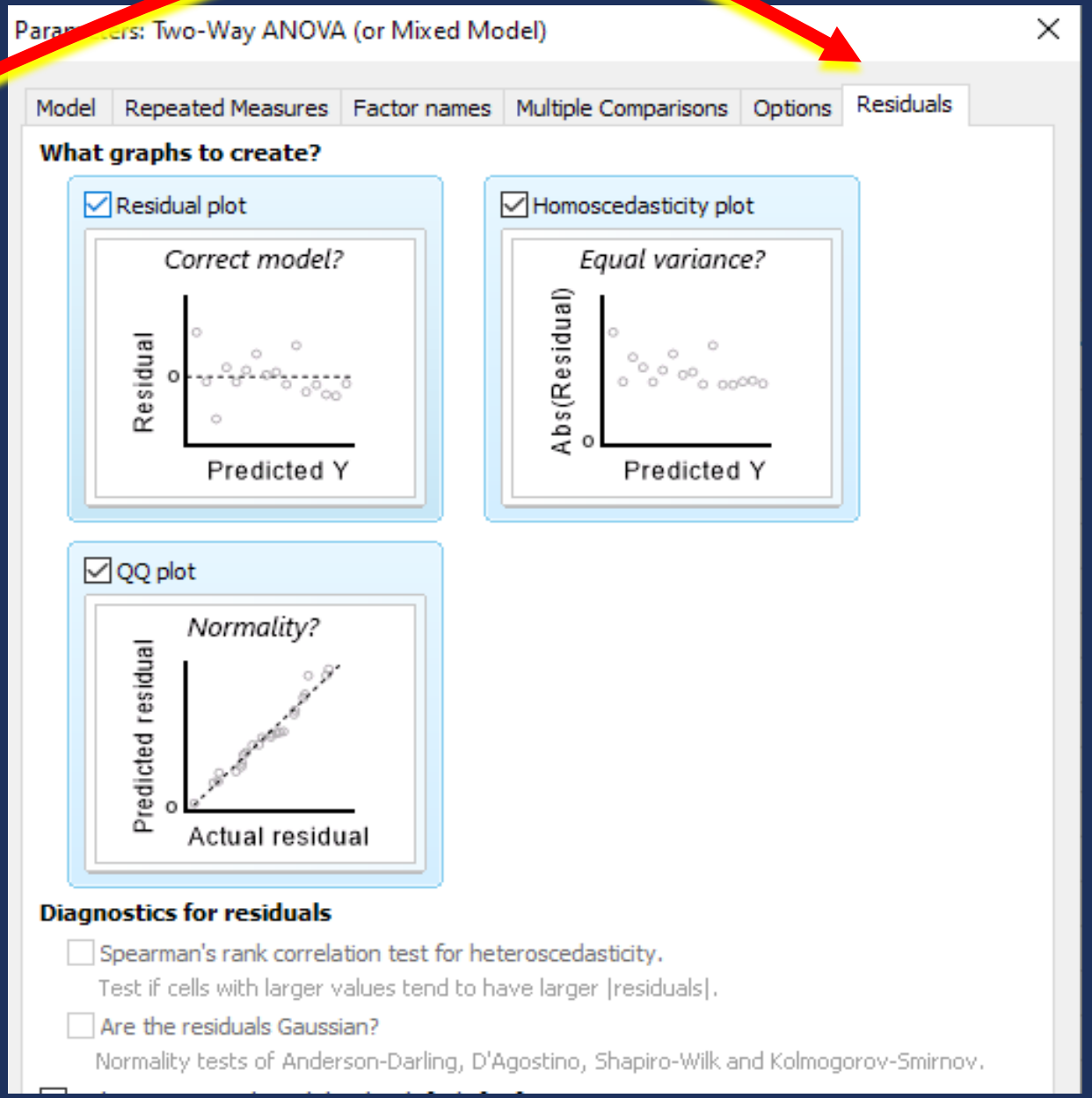
- correcting p-values for the multiple comparisons requested in the previous tab
- available options depend on other options set for the model
- I most often use Tukey adjustment
- Bonferroni is almost always too conservative

not useful



## Residuals

used for diagnostics to determine if the model is inadequate, or if there are assumption violations that should be addressed



# Mixed Model Output

Tabular results | Multiple comparisons | Means

**Mixed-effects analysis**
Tabular results

1

| | | | | | |
|---|---|---|---|---|---|
| 1 | Table Analyzed | original mixed data | | | |
| 2 | | | | | |
| 3 | **Mixed-effects model (REML)** | Matching: Stacked | | | |
| 4 | Assume sphericity? | Yes | | | |
| 5 | Alpha | 0.05 | | | |
| 6 | | | | | |
| 7 | **Fixed effects (type III)** | **P value** | **P value summary** | **Statistically significant (P < 0.05)?** | **F (DFn, DFd)** |
| 8 | Time | <0.0001 | **** | Yes | F (8, 100) = 142.6 |
| 9 | Genotype | 0.0014 | ** | Yes | F (1, 17) = 14.61 |
| 10 | Time x Genotype | <0.0001 | **** | Yes | F (8, 100) = 13.38 |

2

1

| Data summary | |
|---|---|
| Number of columns (Genotype) | 2 |
| Number of rows (Time) | 9 |
| Number of subjects (Mouse) | 19 |
| Number of missing values | 36 |

There is a time by genotype interaction (p<0.0001), meaning that the change over 8 weeks is significantly different for the CON and CKO mice. OR,
The difference in weight between CON and CKO mice depends upon the time point.

- The main effects for time and genotype are NOT interpretable. **Why?**
- We still don't know how the genotypes are different. Did one group increase weight and the other decreased weight? Did one group have no weight change and the other group increased?

these are LS MEANS, not raw data means

Tabular results | Multiple comparisons | Means

**Mixed-effects analysis**
Means

| | Predicted means (LS means) | CON | CKO |
|---|---|---|---|
| 1 | **Predicted means (LS means)** | CON | CKO |
| 2 | 0 | 19.70 | 19.44 |
| 3 | 1 | 22.11 | 20.72 |
| 4 | 2 | 24.08 | 21.34 |
| 5 | 3 | 25.19 | 21.93 |
| 6 | 4 | 27.98 | 23.77 |
| 7 | 5 | 30.55 | 25.13 |
| 8 | 6 | 32.16 | 26.13 |
| 9 | 7 | 34.03 | 26.79 |
| 10 | 8 | 35.37 | 27.64 |

- also known as:
  - LS means
  - least square means
  - adjusted means
  - predicted means
- These are means that are adjusted for the model specifications and take into account missing data.
- **directly correspond to the p-values reported**
- adjust for any between group differences at earlier time points; provide a best estimate of the true impact of the diet

these are LS MEANS with corresponding CIs

Tabular results | Multiple comparisons | Means

**Mixed-effects analysis**
Multiple comparisons

Sidak-adjusted for multiple comparisons

| | | | | | | |
|---|---|---|---|---|---|---|
| 3 | Number of families | 2 | | | | |
| 4 | Number of comparisons per family | 8 | | | | |
| 5 | Alpha | 0.05 | | | | |
| 6 | | | | | | |
| 7 | **Šídák's multiple comparisons test** | **Predicted (LS) mean diff.** | **95.00% CI of diff.** | **Below threshold?** | **Summary** | **Adjusted P Value** |
| 8 | | | | | | |
| 9 | CON | | | | | |
| 10 | 1 vs. 0 | 2.410 | 0.8398 to 3.980 | Yes | *** | 0.0003 |
| 11 | 2 vs. 0 | 4.380 | 2.810 to 5.950 | Yes | **** | <0.0001 |
| 12 | 3 vs. 0 | 5.490 | 3.714 to 7.266 | Yes | **** | <0.0001 |
| 13 | 4 vs. 0 | 8.275 | 6.499 to 10.05 | Yes | **** | <0.0001 |
| 14 | 5 vs. 0 | 10.85 | 9.071 to 12.62 | Yes | **** | <0.0001 |
| 15 | 6 vs. 0 | 12.46 | 10.68 to 14.24 | Yes | **** | <0.0001 |
| 16 | 7 vs. 0 | 14.33 | 12.34 to 16.32 | Yes | **** | <0.0001 |
| 17 | 8 vs. 0 | 15.67 | 13.68 to 17.66 | Yes | **** | <0.0001 |
| 18 | | | | | | |
| 19 | CKO | | | | | |
| 20 | 1 vs. 0 | 1.278 | -0.3774 to 2.933 | No | ns | 0.2413 |
| 21 | 2 vs. 0 | 1.900 | 0.2448 to 3.555 | Yes | * | 0.0147 |
| 22 | 3 vs. 0 | 2.483 | 0.6802 to 4.286 | Yes | ** | 0.0017 |
| 23 | 4 vs. 0 | 4.326 | 2.523 to 6.129 | Yes | **** | <0.0001 |
| 24 | 5 vs. 0 | 5.683 | 3.880 to 7.486 | Yes | **** | <0.0001 |
| 25 | 6 vs. 0 | 6.683 | 4.880 to 8.486 | Yes | **** | <0.0001 |
| 26 | 7 vs. 0 | 7.349 | 5.456 to 9.242 | Yes | **** | <0.0001 |
| 27 | 8 vs. 0 | 8.199 | 6.306 to 10.09 | Yes | **** | <0.0001 |

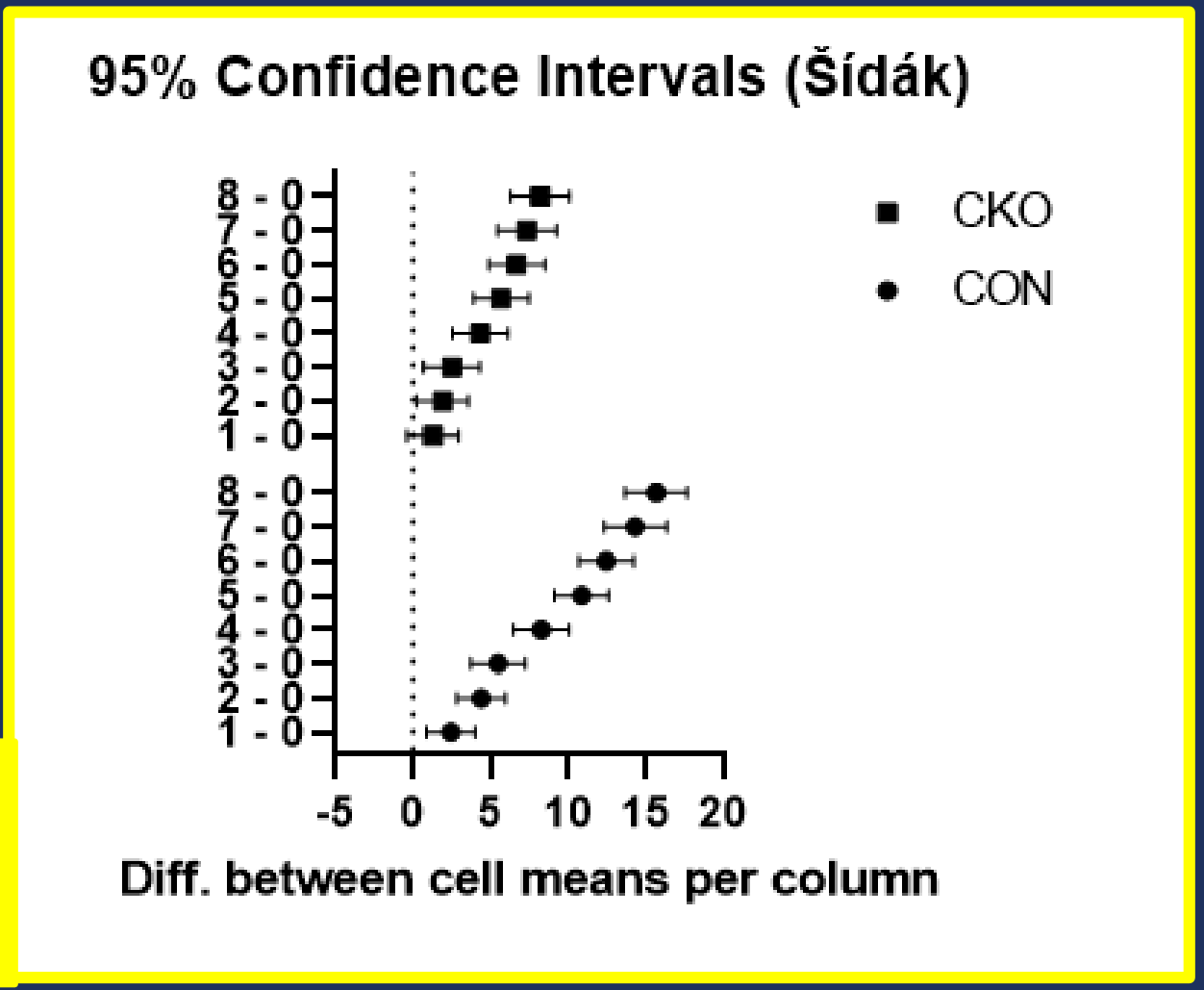
Family 1: CIs and p-values adjusted for 8 comparisons

Family 2: CIs and p-values adjusted for 8 comparisons

**these are LS MEANS with corresponding CIs that are Sidak-adjusted for multiple comparisons**

subset of graphs

- this depicts the mean difference between time 0 and each weekly measurements
- depicts where the differences may be located




**CON have bigger increases in weight from time zero as compared to CKO.**

**these are LS MEANS with corresponding SEs**

TO COMPARE CHANGE between two specific time points in CON vs. CKO:

File > New Data Table and Graph > Column > Enter new table > Enter values calculated elsewhere > Mean, SEM, N

*Enter the data in red boxes. Determine N to enter so the DF for the t-test equals the DF here (100). N = blue box DF ÷ #groups you are comparing in this test + 1)*
*= 100÷2+1=51*

Analyze Data > Column analysis > t tests > Unpaired

| Test details | Predicted (LS) mean 1 | Predicted (LS) mean 2 | Predicted (LS) mean diff. | SE of diff. | N1 | N2 | t | DF |
|---|---|---|---|---|---|---|---|---|
| CON | | | | | | | | |
| 1 vs. 0 | 22.11 | 19.70 | 2.410 | 0.5637 | 10 | 10 | 4.275 | 100.0 |
| 2 vs. 0 | 24.08 | 19.70 | 4.380 | 0.5637 | 10 | 10 | 7.770 | 100.0 |
| 3 vs. 0 | 25.19 | 19.70 | 5.490 | 0.6376 | 7 | 10 | 8.610 | 100.0 |
| 4 vs. 0 | 27.98 | 19.70 | 8.275 | 0.6376 | 7 | 10 | 12.98 | 100.0 |
| 5 vs. 0 | 30.55 | 19.70 | 10.85 | 0.6376 | 7 | 10 | 17.01 | 100.0 |
| 6 vs. 0 | 32.16 | 19.70 | 12.46 | 0.6376 | 7 | 10 | 19.54 | 100.0 |
| 7 vs. 0 | 34.03 | 19.70 | 14.33 | 0.7140 | 5 | 10 | 20.07 | 100.0 |
| 8 vs. 0 | 35.37 | 19.70 | 15.67 | 0.7140 | 5 | 10 | 21.94 | 100.0 |
| | | | | | | | | |
| CKO | | | | | | | | |
| 1 vs. 0 | 20.72 | 19.44 | 1.278 | 0.5942 | 9 | 9 | 2.151 | 100.0 |
| 2 vs. 0 | 21.34 | 19.44 | 1.900 | 0.5942 | 9 | 9 | 3.198 | 100.0 |
| 3 vs. 0 | 21.93 | 19.44 | 2.483 | 0.6472 | 7 | 9 | 3.837 | 100.0 |
| 4 vs. 0 | 23.77 | 19.44 | 4.326 | 0.6472 | 7 | 9 | 6.684 | 100.0 |
| 5 vs. 0 | 25.13 | 19.44 | 5.683 | 0.6472 | 7 | 9 | 8.781 | 100.0 |
| 6 vs. 0 | 26.13 | 19.44 | 6.683 | 0.6472 | 7 | 9 | 10.33 | 100.0 |
| 7 vs. 0 | 26.79 | 19.44 | 7.349 | 0.6796 | 6 | 9 | 10.81 | 100.0 |
| 8 vs. 0 | 27.64 | 19.44 | 8.199 | 0.6796 | 6 | 9 | 12.06 | 100.0 |

| **Unpaired t test** | |
|---|---|
| P value | 0.0031 |
| t, df | t=3.028, df=100 |
| | |
| **How big is the difference?** | |
| Difference between means (F - E) ± SEM | -2.480 ± 0.8190 |
| 95% confidence interval | -4.105 to -0.8550 |

**The increase in weight from time zero to 2 weeks is significantly more for the CON as compared to the CKO mice (0.003).**

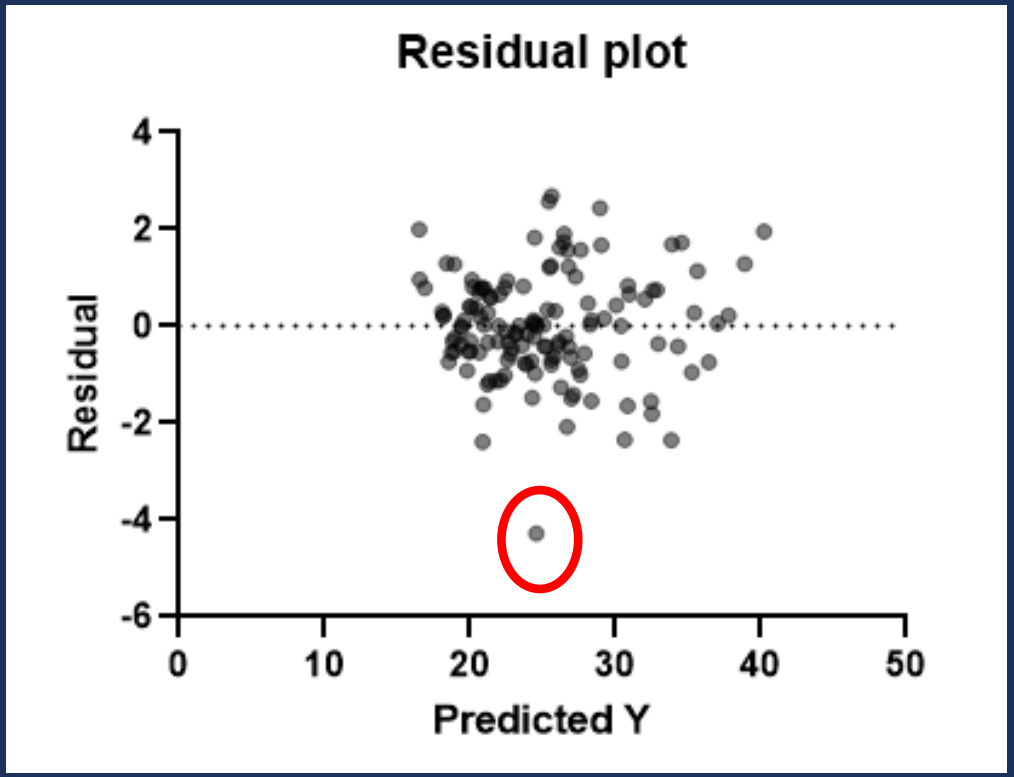
**these are LS MEANS with corresponding CIs**

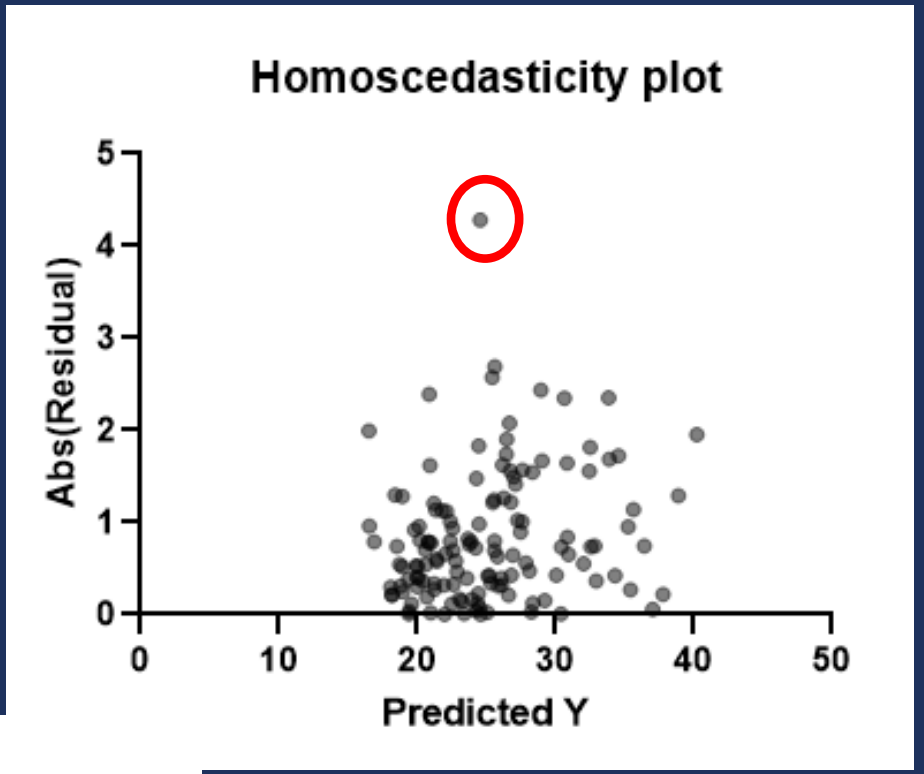
To compare genotypes at time zero:

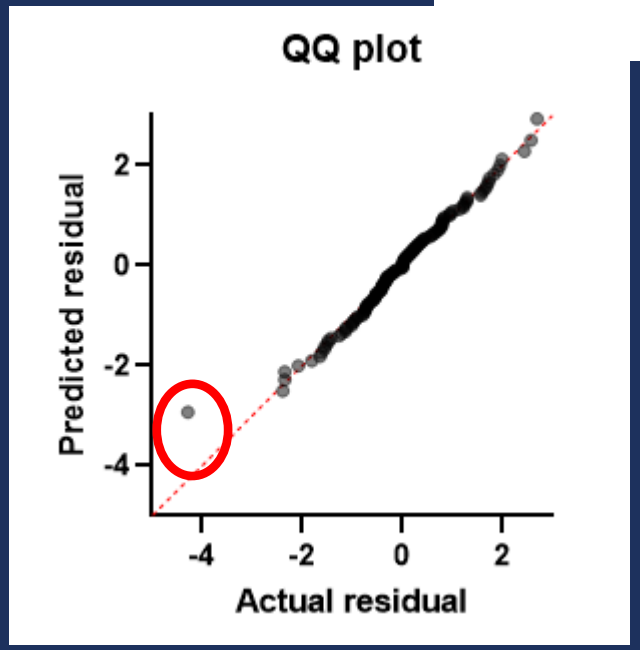
*Change "Multiple Comparisons" to "Compare each cell mean with the other cell mean in that row and no correction for multiple comparisons*

| Uncorrected Fisher's LSD | Predicted (LS) mean diff. | 95.00% CI of diff. | Below threshold? | Summary | Individual P Valu |
|---|---|---|---|---|---|
| | | | | | |
| CKO - CON | | | | | |
| 0 | -0.2556 | -2.686 to 2.175 | No | ns | 0.8354 |
| 1 | -1.388 | -3.818 to 1.043 | No | ns | 0.2604 |
| 2 | -2.736 | -5.166 to -0.3052 | Yes | * | 0.0277 |
| 3 | -3.262 | -5.814 to -0.7101 | Yes | * | 0.0127 |
| 4 | -4.205 | -6.757 to -1.653 | Yes | ** | 0.0014 |
| 5 | -5.419 | -7.971 to -2.867 | Yes | **** | <0.0001 |
| 6 | -6.034 | -8.586 to -3.482 | Yes | **** | <0.0001 |
| 7 | -7.233 | -9.895 to -4.571 | Yes | **** | <0.0001 |
| 8 | -7.723 | -10.39 to -5.061 | Yes | **** | <0.0001 |

**The weight at time zero, before starting the diet, is not significantly different for the CKO and CON mice (p=0.84).**

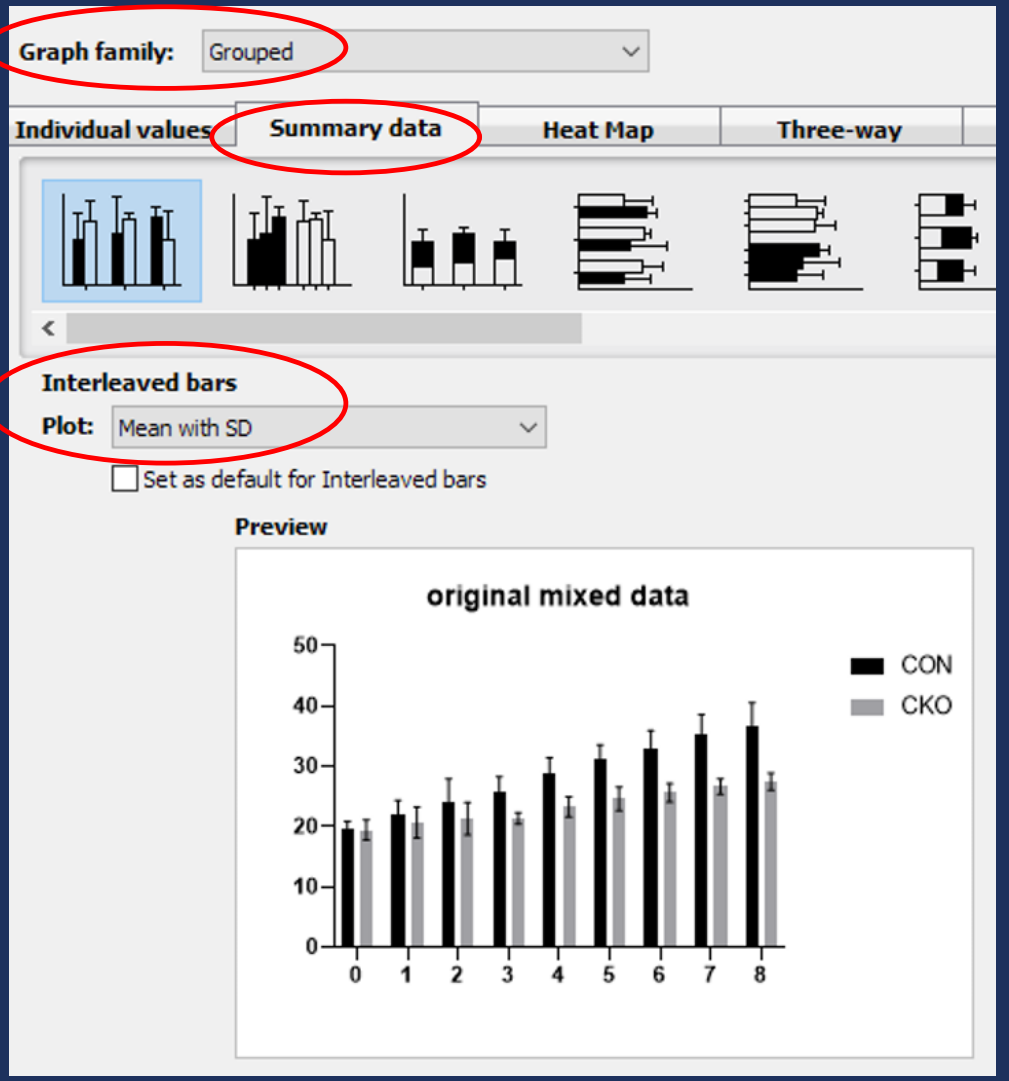



subset of diagnostics: examine for…

- large residuals, meaning that there is a lot of variance not explained by your model
- patterns of association between residuals and predicted
- outliers
- unequal variability across values
- gaps across Predicted Y


There are many graphs and plots available to help with interpretation of the data and results.

# Statistical Methods Section - Components

- avoid ambiguous terms such as “two-way”…spell out the **random and fixed effects and the study design**
- **covariance structure** used
- **options** such as Geisser-Greenhouse correction
- **multiple comparisons** that were examined and the hypotheses that they tested, describe adjustment for multiple comparisons or justification for not adjusting
- describe means/CIs as **unadjusted or adjusted**
- see the Prism ‘Help’ menu for “**How to cite**” the software
- TAKE PICTURES/SCREENSHOTS/NOTES OF THE PRISM SPECIFICATIONS FOR REPRODUCIBILITY

# Mixed Models in Prism

1) available in Prism 8
2) “not for beginners”
3) think of it as repeated measures ANOVA that allows missing data
4) limitations:
    a) can’t define the covariance matrix – assumes compound symmetry
    b) can’t get model fit statistics to compare alternative models
    c) can’t include a covariate
    d) model fitted (LS means) means/SEs not always available
    e) few options to adjust the model

# Caution from Prism

## Notes of caution for statistical novices

Prev Next

Our goal with Prism has always been to make basic biostatistics very accessible and easy. Two-way ANOVA is pushing the limits of "basic biostatistics". Multiple comparisons after two-way ANOVA stretch this definition even more. If you haven't taken the time to really understand two-way ANOVA, it is quite easy to be misled by the results. Beware!

- Two-way ANOVA is not a topic that is easy to master. In addition to reading textbooks, also consider getting help.from someone with more experience.
- Prism also offers to fit a mixed model to repeated measures data. Understanding this fully is even more complicated.
- Before getting lost in the many choices for multiple comparisons, first articulate clearly the scientific goals of the study. Don't articulate your goals in terms of ANOVA (looking for interactions) and avoid the word "significant" which often leads to muddled thinking. Figure out what you really want to know. Then figure out the best statistical approach to getting the answer.
- In Prism, the two-way ANOVA analysis can be used when, as the name suggests, there are two factors. None, one, or both of the factors can be repeated measures.

# Know When to Get Help

Division of Biostatistics

Biostatistical Consulting Service

https://biostatistics.wustl.edu/consulting/

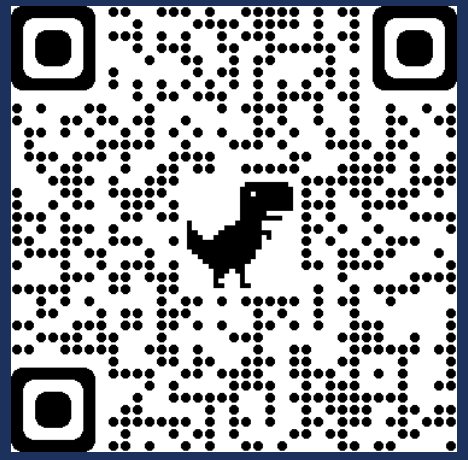

http://bit.ly/wubios

# Statistical Resources

- UCLA tutorials & examples with various software packages
  https://stats.idre.ucla.edu/
- Vanderbilt Biostatistics for Biomedical Research
  https://hbiostat.org/bbr/?preview=true
- Ware JH, Harrington D, Hunter DJ, D'Agostino RB. Missing Data. N Engl J Med. 2012;367(14):1353–4.
  https://www.nejm.org/doi/full/10.1056/NEJMsm1210043
- Matthews JN, Altman DG, Campbell MJ, Royston P. (1990). Analysis of serial measurements in medical research. *BMJ*, *300*, 230–5.
  https://doi.org/10.1136/bmj.300.6719.230

# July 30 Workshop: Obtain a repeated measures dataset.

1) Consider the experimental design and analytic model:
    1a. What is your primary research question?
    1b. In your model, specify the p-value(s) that tests the primary research question.
    1c. What is your conclusion? What evidence does the model provide to support your conclusion?
2) Describe the data, statistical methods that you used, and the results:
    2a. Create a table/graph that illustrates the important feature(s) of the data and the results.
    2b. Describe the statistical methods, with details about model specifications or options that you used.
3) Assumptions: How were the assumptions evaluated? If an assumption was violated, describe your procedure for dealing with this.
4) Multiplicity: Do you report p-values for comparisons that are adjusted for multiple testing? If so, why did you choose to adjust and how did you choose the method of adjustment? If no, why not?
5) Challenges? Lessons learned?



Scan QR code, scroll to the bottom of the page, sign up by Monday. Deb will assign groups.

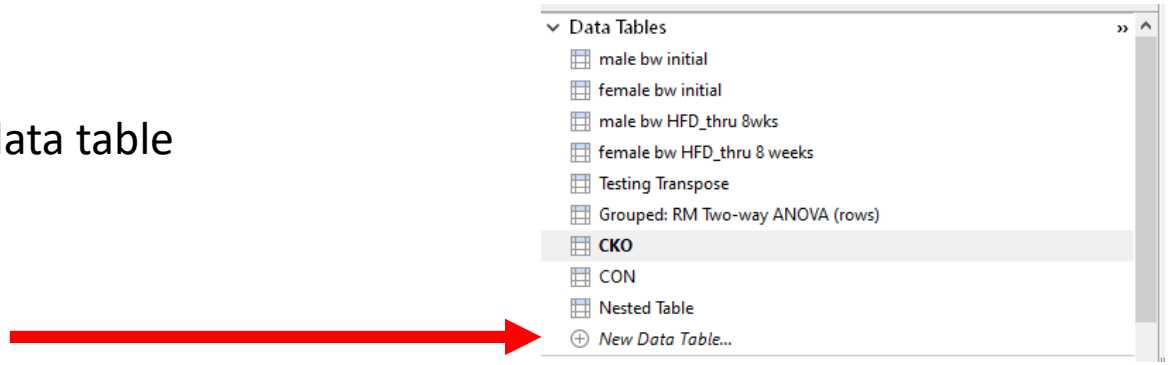
# Appendix A (author: Mansi Agarwal)

**The Problem:**

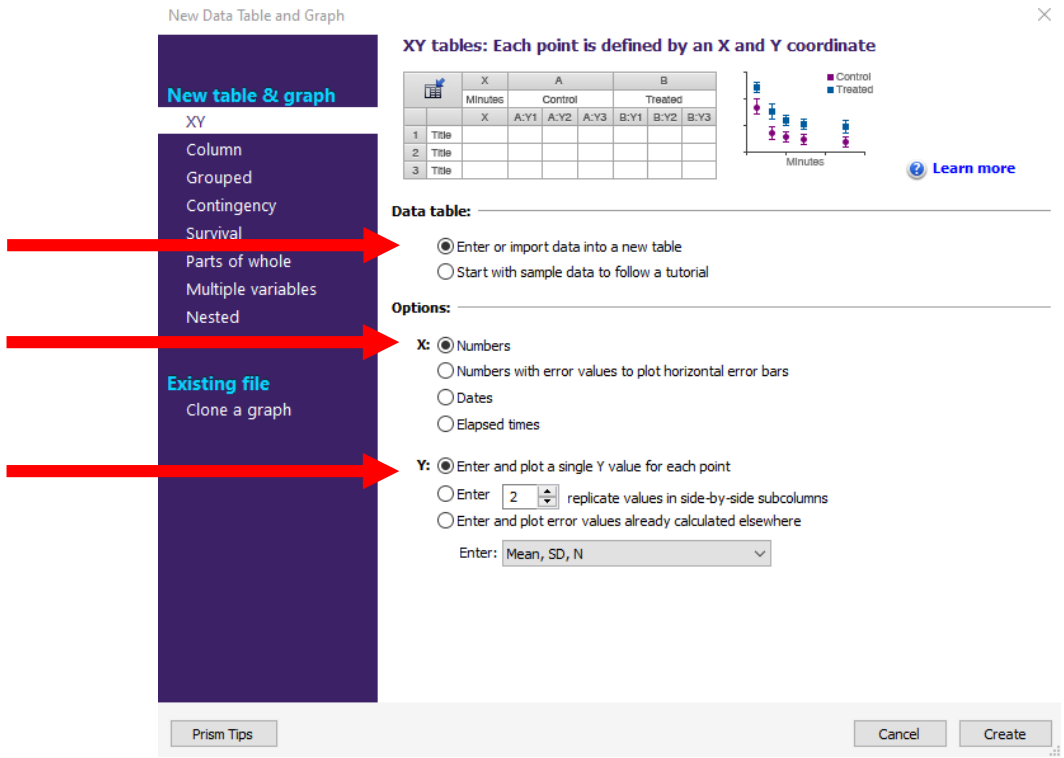
1 - The transform and transpose functions in Prism do not work with subcolumns.
2 - Repeated measures analyses can only be done with subcolumns.
3 - Correlations can only be done with columns (not subcolumns).
4 - Normality can be done with XY Tables, Grouped Tables, and Nested Tables.

**Solution: Convert each column into its own data table to check normality and correlation across time.**

Step 1: Create a new data table




Step 2: Select the following options



**Solution: Convert each column into its own data table to check normality and correlation across time.**

Step 3: Type in your X Title (ID) and X values

| Table format: XY | X | Group A | Group B | Group C | Group D |
|---|---|---|---|---|---|
| | ID | Title | Title | Title | Title |
| | X | Y | Y | Y | Y |
| 1 Title | 1 | | | | |
| 2 Title | 2 | | | | |
| 3 Title | 3 | | | | |
| 4 Title | 4 | | | | |
| 5 Title | 5 | | | | |
| 6 Title | 6 | | | | |
| 7 Title | 7 | | | | |
| 8 Title | 8 | | | | |
| 9 Title | 9 | | | | |
| 10 Title | | | | | |

Step 4: Type in your Y Titles (Time 0, Time 1, Time 2, etc. for each Y column.

| Table format: XY | X | Group A | Group B | Group C | Group D | Group E | Group F | Group G | Group H | Group I |
|---|---|---|---|---|---|---|---|---|---|---|
| | ID | Time 0 | Time 1 | Time 2 | Time 3 | Time 4 | Time 5 | Time 6 | Time 7 | Time 8 |
| | X | Y | Y | Y | Y | Y | Y | Y | Y | Y |
| 1 Title | 1 | | | | | | | | | |
| 2 Title | 2 | | | | | | | | | |
| 3 Title | 3 | | | | | | | | | |
| 4 Title | 4 | | | | | | | | | |

**Solution: Convert each column into its own data table to check normality and correlation across time.**

Step 5: Go back to your original data (with subcolumns) and copy the data (right click -> copy) for the column that you are going to transpose.

| Group B | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CKO | | | | | | | | | |
| B:Y1 | B:Y2 | B:Y3 | B:Y4 | B:Y5 | B:Y6 | B:Y7 | B:Y8 | B:Y9 | B:Y10 |
| 19.4 | 20.2 | 21.1 | 18.2 | 17.8 | 19.4 | 17.7 | 18.4 | 22.8 | |
| 20.0 | 21.0 | 22.0 | 19.5 | 18.9 | 20.9 | 18.4 | 19.0 | 26.8 | |
| 20.7 | 21.6 | 22.7 | 20.1 | 20.8 | 20.2 | 18.5 | 19.7 | 27.8 | |
| 21.4 | 22.0 | 22.3 | 21.5 | 21.8 | 21.6 | 19.4 | | | |
| 24.3 | 23.1 | 26.3 | 22.9 | 22.4 | 23.0 | 20.9 | | | |
| 28.3 | 24.6 | 25.2 | 24.2 | 23.5 | 24.7 | 21.9 | | | |
| 26.4 | 24.8 | 26.4 | 28.0 | 24.8 | 25.8 | 23.2 | | | |
| 28.3 | 25.0 | 26.6 | 25.7 | 26.2 | 28.0 | | | | |
| 28.6 | 25.7 | 26.8 | 26.3 | 28.3 | 29.2 | | | | |

Step 6: Click on the 1st data box and paste transpose the data (right click -> paste transpose -> paste data).

| Table format: XY | | X | Group A | Group B | Group C | Group D |
|---|---|---|---|---|---|---|
| | | ID | Time 0 | Time 1 | Time 2 | Time 3 |
| | | X | Y | Y | Y | Y |
| 1 | Title | 1 | | | | |
| 2 | Title | 2 | | | | |
| 3 | Title | 3 | | | | |
| 4 | Title | 4 | | | | |
| 5 | Title | 5 | | | | |
| 6 | Title | 6 | | | | |
| 7 | Title | 7 | | | | |
| 8 | Title | 8 | | | | |
| 9 | Title | 9 | | | | |

**Solution: Convert each column into its own data table to check normality and correlation across time.**

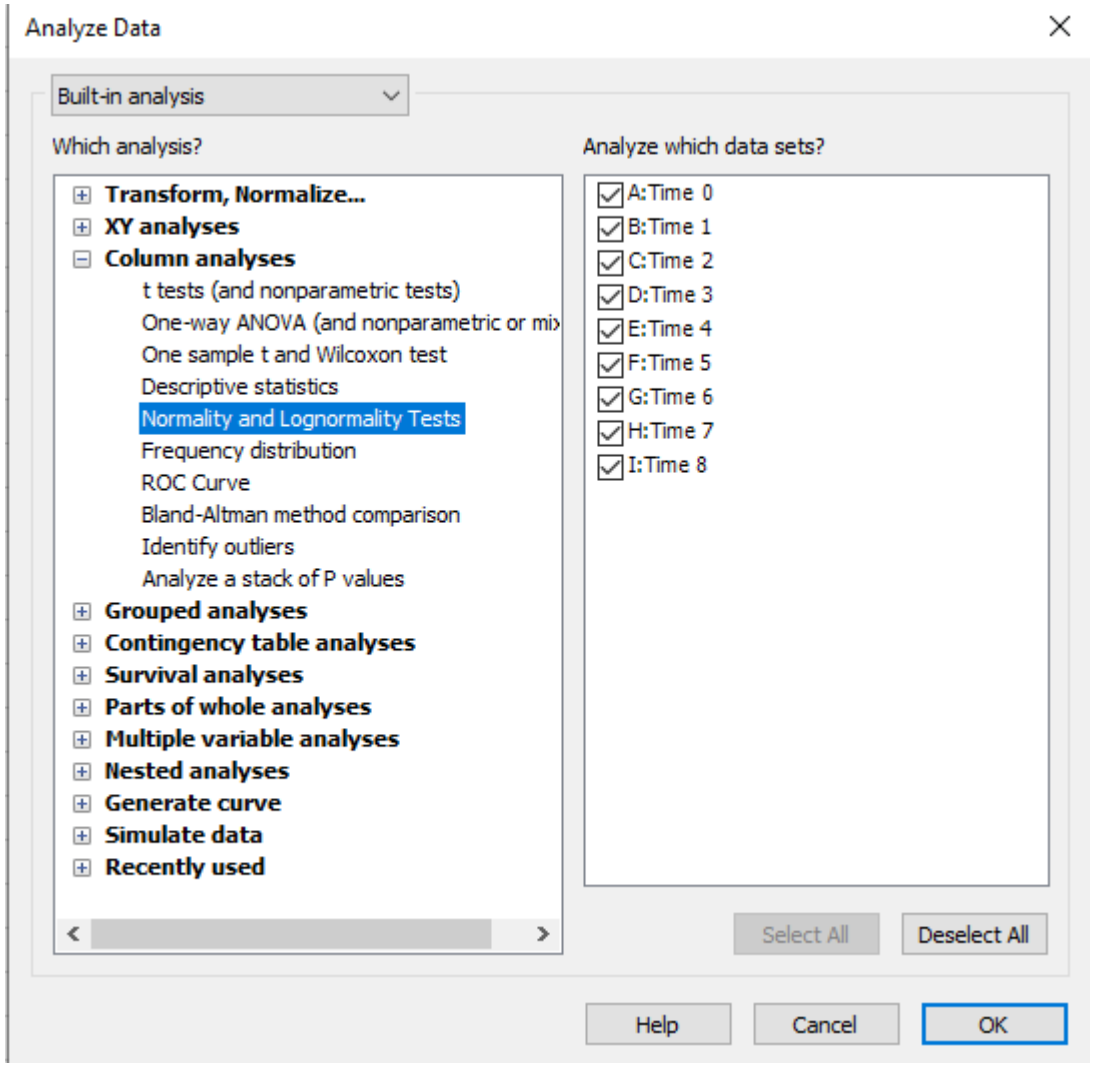
Transposed data for CKO:

| Table format: XY | X | Group A | Group B | Group C | Group D | Group E | Group F | Group G | Group H | Group I |
|---|---|---|---|---|---|---|---|---|---|---|
| | ID | Time 0 | Time 1 | Time 2 | Time 3 | Time 4 | Time 5 | Time 6 | Time 7 | Time 8 |
| | X | Y | Y | Y | Y | Y | Y | Y | Y | Y |
| 1 Title | 1 | 19.4 | 20.0 | 20.7 | 21.4 | 24.3 | 28.3 | 26.4 | 28.3 | 28.6 |
| 2 Title | 2 | 20.2 | 21.0 | 21.6 | 22.0 | 23.1 | 24.6 | 24.8 | 25.0 | 25.7 |
| 3 Title | 3 | 21.1 | 22.0 | 22.7 | 22.3 | 26.3 | 25.2 | 26.4 | 26.6 | 26.8 |
| 4 Title | 4 | 18.2 | 19.5 | 20.1 | 21.5 | 22.9 | 24.2 | 28.0 | 25.7 | 26.3 |
| 5 Title | 5 | 17.8 | 18.9 | 20.8 | 21.8 | 22.4 | 23.5 | 24.8 | 26.2 | 28.3 |
| 6 Title | 6 | 19.4 | 20.9 | 20.2 | 21.6 | 23.0 | 24.7 | 25.8 | 28.0 | 29.2 |
| 7 Title | 7 | 17.7 | 18.4 | 18.5 | 19.4 | 20.9 | 21.9 | 23.2 | | |
| 8 Title | 8 | 18.4 | 19.0 | 19.7 | | | | | | |
| 9 Title | 9 | 22.8 | 26.8 | 27.8 | | | | | | |

## Check normality for CKO using the newly transposed CKO data table.

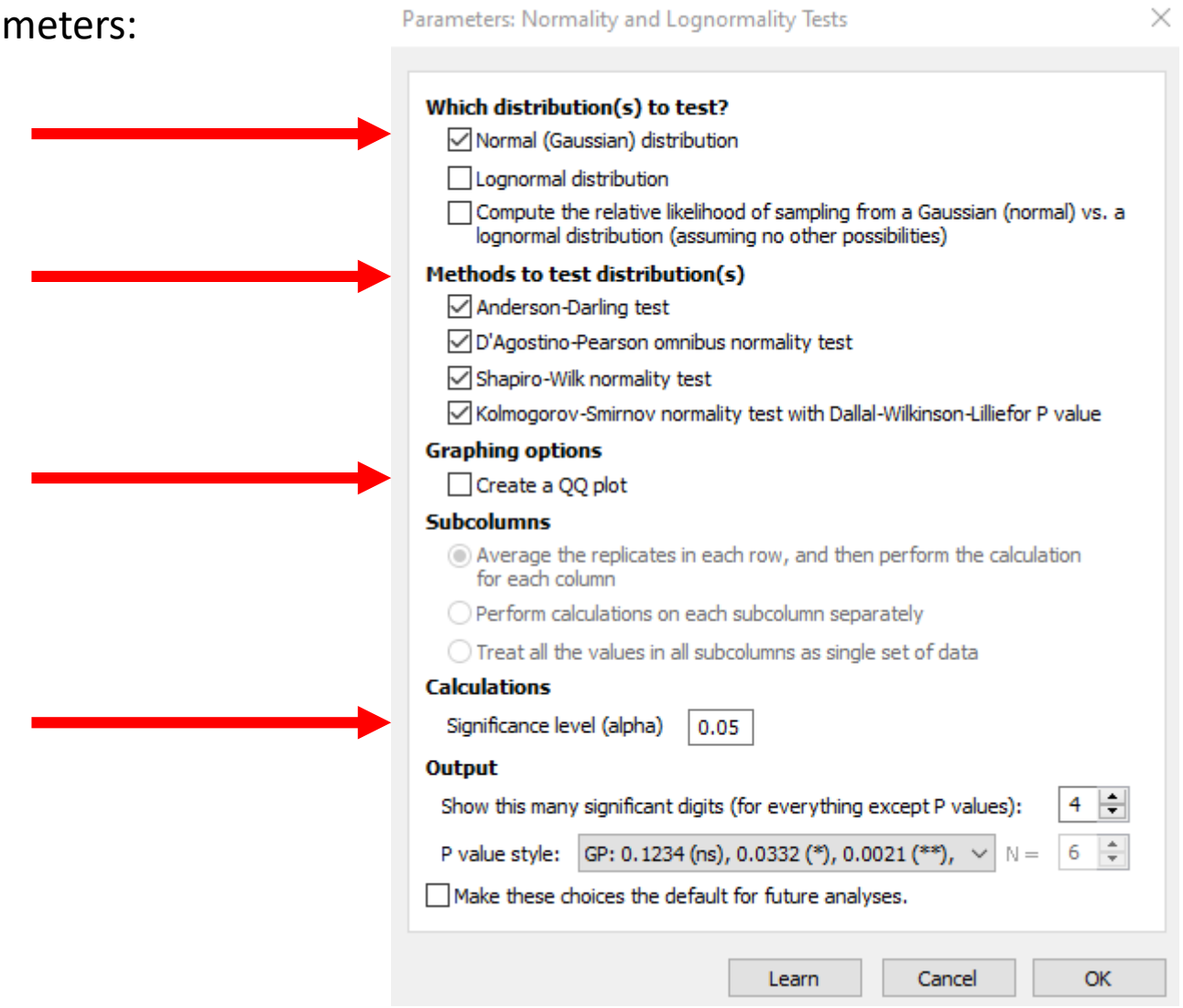
Step 1: Click on Analyze Data on the top left -> Column Analysis -> Normality and Lognormality Tests

Step 2: Select all time points.



## Check normality for CKO using the newly transposed CKO data table.

Step 3: Select the following parameters:




*Note: If using a grouped or nested table, you can select to calculate normality with each subcolumn separately.

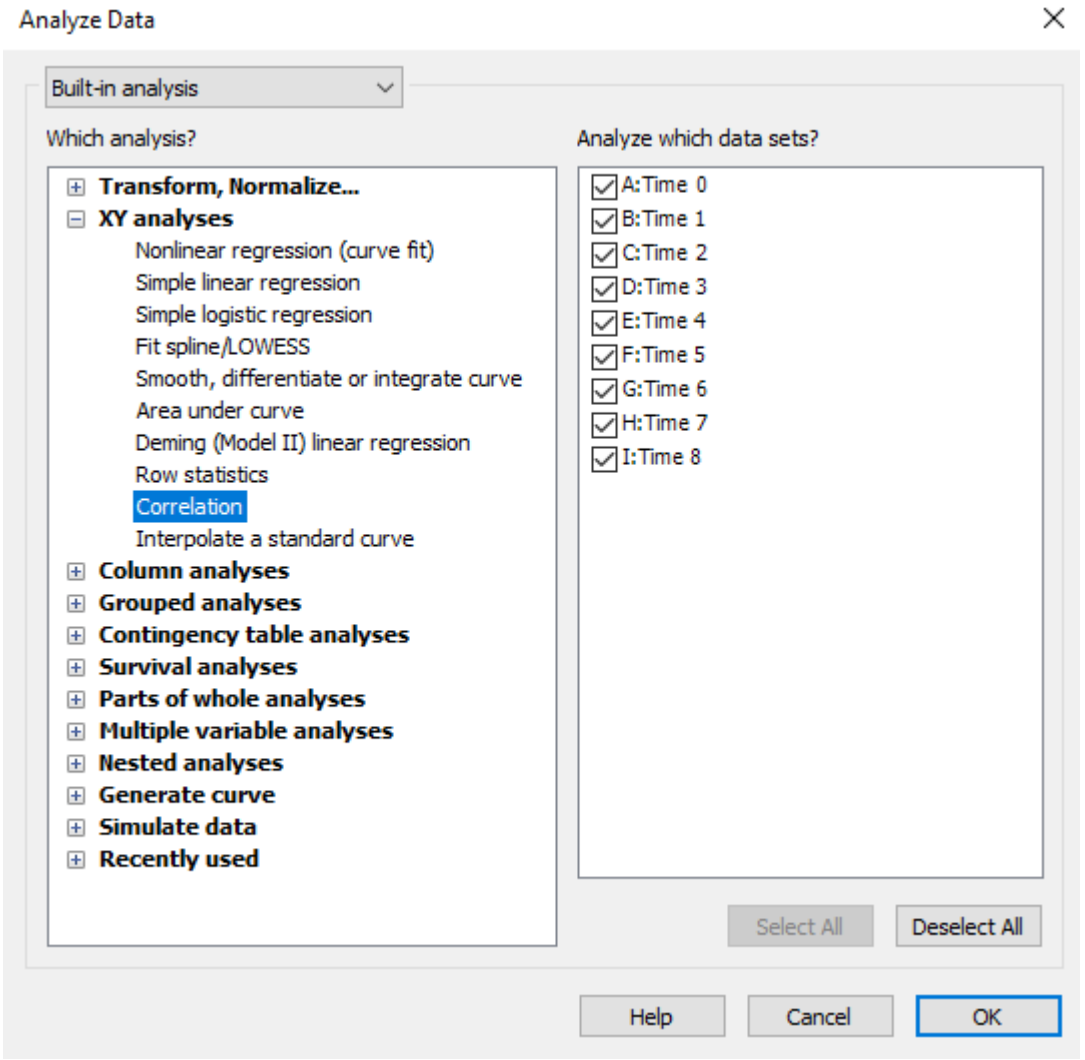
# Check normality for CKO using the newly transposed CKO data table.

| | Normality and Lognormality Tests | A<br>Time 0 | B<br>Time 1 | C<br>Time 2 | D<br>Time 3 | E<br>Time 4 | F<br>Time 5 | G<br>Time 6 | H<br>Time 7 | I<br>Time 8 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | **Test for normal distribution** | | | | | | | | | |
| 2 | **Anderson-Darling test** | | | | | | | | | |
| 3 | A2* | 0.3572 | 0.7145 | 0.7981 | N too small | N too small | N too small | N too small | N too small | N too small |
| 4 | P value | 0.3687 | 0.0397 | 0.0234 | | | | | | |
| 5 | Passed normality test (alpha=0.05)' | Yes | No | No | | | | | | |
| 6 | P value summary | ns | * | * | | | | | | |
| 7 | | | | | | | | | | |
| 8 | **D'Agostino & Pearson test** | | | | | | | | | |
| 9 | K2 | 2.207 | 11.87 | 12.82 | N too small | N too small | N too small | N too small | N too small | N too small |
| 10 | P value | 0.3317 | 0.0026 | 0.0016 | | | | | | |
| 11 | Passed normality test (alpha=0.05)' | Yes | No | No | | | | | | |
| 12 | P value summary | ns | ** | ** | | | | | | |
| 13 | | | | | | | | | | |
| 14 | **Shapiro-Wilk test** | | | | | | | | | |
| 15 | W | 0.9071 | 0.8016 | 0.7984 | 0.7677 | 0.9345 | 0.9209 | 0.9708 | 0.9381 | 0.9256 |
| 16 | P value | 0.2958 | 0.0213 | 0.0196 | 0.0193 | 0.5901 | 0.4764 | 0.9038 | 0.6443 | 0.5467 |
| 17 | Passed normality test (alpha=0.05)' | Yes | No | No | No | Yes | Yes | Yes | Yes | Yes |
| 18 | P value summary | ns | * | * | * | ns | ns | ns | ns | ns |
| 19 | | | | | | | | | | |
| 20 | **Kolmogorov-Smirnov test** | | | | | | | | | |
| 21 | KS distance | 0.1771 | 0.2346 | 0.2468 | 0.3451 | 0.2550 | 0.2416 | 0.1646 | 0.1879 | 0.2191 |
| 22 | P value | >0.1000 | >0.1000 | >0.1000 | 0.0117 | >0.1000 | >0.1000 | >0.1000 | >0.1000 | >0.1000 |
| 23 | Passed normality test (alpha=0.05)' | Yes | Yes | Yes | No | Yes | Yes | Yes | Yes | Yes |
| 24 | P value summary | ns | ns | ns | * | ns | ns | ns | ns | ns |
| 25 | | | | | | | | | | |
| 26 | **Number of values** | 9 | 9 | 9 | 7 | 7 | 7 | 7 | 6 | 6 |

## Check correlations between time points for CKO data table.

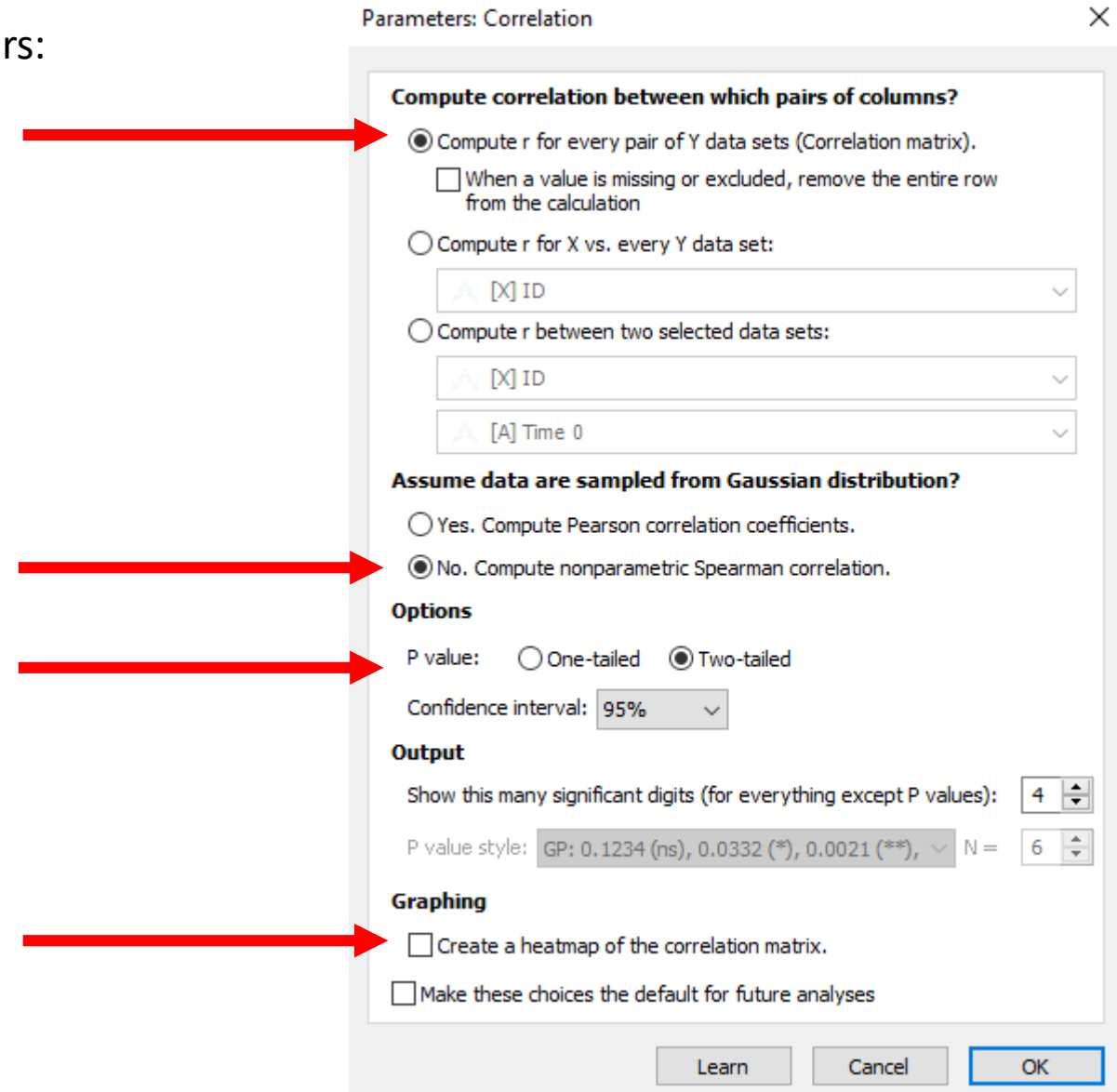
Step 1: Click on Analyze Data on the top left -> XY analyses -> Correlation

Step 2: Select all time points.



## Check correlations between time points for CKO data table.

Step 3: Select the following parameters:



## Check correlations between time points for CKO data table.

Spearman r | P values | Sample size | Confidence interval of rs

| Correlation Spearman r | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| | Time 0 | Time 1 | Time 2 | Time 3 | Time 4 | Time 5 | Time 6 | Time 7 | Time 8 |
| 1 | Time 0 | 1.000 | 0.979 | 0.812 | 0.721 | 0.937 | 0.793 | 0.413 | 0.058 | -0.232 |
| 2 | Time 1 | 0.979 | 1.000 | 0.817 | 0.750 | 0.893 | 0.750 | 0.382 | 0.029 | -0.200 |
| 3 | Time 2 | 0.812 | 0.817 | 1.000 | 0.893 | 0.714 | 0.500 | 0.109 | -0.143 | -0.314 |
| 4 | Time 3 | 0.721 | 0.750 | 0.893 | 1.000 | 0.536 | 0.286 | 0.109 | -0.429 | -0.429 |
| 5 | Time 4 | 0.937 | 0.893 | 0.714 | 0.536 | 1.000 | 0.929 | 0.546 | 0.371 | -0.029 |
| 6 | Time 5 | 0.793 | 0.750 | 0.500 | 0.286 | 0.929 | 1.000 | 0.600 | 0.714 | 0.371 |
| 7 | Time 6 | 0.413 | 0.382 | 0.109 | 0.109 | 0.546 | 0.600 | 1.000 | 0.265 | 0.000 |
| 8 | Time 7 | 0.058 | 0.029 | -0.143 | -0.429 | 0.371 | 0.714 | 0.265 | 1.000 | 0.886 |
| 9 | Time 8 | -0.232 | -0.200 | -0.314 | -0.429 | -0.029 | 0.371 | 0.000 | 0.886 | 1.000 |