Analysis of Variance

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Introduction

Cancer Vaccine

- The human epidermal growth factor receptor (HER) family, consisting of HER1 (also known as EGFR), HER2, HER3, and HER4, drives the progression of many epithelial malignancies;
- HER3 is overexpressed in breast, lung, gastric, head and neck, and ovarian cancers and melanoma, associating with poor prognosis;
- Therefore, HER3 is an attractive therapeutic target;
- Challenges for immunotherapies, in general, and cancer vaccines in particular are host and tumor factors that limit antitumor immune responses;
- Among these are the interaction of CTLA4 on activated T cells and CD80/CD86 on antigen presenting cells, which limits the proliferation of antigen-specific T cells.
- Upregulation of other immune checkpoint ligands such as PD-L1 on tumor cells or infiltrating immune cells and their receptors such as PD-1 on effector T cells limit antitumor T-cell function.

Introduction

Cancer Vaccine

- Osada, T., Morse, M. A., Hobeika, A., Diniz, M. A., Gwin, W. R., Hartman, Z., ... & Kaneko, K. (2017). Vaccination targeting human HER3 alters the phenotype of infiltrating T cells and responses to immune checkpoint inhibition. OncoImmunology, e1315495.
  - Human HER3 transgenic mouse models of breast cancer;
  - A recombinant adenoviral vector expressing full length human HER3 (Ad-HER3-FL) was created;
  - In addition, a combination of the Ad-HER3-FL vaccine with either anti-CTLA4 or anti-PD-L1 antibodies were studied;
  - Questions:
    - Does the vaccine Ad-HER3-FL shrink tumor size?
    - Do the checkpoint inhibitors anti-CTLA4 pr anti-PD-L1 shrink tumor size?
    - Which combination of vaccine and antibodies is the most effective shrinking tumor size?

Introduction

Prevention model

- Mice were first immunized with the Ad-HER3-FL vaccine, tumor was then implanted, and tumor implantation was followed by anti-PD-1 or anti-PD-L1 antibody administration;
- Groups:
  - Ad-GFP + IgG;
  - Ad-GFP + anti-PD1;
  - Ad-GFP + anti-PD-L1;
  - Ad-HER3-FL + IgG;
  - Ad-HER3-FL + anti-PD1;
  - Ad-HER3-FL + anti-PD-L1.

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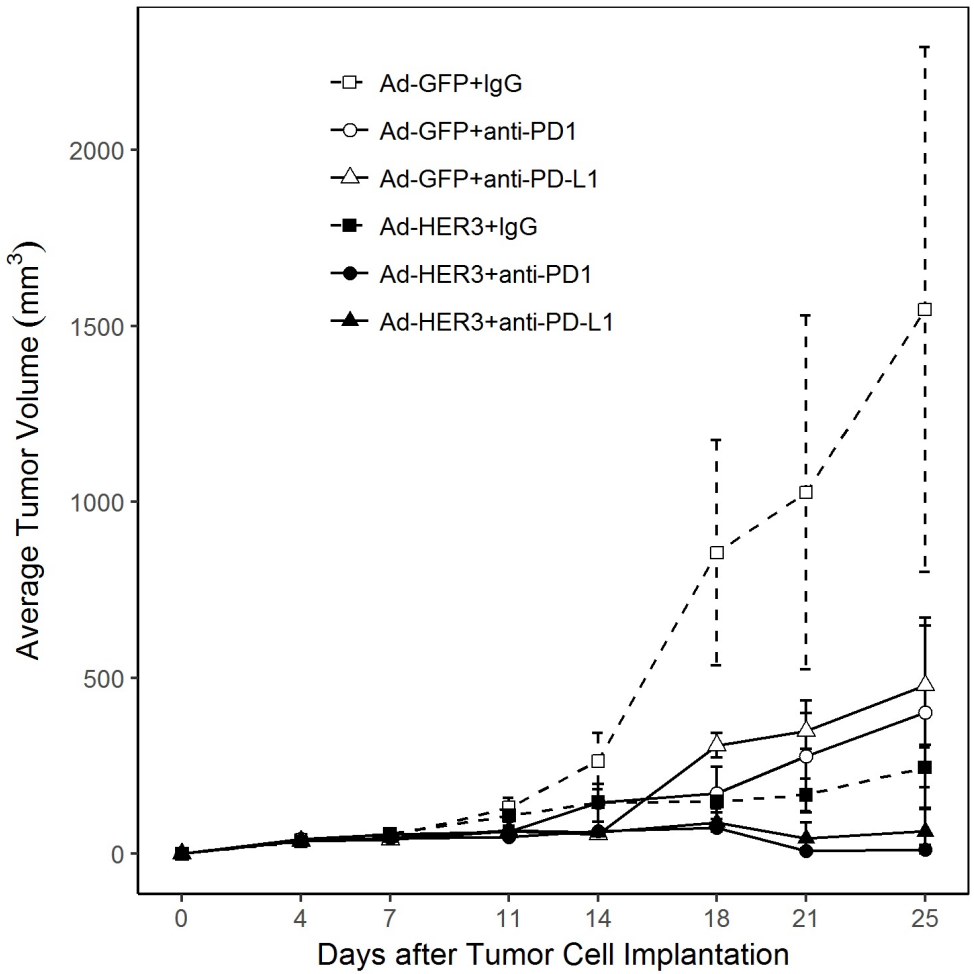
  6. Ad-HER-FL + anti-PD1 vs Ad-HER3-FL + anti-PD-L1.
- FWER $= 1 - (1 - 0.05)^6 = 0.2649$

Introduction

Prevention model



Mean $\pm$ 2 SE

Introduction

Treatment model

- Tumor-bearing mice were immunized with the Ad-HER3-FL vaccine followed by anti-PD-L1 or anti-CTLA4 antibody administration;
- Groups:
  - Ad-GFP + IgG;
  - Ad-HER3-FL + IgG;
  - Ad-HER3-FL + anti-PD-L1;
  - Ad-HER3-FL + anti-CTLA4;
  - Ad-HER3-FL + anti-PD-L1 + anti-CTLA4.

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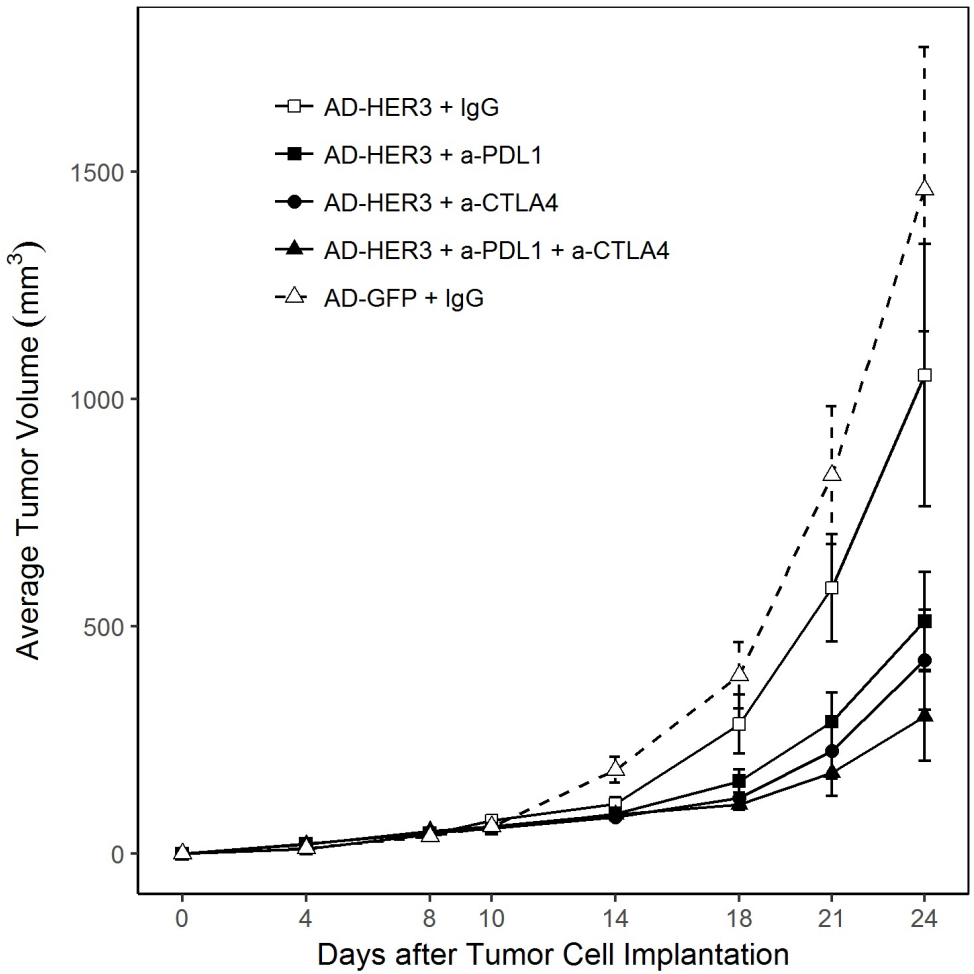
  7. Ad-HER3-FL + anti-CTLA4 vs Ad-HER3-FL + anti-PD-L1 + anti-CTLA4.
- FWER $= 1 - (1 - 0.05)^7 = 0.3016$

Introduction

Treatment model



Mean $\pm$ 2 SE

Introduction

Endpoints

- Investigators are interested in the tumor growth rate;
- A common strategy is to compare tumor volume at each time point, without taking into account multiple testing;
- There are statistical methods that consider the repeated measures over time that require more complex statistical methods;

Alternative strategy

- All mice do not have tumor in the beginning of the experiment, therefore, the tumor size after the study period is a proxy to tumor growth rate;
- Another proxy to tumor rate growth is the area under the curve;
- If tumor volume at baseline is not homogeneous, then a possible solution is to standardize by baseline tumor volume.

Analysis of Variance

Notation

- X_{Ai}: tumor size growth curve for mice i from the group A;
- X_{Bi}: tumor size growth curve for mice i from the group B;
- X_{Ci}: tumor size growth curve for mice i from the group C;

Assumptions

- $X_{A1}, \ldots, X_{An_A} \sim \text{Normal}(\mu_A, \sigma_A^2)$;
- $X_{B1}, \ldots, X_{Bn_B} \sim \text{Normal}(\mu_B, \sigma_B^2)$;
- $X_{C1}, \ldots, X_{Cn_C} \sim \text{Normal}(\mu_C, \sigma_C^2)$;
- Independent samples;
- $\sigma_A^2 = \sigma_B^2 = \sigma_C^2 = \sigma^2$.

Analysis of Variance

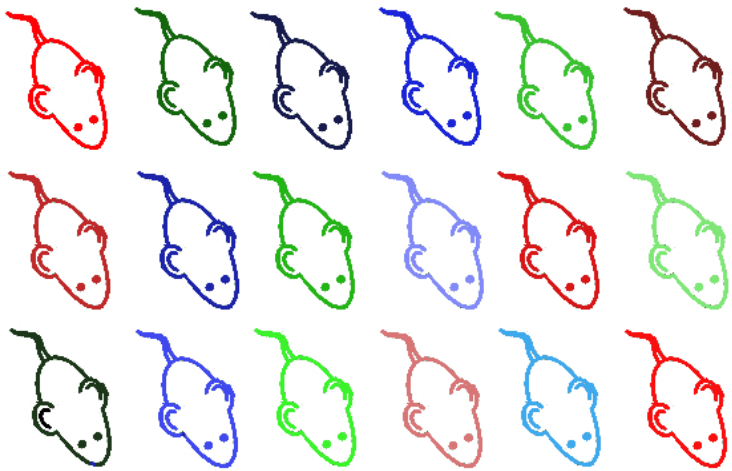
Hypothesis

- $H_0 : \mu_A = \mu_B = \mu_C$ (all means are equal);
- $H_1 : \exists \mu_i \neq \mu_j$ for $i \neq j$ (at least one mean is different).

Multiple comparisons

- It is possible to verify that all the means are equal if we compare means pairwise;
- Three comparisons will be performed, then a multiple comparison procedure should be applied. Otherwise, the error type I inflates to 22.6% if each test has a significance level of 5%;
- There are specific procedures that have higher power for multiple groups than pairwise comparisons with p-values corrections.

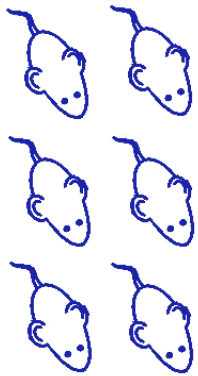
What are the sources of variability?



Total Variability

What are the sources of variability?

A



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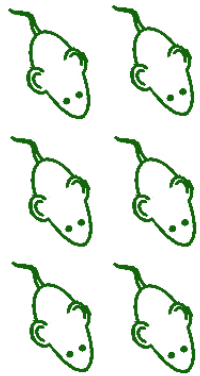
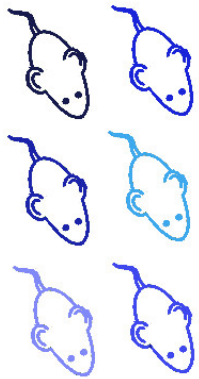


C

Variability between groups

What are the sources of variability?

A



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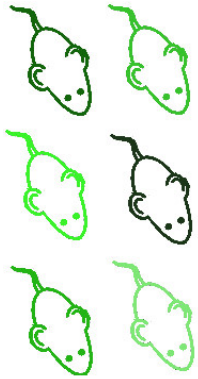

C

Variability within groups

What are the sources of variability?

Total Variability = Within-group variability + Between-group variability

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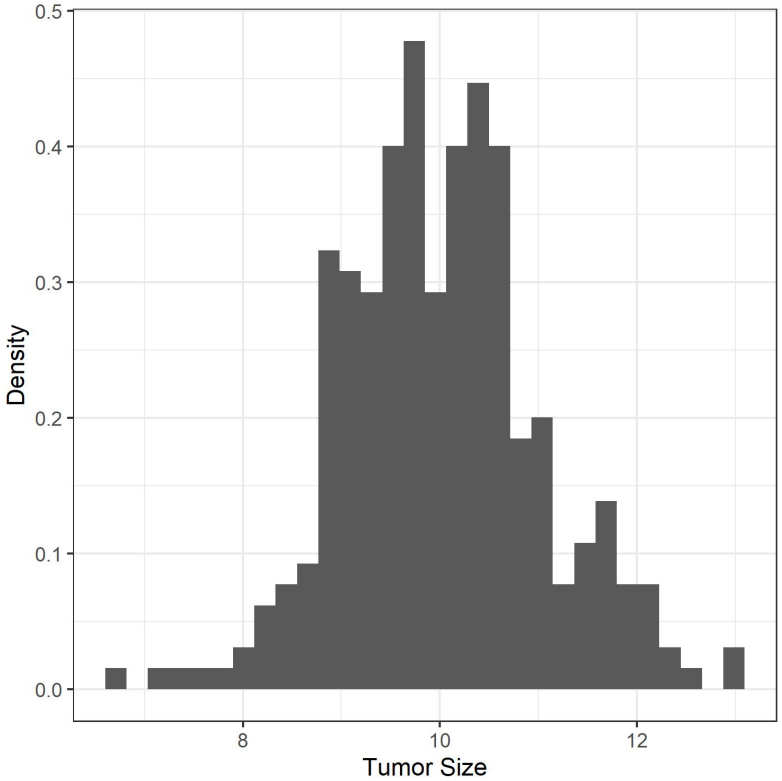
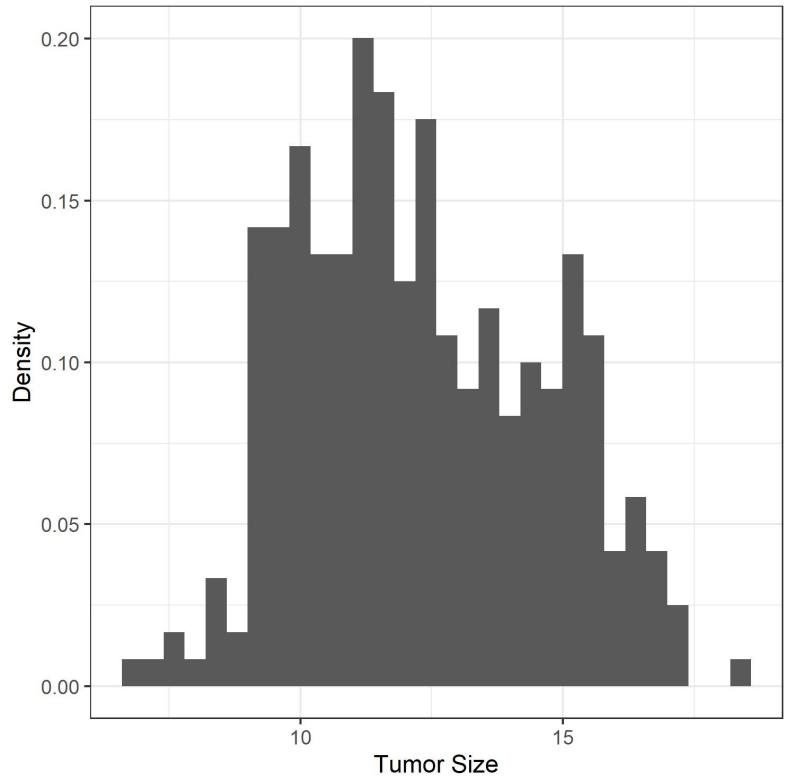
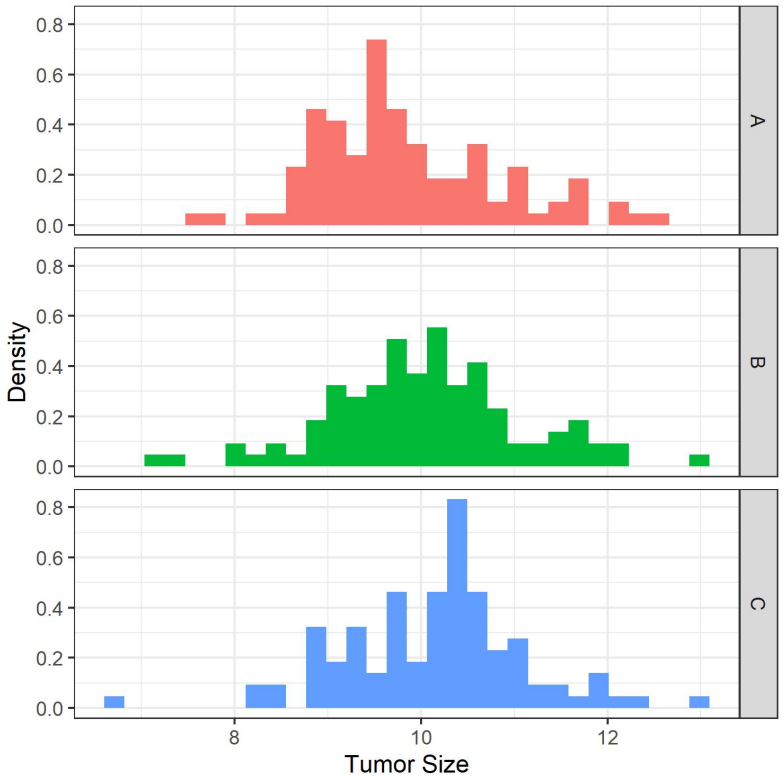


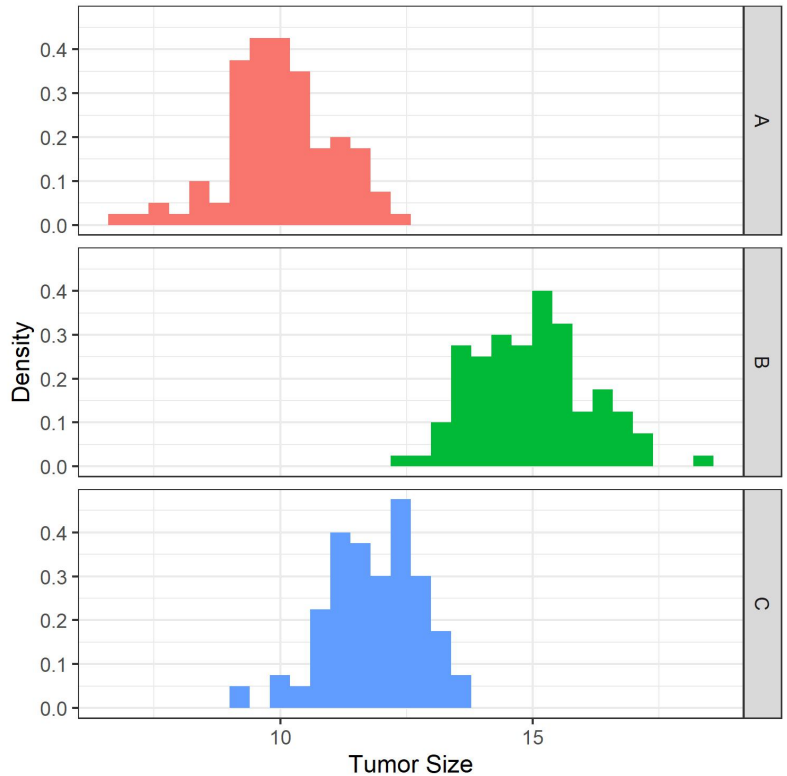
Total Variability = 1



Total Variability = 5

What are the sources of variability?





Within-group variability = 1

Between-group varibaility = Total variability - Within-group variability

What are the sources of variability?

Within-group variability

- If the null hypothesis is true, then three samples can be considered as only one group;
- The sampling variance for the total sample can be calculated,

$$
\begin{aligned}
MSE &= \frac{1}{n-3}\sum_{i=1}^{3}\sum_{j=1}^{n_i}(X_{ij}-\bar{X}_i)^2 \\
&= \frac{1}{n-3}\left(\sum_{j=1}^{n_A}(X_{Aj}-\bar{X}_A)^2+\sum_{j=1}^{n_B}(X_{Bj}-\bar{X}_B)^2+\sum_{j=1}^{n_C}(X_{Cj}-\bar{X}_C)^2\right)
\end{aligned}
$$

- The expected value for MSE is σ^2, i.e., MSE is a good estimator for σ^2.

What are the sources of variability?

Between-group variability

- If the null hypothesis is true, then three averages $(\bar{X}_A, \bar{X}_B, \bar{X}_C)$ can be considered as samples from $\bar{X} \sim N\left(\mu, \frac{\sigma^2}{n}\right)$;
- The sampling variance for the averages can be calculated,

$$MSB = \frac{1}{3-1}\sum_{i=1}^{3}(\bar{X}_i - \bar{X})^2$$

- The expected value for MSE is $\frac{\sigma^2}{n}$. Then, nMSB is a good estimator for σ^2.

Analysis of Variance

Test Statistic

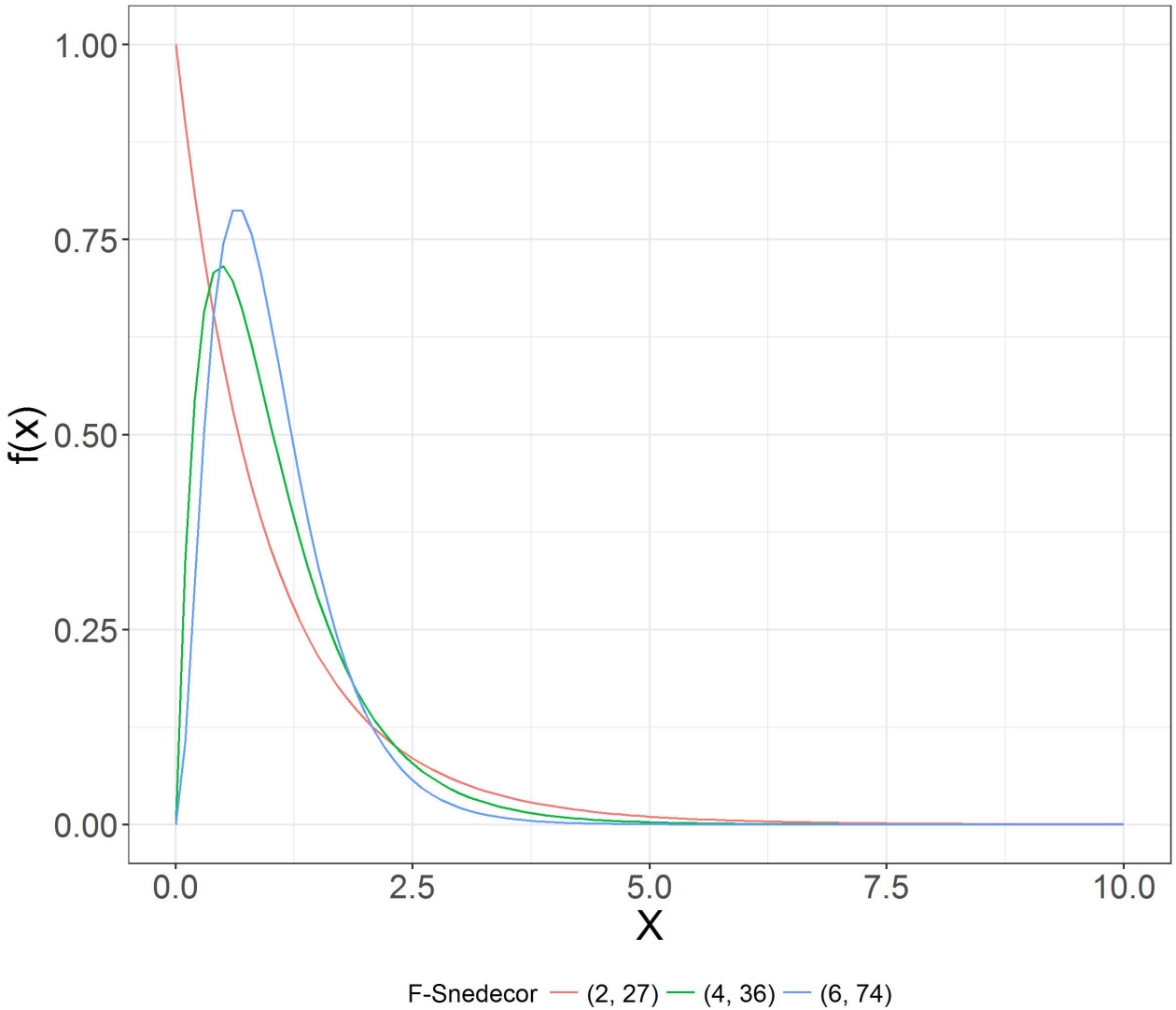
- There are several ways to present:

$$\begin{aligned} \text{F-ratio} &= \frac{\text{variability of the group means}}{\text{variability of the sample}} \\ &= \frac{\text{between-group variability}}{\text{within-group variability}} \\ &= \frac{\text{explained variance}}{\text{unexplained variance}} \end{aligned}$$

- F-ratio $\sim F - Snedecor_{g-1,n-g}$ where n is the total sample size and g is the number of groups;
- Under the H_0, F-ratio will be closer to 1;
- p-value = P(F > k), where k = F-ratio evaluated for a specific sample.

Analysis of Variance

Test Statistic



Analysis of Variance

How to evaluate the assumptions?

- We could evaluate the normality for each our groups as well as the homoscedasticity;
- An easier way is to rewrite the probabilistic model:

$$X_{ij} = \mu + \alpha_j + \epsilon_{ij}$$

where

- μ is a general effect for all groups;
- α_j is a specific effect for the group j for $j = A, B, C$;
- $\epsilon_i j \sim Normal(0, \sigma^2)$;

- In this way, $\mu_j = \mu + \alpha_j$.

Analysis of Variance

How to evaluate the assumptions?

- Considering a sample, it is possible to estimate μ and α_j for $j = A, B, C$ as follow

$$\hat{X}_{ij} = \hat{\mu} + \hat{\alpha}_j$$

where

  - $\hat{\mu} = \bar{X}$;
  - $\hat{\alpha}_j = \bar{X}_j - \bar{X}$ for $j = A, B, C$;

- In this way, residuals can be calculated,

$$e_{ij} = X_{ij} - \hat{X}_{ij}$$

such that residuals are possible estimates for ϵ_{ij};

- The assumptions can be verified using the residuals.

Analysis of Variance

Alternatives

- Welch ANOVA if the homoscedasticity is not verified;
- Kruskal-Wallis if the normality is not verified. The null hypothesis will be defined in terms of medians;
- If the normality and homoscedasticiy are not verified, Kruskal-Wallis is still applied. However, the hypothesis will be defined based on the distributions of probability, i.e.,
  - $H_0 : P(X_j > X_i) = P(X_j < X_i)$ for all $i \neq j$
  - $H_1 : P(X_j > X_i) \neq P(X_j < X_i)$ for at least one $i \neq j$.

Analysis of Variance

What is the follow-up question?

- If p-value is smaller than 0.05, then the null hypothesis is reject in favor of the alternative hypothesis;

Analysis of Variance

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Analysis of Variance

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- If p-value is smaller than 0.05, then the null hypothesis is reject in favor of the alternative hypothesis;
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- Which groups are different?

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What is the follow-up question?

- If p-value is smaller than 0.05, then the null hypothesis is reject in favor of the alternative hypothesis;
- We have enough evidence to say that there is at least one group that is different to the others.
- Which groups are different?
- Post-Hoc tests.

ANOVA

Post-Hoc tests

- Pairwise t-test using a pooled variance from ANOVA:
  - It requires some multiple comparisons correction like Bonferroni or Holm;
- Tukey Honest Significance test:
  - It performs all comparisons;
  - It is essentially a t-test, but rearranging the means for each comparison such that the FWER is corrected;
- Dunnet:
  - It performs all comparisons against a control group.

Welch ANOVA

Post-Hoc tests

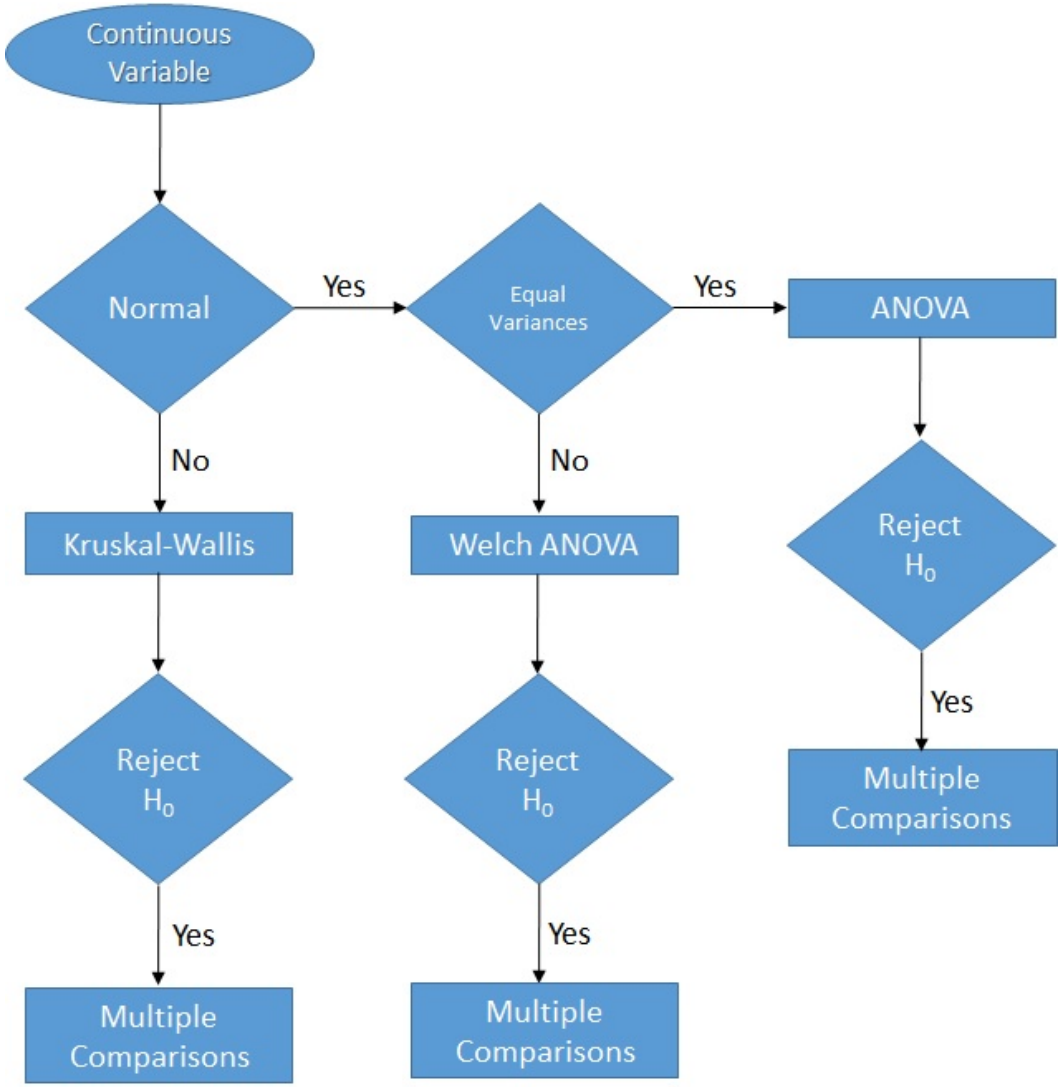
- Pairwise t-test using different variances:
  - It requires some multiple comparisons correction like Bonferroni or Holm;
- Games-Howell test:
  - It performs all comparisons similarly the Tukey HS test.

Kruskal-Wallis

Post-Hoc tests

- Dunn's test:
  - It has the assumption of the same shape;
  - It performs comparisons taking in account the pooled variance from Kruskal-Wallis;
  - It requires multiple comparisons correction such as Bonferroni or Holm;
- Conover's test:
  - It has the assumption of the same shape;
  - It requires multiple comparisons correction such as Bonferroni or Holm;
  - It is stricly more powerful than Dunn's and Kruskal-Nemenyi test;
- Munzel-Hothorn test:
  - It does not have the assumption of the same shape.

Analysis of Variance



Analysis of Variance

Sources of Variation

- One-Way ANOVA
  - Vaccine + Checkpoint inhibitor;
  - Total variability = Treatment variability + Within Variability;
- Two-Way ANOVA
  - Vaccine (main effect);
  - Checkpoint inhibitor (main effect);
  - Vaccine $\times$ Checkpoint inhibitor (interaction);
  - Total variability = Vaccine variability + Checkpoint inhibitor Variability + Within Variability;

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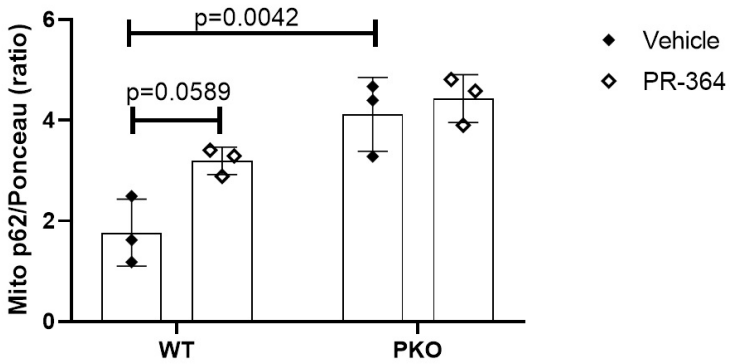
Two-way Analysis of Variance

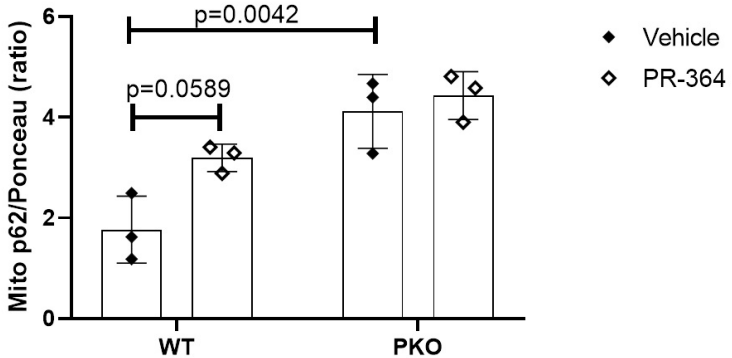
Example 01



- Two mouse type: Wild Type (WT) and Parkin (PKO);
- Two treatments: PR-364 and Vehicle
- Hypothesis: WT mice will show accumulation of p62 protein when receiving PR-364, while this will not happen with PKO mice;
- How can we test this hypothesis?

Two-way Analysis of Variance

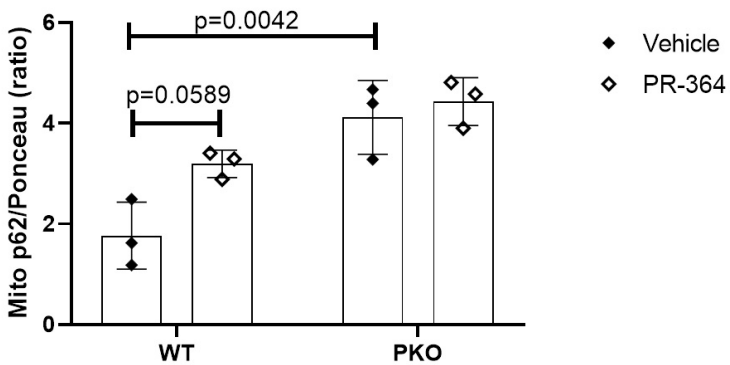
Example 01



- Investigator approach:
  - WT+Vehicle vs WT+PR364;
  - PKO+Vehicle vs PKO+PR-364;
  - WT+PR364 vs PKO+PR-364;
  - WT+Vehicle vs PKO+Vehicle.

Two-way Analysis of Variance

Example 01



- Four groups: WT+Vehicle, WT+PR364, PKO+Vehicle, PKO+PR-364;
- Is there differences between WT and PKO?
  - (WT+Vehicle + WT+PR364) - (PKO+Vehicle + PKO+PR-364);
- Is there differences between PR364 and Vehicle?
  - (WT+PR364 + PKO+PR-364) - (WT+Vehicle + PKO+Vehicle).

Two-way Analysis of Variance

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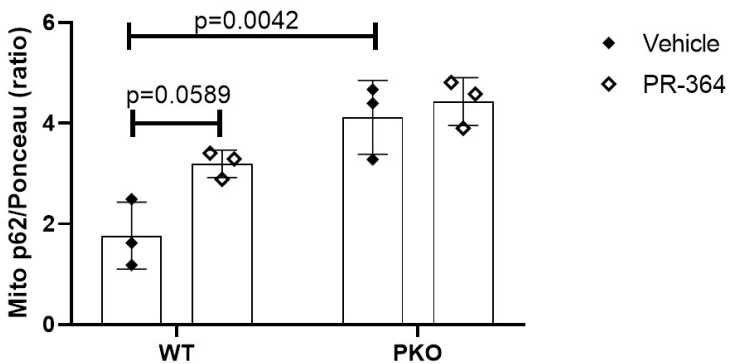

- Is the difference between PR364 and Vehicle different between WT and PKO?
  - For WT, difference between PR364 and Vehicle:
    WT+PR364 - WT+Vehicle;
  - For PKO, difference between PR364 and Vehicle:
    PKO+PR-364 - PKO+Vehicle;
  - The difference of the differences between WT and PKO:
    (WT+PR364 - WT+Vehicle) -
    (PKO+PR-364 - PKO+Vehicle).