Lab 11: After ANOVA—*post hoc* analyses

Introduction and Objective:

We learned how to perform ANOVA tests to compare multiple means. During the very recent lecture, we also discussed if we found the differences between the means was statistically different from the result of ANOVA tests, we can perform additional tests, the *post hoc* tests, to further investigate which two means are different from each other.

In today's lab, we will learn how to use R to perform two *post hoc* tests: Dunnett's test and Tukey's test.

The following files you should have received and are needed for today's class:

1) ExampleInLecture13.csv
2) DAC_VC.csv (This is the same file you used in Lab 10.)
3) PlaceboAnd3Drugs.csv (This is the same file you used in Lab 10.)

All the R command lines are in **bold**; all the notes are following a #; all the R results directly follow the R codes/command lines and are not in bold or following a #.

setwd("C:/R")

The package "multcomp" is very useful for performing multiple comparisons (for many people, *post hoc* test is the synonym for multiple comparisons.)

Remember that there are two ways to install a package. Here we choose to type the command install.packages().

install.packages("multcomp")

Just to remind you that once we install a package, we have to activate it before we can use it in a new R session. We use the function library() to activate it.

library(multcomp)

We may also activate another package, ggplot2, which we downloaded before.

Challenge Question 1:

Do you still remember what the package, ggplot2, is used for? (The answer key is at the end of the notes.)

library(ggplot2)

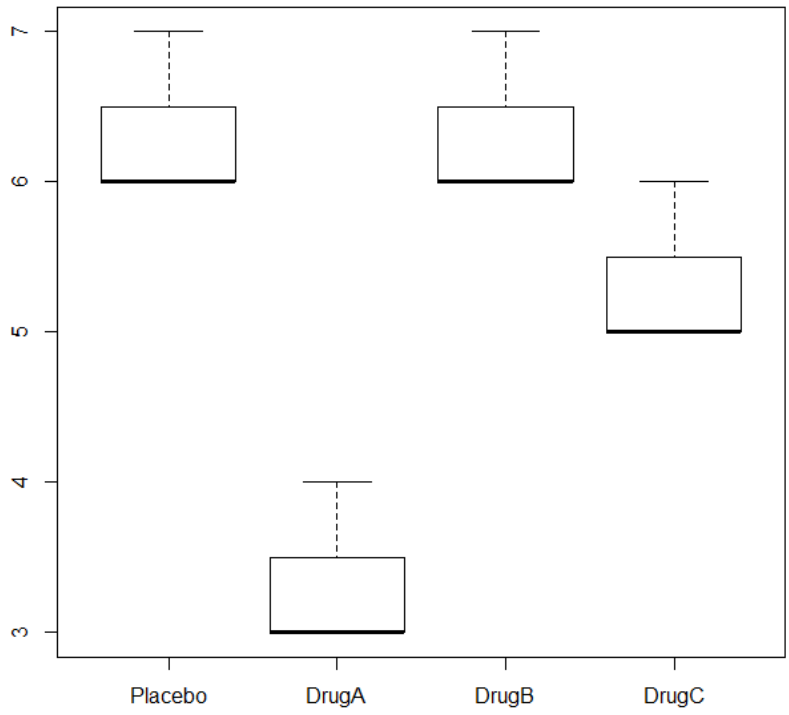
I. Dunnett's test

As we talked about it in the lecture, after the ANOVA test, if you find the difference between the means is statistically significant, of course, you can simply plot the data to visualize which mean is different from the others.

a<-read.csv("ExampleInLecture13.csv")

View(a)

boxplot(a)



Even from this graph, we clearly see the mean of "DrugA" is very different from the means of the other treatments. However, we cannot say the difference is statistically significant or not.

Without performing an additional test, we cannot say the difference between the mean of "DrugA" and the mean of the "Placebo" is statistically significant.

As we learned, we can perform additional tests to examine whether this difference is statistically different or not. Of course, for these additional *post hoc* tests, in particular, the Tukey's or Dunnett's test, we have to plan it ahead of looking at the data.

For this example, which is a typical experiment a biologist or medical researcher would perform, it has a control group, the placebo. As we talked about it in the lecture, for this kind of experiment, we should design Dunnett's test as the *post hoc* test after the ANOVA in case the ANOVA test shows the difference between the means is statistically significant. Because if there are N groups, and one of them is a reference, Dunnett's test simply compares the other groups with the reference. There would only be N-1 comparisons, which generates smaller CIs than the ones from Tukey's test, and thus has more power to detect the differences (later on, after we performed the Tukey's test, you may compare the results from these two tests, and have a better idea of this).

Now, let's perform the Dunnett's test:

The first part is the same as what we did for the ANOVA test:

dim(a)

[1] 3 4

Pla<-a[,1]

Pla

[1] 6 7 6

DA<-a[,2]

DA

[1] 3 4 3

DB<-a[,3]

DB

[1] 6 6 7

DC<-a[,4]

DC

[1] 6 5 5

TumorSize<-c(Pla,DA,DB,DC)

TumorSize

[1] 6 7 6 3 4 3 6 6 7 6 5 5

Treatments<-c(rep("Control",3),rep("DrugA",3),rep("DrugB",3),rep("DrugC",3))

Treatments

[1] "Control" "Control" "Control" "DrugA" "DrugA" "DrugA" "DrugB"

[8] "DrugB" "DrugB" "DrugC" "DrugC" "DrugC"

b<-data.frame(TumorSize,Treatments)

b$Treatments<-as.factor(b$Treatments)

For some RStudio versions, especially the latest version, it may not recognize the variable "Treatments" as a factor after you created the data frame and deposited it in object “b”. By tying the above command line, you forced the variable to be recognized as a factor. It likes what we did previously to use as.matrix() to force some data to become a matrix or use as.numeric() to force the data values to be recognized as numbers.

b

	TumorSize	Treatments
1	6	Control
2	7	Control
3	6	Control
4	3	DrugA
5	4	DrugA
6	3	DrugA
7	6	DrugB

8 6 DrugB

9 7 DrugB

10 6 DrugC

11 5 DrugC

12 5 DrugC

c<-aov(TumorSize~Treatments,data=b)

c

Call:

aov(formula = TumorSize ~ Treatments, data = b)

Terms:

Treatments Residuals

Sum of Squares 18.000000 2.666667

Deg. of Freedom 3 8

Residual standard error: 0.5773503

Estimated effects may be unbalanced

summary(c)

Df Sum Sq Mean Sq F value Pr(>F)

Treatments 3 18.000 6.000 18 0.000646 ***

Residuals 8 2.667 0.333

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Since 0.000646 is less than 0.05 (if we set our significant level at 0.05), we can tell the difference between the 4 means is statistically significant.

library(multcomp)

Actually, we have already activated the package “multcomp”. The reason that we active it here again is simply to emphasize that this is the package we use to perform Dunnett’s test.

d<-glht(c,linfct=mcp(Treatments="Dunnett"))

The function glht() stands for “general linear hypothesis test”. Because the ANOVA test basically is fitting your result with the model of ANOVA.

After the bracket and before the comma, you have to input the result of the ANOVA test, which is the object “c” here.

The “linfct” is a specification of the linear hypotheses to be tested. Here we tell R the specific linear hypothesis we are going to test is the “Dunnett” test of multiple comparisons (“mcp()”).

d

This only tells you the mathematical differences of the means.

General Linear Hypotheses

Multiple Comparisons of Means: Dunnett Contrasts

Linear Hypotheses:

Estimate

DrugA - Control == 0 -3.000e+00

DrugB - Control == 0 -2.434e-15

DrugC - Control == 0 -1.000e+00

Challenge Question 2:

What if we used “Placebo” instead of “Control” when we name the control group and then performed the one-way ANOVA and followed by Dunnett’s test? (The answer key is at the end of this note.)

To find the confidence intervals, you have to type:

confint(d)

This gives you the confidence intervals. From these CIs, you will be able to make conclusions.

Simultaneous Confidence Intervals

Multiple Comparisons of Means: Dunnett Contrasts

Fit: aov(formula = TumorSize ~ Treatments, data = b)

Quantile = 2.8779

95% family-wise confidence level

Linear Hypotheses:

	Estimate	lwr	upr
DrugA - Control == 0	-3.000e+00	-4.357e+00	-1.643e+00
DrugB - Control == 0	-2.434e-15	-1.357e+00	1.357e+00
DrugC - Control == 0	-1.000e+00	-2.357e+00	3.566e-01

summary(d)

This will give you the P values, which help you to make crisp decisions.

Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: Dunnett Contrasts

Fit: aov(formula = TumorSize ~ Treatments, data = b)

Linear Hypotheses:

	Estimate	Std. Error	t value	Pr(>|t|)
DrugA - Control == 0	-3.000e+00	4.714e-01	-6.364	<0.001 ***
DrugB - Control == 0	-2.434e-15	4.714e-01	0.000	1.000

DrugC - Control == 0 -1.000e+00 4.714e-01 -2.121   0.154

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Adjusted p values reported -- single-step method)

II. Tukey-Kramer test (Tukey’s test or Tukey's HSD (Honest Significant Difference) test)

Tukey’s test is very conservative. It compares every mean with every other mean. So if there are N groups, there will be N(N-1)/2 comparisons. This test generates fewer type I errors than performing a pair-wise t-test. However, because the number of tests is larger than a Dunnett’s test would do, it generates larger CIs and reduces the power to detect differences.

Although for the example, which we used in the lecture, we should use Dunnett's test as we discussed, here we use Tukey’s test anyway to show how to perform a Tukey’s test by using R.

There are two ways we can do a Tukey’s test:

1. Use the “multcomp” package

(e<-glht(c,linfct=mcp(Treatments="Tukey")))

General Linear Hypotheses

Multiple Comparisons of Means: Tukey Contrasts

Linear Hypotheses:

Estimate

DrugA - Control == 0 -3.000e+00

DrugB - Control == 0 -2.434e-15

DrugC - Control == 0 -1.000e+00

DrugB - DrugA == 0 3.000e+00

DrugC - DrugA == 0 2.000e+00

DrugC - DrugB == 0 -1.000e+00

confint(e)

Simultaneous Confidence Intervals

Multiple Comparisons of Means: Tukey Contrasts

Fit: aov(formula = TumorSize ~ Treatments, data = b)

Quantile = 3.2013

95% family-wise confidence level

Linear Hypotheses:

	Estimate	lwr	upr
DrugA - Control == 0	-3.000e+00	-4.509e+00	-1.491e+00
DrugB - Control == 0	-2.434e-15	-1.509e+00	1.509e+00
DrugC - Control == 0	-1.000e+00	-2.509e+00	5.091e-01
DrugB - DrugA == 0	3.000e+00	1.491e+00	4.509e+00
DrugC - DrugA == 0	2.000e+00	4.909e-01	3.509e+00
DrugC - DrugB == 0	-1.000e+00	-2.509e+00	5.091e-01

summary(e)

Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: Tukey Contrasts

Fit: aov(formula = TumorSize ~ Treatments, data = b)

Linear Hypotheses:

	Estimate	Std. Error	t value	Pr(>|t|)
DrugA - Control == 0	-3.000e+00	4.714e-01	-6.364	< 0.001 ***
DrugB - Control == 0	-2.434e-15	4.714e-01	0.000	1.00000

DrugC - Control == 0 -1.000e+00 4.714e-01 -2.121 0.22539

DrugB - DrugA == 0   3.000e+00 4.714e-01   6.364 0.00106 **

DrugC - DrugA == 0   2.000e+00 4.714e-01   4.243 0.01199 *

DrugC - DrugB == 0   -1.000e+00 4.714e-01 -2.121 0.22524

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

(Adjusted p values reported -- single-step method)

2. Use the R built-in function TukeyHSD

(f<-TukeyHSD(c,ordered=T))

Tukey multiple comparisons of means

95% family-wise confidence level

factor levels have been ordered

Fit: aov(formula = TumorSize ~ Treatments, data = b)

$Treatments

	diff	lwr	upr	p adj
DrugC-DrugA	2.000000e+00	0.4903968	3.509603	0.0120685
DrugB-DrugA	3.000000e+00	1.4903968	4.509603	0.0009833
Control-DrugA	3.000000e+00	1.4903968	4.509603	0.0009833
DrugB-DrugC	1.000000e+00	-0.5096032	2.509603	0.2252476
Control-DrugC	1.000000e+00	-0.5096032	2.509603	0.2252476
Control-DrugB	8.881784e-16	-1.5096032	1.509603	1.0000000

Answer Keys to the Challenge Questions:

Challenge Question 1:

The R package ggplot2 is used for plotting data and generating high-quality figures.

Challenge Question 2:

This is a very good and important question. If you used "Placebo" instead of "Control" to name the control group and did the Dunnett's test after ANOVA, you would have seen R used "DrugA" as the reference group, and all the other groups were compared with "DrugA". Why? Because when R performs Dunnett's test, it uses the group having the lowest alphabetical rank as the reference group. Thus, it is important to keep it in mind that you have to make sure the reference group has the lowest alphabetical rank.

Groupwork assignment 8 (Part 2):

1. A colon cancer cell line was treated with decitabine (DAC) and vitamin C (VC). The concentration of DAC was always 1nM. There are three different concentrations of VC. Each treatment had 6 biological repeats. Reactivation of an epigenetically silenced gene was measured by qPCR, delta CT was reported in the table that summarized the results. The following table is the file (DAC_VC.csv) you downloaded before.

DAC_VC1	DAC_VC2	DAC_VC3
4	6	11
3	8	12
4	11	13
5	9	7
6	8	8
8	12	9

Previously, to answer the question that whether or not the expression level of a gene of interest in a colon cancer cell line was different resulted from 3 different treatments, we performed a one-way ANOVA test to compare the 3 means of the delta CT of the gene of interest.

From our one-way ANOVA test, we know the P-value is less than 0.05, and the difference between the means is statistically significant. However, from the ANOVA test, we do not know which two means are different from each other.

Assume you have designed a post hoc test in your experiment design. Please perform this post hoc test to find out which means are different from the others.

1) Which post hoc test would you perform? (1pt)

2) Show your R commands and results. (3pts)
3) According to your result, which mean(s) is/are significantly different from the others? (1pt)

2. Previously, we did one-way ANOVA test on the data from the following problem as well:

A pharmaceutical company tested 3 drugs together with a placebo to see whether the putative drugs have an anti-cancer function. The experiments were done in the same kinds of mice. Before the study, the mice had tumor xenograft at the same size. After the treatments, the tumor size (in mm^3) was reported in the following table:

Placebo	DrugA	DrugB	DrugC
620	321	568	623
733	367	562	489
665	310	658	456
692	289	632	398
638	297	523	527
712	378	489	556
701	432	386	423
682	356	612	439
633	286		412
678			432

You downloaded the file contains the above data before. The file name is *PlaceboAnd3Drugs.csv*

From the ANOVA test, we know the difference between the means is statistically significant.

Assume you have designed a *post hoc* test in your experiment design. Please perform this *post hoc* test to find out which means are different from the others.

1) Which post hoc test would you perform? (1pt)

2) Show your R commands and results. (3pts)

3) According to your result, which mean(s) is/are significantly different from the others? (1pt)