Parametric Statistical Inference for Comparing Means and Variances

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Received: May 3, 2020
Accepted: June 3, 2020
Published: July 21, 2020

Citation: Ledolter J, Gramlich OW, Kardon RH. Parametric statistical inference for comparing means and variances. Invest Ophthalmol Vis Sci. 2020;61(8):25.
https://doi.org/10.1167/iovs.61.8.25

PURPOSE. The purpose of this tutorial is to provide visual scientists with various approaches for comparing two or more groups of data using parametric statistical tests, which require that the distribution of data within each group is normal (Gaussian). Non-parametric tests are used for inference when the sample data are not normally distributed or the sample is too small to assess its true distribution.

METHODS. Methods are reviewed using retinal thickness, as measured by optical coherence tomography (OCT), as an example for comparing two or more group means. The following parametric statistical approaches are presented for different situations: two-sample t-test, Analysis of Variance (ANOVA), paired t-test, and the analysis of repeated measures data using a linear mixed-effects model approach.

RESULTS. Analyzing differences between means using various approaches is demonstrated, and follow-up procedures to analyze pairwise differences between means when there are more than two comparison groups are discussed. The assumption of equal variance between groups and methods to test for equal variances are examined. Examples of repeated measures analysis for right and left eyes on subjects, across spatial segments within the same eye (e.g., quadrants of each retina), and over time are given.

CONCLUSIONS. This tutorial outlines parametric inference tests for comparing means of two or more groups and discusses how to interpret the output from statistical software packages. Critical assumptions made by the tests and ways of checking these assumptions are discussed. Efficient study designs increase the likelihood of detecting differences between groups if such differences exist. Situations commonly encountered by vision scientists involve repeated measures from the same subject over time, measurements on both right and left eyes from the same subject, and measurements from different locations within the same eye. Repeated measurements are usually correlated, and the statistical analysis needs to account for the correlation. Doing this the right way helps to ensure rigor so that the results can be repeated and validated.

Keywords: statistical methods, parametric inference, ANOVA, repeated measurements, variance components

This tutorial deals with statistical parametric tests for inference, such as comparing the means of two or more groups. Parametric tests refer to those that make assumptions about the distribution of the data, most commonly assuming that observations follow normal (Gaussian) distributions or that observations can be mathematically transformed to a normal distribution (e.g., log transformation). Non-parametric tests are used for inference when the sample data are not normally distributed or the sample is too small to assess its true distribution and will be covered in a separate tutorial.

For this tutorial on parametric statistical inference, optical coherence tomography thickness measurements of the inner retinal layers recorded in eyes of control mice and mice with optic neuritis produced by experimental autoimmune encephalitis (EAE) serve as illustration. For brevity, we refer to the measured response as retinal thickness. We have explained the goals of this study in another tutorial on the display of data,1 and they are summarized here. There are three treatment groups: control mice, diseased mice (EAE) with optic neuritis, and treated diseased mice (EAE + treatment). For the purpose of this tutorial, we consider only mice with measurements made on both eyes. This leaves us with 15, 12, and six subjects (mice) in the three groups, respectively. For the various statistical analyses in this tutorial, the variance ($s^2$) is defined as the sum of the squared differences of each sample from their sample mean, which is then divided by the number of samples minus 1 (subtracting 1 corrects for the sample bias). The standard deviation is the square root of the variance. The software programs Prism 8 (GraphPad, San Diego, CA, USA) and Minitab (State College, PA, USA) were used to generate the graphs shown in this tutorial.
This tutorial analyzes the average inner retinal thickness of subjects by averaging the measurements on right and left eyes. It also analyzes the inner retinal thickness of mice, but incorporates the correlation between right and left eye measurements on the same subject.

ANALYSIS OF THE AVERAGE RETINAL THICKNESS OF SUBJECTS AFTER COMBINING THEIR MEASUREMENTS ON RIGHT AND LEFT EYES

Comparing Means of Two Treatment Groups: Two-Sample t-Test

First we discuss whether there is a difference between the average retinal thickness of control and diseased mice after EAE-induced optic neuritis. We compare the two groups A = control and B = EAE. Measurements in these two groups are independent, as each group contains different mice. The two-sample t-test relates the difference of the sample means $y_A - y_B$ to its estimated standard error, $se_{y_A-y_B} = \sqrt{\frac{s_A^2}{n_A} + \frac{s_B^2}{n_B}}$. Here, $n_A, y_A, s_A$ and $n_B, y_B, s_B$ are the sample size, mean, and standard deviation for each of the two groups.

Under the null hypothesis of no difference between the two means, the ratio $\frac{y_A - y_B}{se_{y_A-y_B}}$ is well-approximated by a $t$-distribution, with its degrees of freedom $df = n_A + n_B - 2$. The $t$ value is then compared to the critical value from the $t$-distribution.

Comparing Means of Two or More (Independent) Treatment Groups: One-Way ANOVA

The one-way analysis of variance can be used to compare two or more means. Assume that there are $k$ groups (for our illustration, $k = 3$) with observations $y_{ij}$ for $i = 1, 2, \ldots, k$ and $j = 1, 2, \ldots, n_i$ (number of observations in the $i$th group). The ANOVA table partitions the sum of squared deviations of the $n = \sum n_i$ observations from their overall mean, $\bar{y}$, into two components: the between-group (or treatment) sum of squares, $SSB = \sum_{i=1}^{k} n_i (y_{i.} - \bar{y})^2$, expressing the variability of the group means $y_{i.}$ from the overall mean $\bar{y}$, and the within-group (or residual) sum of squares, $SSW = \sum_{i=1}^{k} \sum_{j=1}^{n_i} (y_{ij} - y_{i.})^2 = \sum_{i=1}^{k} (n_i - 1)s_i^2$, adding up all within-group variances, $s_i^2$. The ratio of the resulting mean squares (where mean squares are obtained by dividing sums of squares by their degrees of freedom), $F = \frac{SSB/(k-1)}{SSW/(n-k)}$, serves as the statistic for testing the null hypothesis that all group means are equal. The probability value for testing this hypothesis can be obtained from the $F$-distribution. Small probability values (smaller than 0.05 or 0.10) indicate that the null hypothesis should be rejected.

The ANOVA assumes that all measurements are independent. This is the case here, as we have different subjects in the three groups. Note that independence could not be assumed if both right and left eyes were included, as right and left eye observations from the same subject are most likely correlated; we will discuss later how to handle this situation.

The ANOVA assumes that the variances of the treatment groups are the same. Its conclusions may be misleading if the variances are different. Box showed that the $F$-test is sensitive to violations of the equal variance assumption, especially if the sample sizes in the groups are different. The $F$-test is less affected by unequal variances if the sample sizes are equal. Although the $F$-test assumes normality, it is robust to non-normality as long as the sample sizes are reasonably large (e.g., 30 samples per group).

For only two treatment groups, the ANOVA approach reduces to the two-sample $t$-test that uses the pooled variance. Earlier we had recommended the Welch approximation, which uses a different standard error calculation for the difference of two sample means, as it does not assume equal variances. Useful tests for the equality of variances are discussed later.

If the null hypothesis of equal group means is rejected when there are more than two treatment groups, then follow-up tests are needed to determine which of the treatment groups differ from the others using pairwise comparisons. For three groups, one calculates three pairwise (multiple) comparisons and three confidence intervals for each pairwise difference of two means. The significance level of individual pairwise tests needs to be adjusted for the number of comparisons being made. Under the null...
TABLE 2. Subject Average Retinal Thickness (in μm): One-Way ANOVA with Three Groups (Control, EAE, EAE + Treatment) and Tukey’s Multiple Comparison Tests

| Summary                        | Control | EAE   | EAE + Treatment |
|-------------------------------|---------|-------|-----------------|
| Number of values              | 15      | 12    | 6               |
| Mean                          | 66.21   | 59.81 | 61.65           |
| SD                            | 3.390   | 3.721 | 2.780           |

ANOVA Table

|                     | Sum of Squares | Degrees of Freedom | Mean Square | F(DFn, DFd) | P       |
|---------------------|----------------|--------------------|-------------|-------------|---------|
| Treatment           | 287.2          | 2                  | 143.6       | F(2, 30) = 12.25 | 0.0001  |
| Residual            | 351.8          | 30                 | 11.73       | —           | —       |
| Total               | 639.0          | 32                 | —           | —           | —       |

Tukey’s Multiple Comparisons Test

| Mean Difference      | 95% CI of Difference | Significant? | Adjusted P |
|----------------------|----------------------|--------------|------------|
| Control vs. EAE      | 6.396                | 3.126–9.665  | Yes        | *** 0.0001 |
| Control vs. EAE + treatment | 4.562                | 0.4847–8.640 | Yes        | * 0.0258   |
| EAE vs. EAE + treatment | –1.833               | –6.054 to 2.388 | No        | ns 0.5392 |

* Analysis with GraphPad Prism8. Dfn, degrees of freedom numerator; Dfd, degrees of freedom denominator.

hypothesis of no treatment effects, we set the error that one or more of these multiple pairwise comparisons are falsely significant at a given significance level, such as $\alpha = 0.05$. To achieve this, one must lengthen individual confidence intervals and increase individual probability values. This is exactly what the Tukey multiple comparison procedure4 does (Table 2, Fig. 1). Many other multiple comparison procedures are available (Bonferroni, Scheffe, Sidak, Holm, Dunnett, Benjamini–Hochberg), but their discussion would go beyond this introduction. For a discussion...
of the general statistical theory of multiple comparisons, see Hsu.5

The ANOVA results in Table 2 show that mean retinal thickness differs significantly across the three treatment groups (P = 0.0001). Tukey pairwise comparisons show differences between the group means of thickness for control and EAE and for control and EAE + treatment. The means of EAE and EAE + treatment are not significantly different.

**Comparing Variances of Two or More (Independent) Treatment Groups: Bartlett, Levine, and Brown–Forsythe Tests**

As stated above, ANOVA testing assumes that the group variances are equal. How does one test for equal variances? Bartlett’s test6 (see Snedecor and Cochran7) is employed for testing if two or more samples are from populations with equal variances. Equal variances across populations are referred to as homoscedasticity or homogeneity of variances. The Bartlett test compares each group variance with the pooled variance and is sensitive to departures from normality. The tests by Levene8 and Brown and Forsythe9 are good alternatives that are less sensitive to departures from normality. These tests make use of the results of a one-way ANOVA on the absolute value of the difference between measurements and their respective group mean (Levene test) or their group median (for the Brown–Forsythe test).

We apply these tests to the average retinal thickness data. We cannot reject the hypothesis that all three variances are the same, so we can be more confident in our interpretation of the ANOVA results, as the variances of the groups appear to be similar (Table 3). If one of the tests shows unequal variance but the other test does not, then one needs to evaluate how significant the P value was in rejecting the null hypothesis of equal variance. If a fair amount of uncertainty remains, then alternative approaches are discussed in the next section.

**Approaches to Take When Variances Are Different**

A finding of unequal variances is not just a nuisance (because it puts into question the results from the ANOVA on means) but it also provides an opportunity to learn something more about the data. Discovering that particular groups have different variances gives valuable insights.

Transforming measurements usually helps to satisfy the requirement that variances are equal. Box and Cox10 discussed transformations that stabilize the variability so that the variances in the groups are the same. A logarithmic transformation is indicated when the standard deviation in a group is proportional to the group mean; a square root transformation is indicated when the variance is proportional to the mean. Reciprocal transformations are useful if one studies the time from the onset of a disease (or of a treatment) to a certain failure event such as death or blindness. The reciprocal of time to death, which expresses the rate of dying, often stabilizes group variances. For details, see Box et al.11

If one cannot find a variance-stabilizing transformation, one can proceed with the Welch approximation of pairwise two-sample comparisons. For nearly equal and moderately large sample sizes, the assumption of equal standard deviations is not a crucial assumption, and moderate violations of equal variances can be ignored. Another alternative would be to use nonparametric procedures (they are covered in a different tutorial).

**Analysis of Retinal Thickness Using Both Right and Left Eye Measurements of Each Subject**

**Comparing Means of Two Repeated Measurements: Paired t-Test**

In the earlier two-sample comparison, different subjects were assigned to each of two treatment groups. Often it is more efficient to design the experiment such that a treatment (or induction of a disease phenotype, as in this example) is applied to the same subject. For our example, each mouse could be observed both under its initial healthy condition and after having been exposed to a multiple sclerosis phenotype EAE protocol. Measurements are then available on the same mouse under both conditions, and one can control for (remove) the subject effect that exists. A within-subject comparison of the effectiveness of a treatment or drug is subject to fewer interfering variables than a comparison across subjects. The same is true for the comparison of right and left eyes when both measurements come from the same subject and only one eye is treated, with the other eye acting as a within-subject control. The large subject effect that affects both eyes in a similar way can be removed, resulting in an increase of the precision of the comparison, potentially making it more sensitive to detecting an effect, if one exists.

The paired t-test considers treatment differences, d, on n different subjects and compares the sample mean (d) to its standard error, se_{d} = se_{d}/√{n}. Under the null hypothesis of no difference, the ratio (test statistic) d/se_{d} has a t-distribution with n – 1 degrees of freedom, and confidence intervals and probability values can be calculated. Small probability values (usually smaller than 0.05 or 0.10) would indicate that the null hypothesis should be rejected.

For illustration, we use the right eye (OD) and left eye (OS) retinal thickness measurements from the 15 mice of the control group. Figure 2 demonstrates considerable between-subject variability; the intercepts of the lines that connect measurements from the same subject differ considerably. Pairing the observations and working with changes on the same subject removes the subject variability and makes the analysis more precise. Table 4 indicates that there is no difference in the average retinal thickness of right and left eyes. We had expected this result, as neither eye was treated. However, if one wanted to test a treatment that is given to
just one eye without affecting the other, such a paired treatment comparison between the two eyes would be a desirable analysis plan.

Correlation Between Repeated Measurements on the Same Subject

The two-sample t-test in Table 1 and the ANOVA in Table 2 used subject averages of the thickness of the right and left eyes. Switching to eyes as the unit of observation, it is tempting to run the same tests with twice the number of observations in each group, as now each subject provides two observations. But, if eyes on the same subject are correlated (in our illustration with 33 subjects, the correlation between OD and OS retinal thickness is very large: $r = 0.90$), this amounts to “cheating,” as correlated observations carry less information than independent ones. By artificially inflating the number of observations and inappropriately reducing standard errors, the probability values appear more significant than they actually are.

Suppose that measurements on the right and left eye are perfectly correlated. Adding perfect replicates does not change the group means and the standard deviations that we obtained from the analysis of subject averages; however, with perfect replicates, the earlier standard error of the difference of the two group means gets divided by $\sqrt{2}$, which increases the test statistic and makes the difference appear more significant than it actually is. The earlier ANOVA is equally affected. Adding replicates increases the between-group mean square by a factor of 2 but does not affect the within-group mean square, thus increasing the F-test statistic. This shows that a strategy of adding more and more perfect replicates to each observation makes even the smallest difference significant. One cannot ignore the correlation among measurements on the same subject! The following two sections show how this correlation can be incorporated into the analysis.

Analysis of Repeated Measures Data

Many studies involve repeated measurements on each subject. Here we have 15 healthy control mice, 12 diseased mice (EAE), and 6 treated diseased mice (EAE + treatment), and we have repeated measurements on each subject: measurements on the left and right eye. But, repeated measurements may also reflect measurements over time or across spatial segments (e.g., quadrants of each retina). The objective is to study the effects of the two factors, treatment and eye. Repeated measurements on the same subject can be expected to be dependent, as a subject that measures high on one eye tends to also measure high on the other. The correlation must be incorporated into the analysis. This makes the analysis different from that of a completely randomized two-factor experiment where all observations are assumed independent.

The model for data from such a repeated measures experiment represents the observation $Y_{ijk}$ on subject $i$ in treatment group $j$ and eye $k$ according to

$$Y_{ijk} = \alpha + \beta_j + \pi_{i(j)} + \gamma_k + \beta'_{jk} + \varepsilon_{ijk}$$

where

- $\alpha$ is an intercept.
- $\beta_j$ in this example represents (three) fixed differential treatment effects, with $\beta_1 + \beta_2 + \beta_3 = 0$. With this restriction, treatment effects are expressed as devia-
Pharmacologic treatment is widely used to control intraocular inflammation associated with uveitis. We recently compared a new oral ivermectin formulation with intravitreal corticosteroids in a prospective randomized, double-masked trial. Here we report the visual outcome of this study and provide a comprehensive review of previous studies on the use of ivermectin in uveitis.

In the study, we enrolled 42 patients with active anterior uveitis and baseline visual acuity (VA) of 20/40 or worse. Patients were randomized to receive either intravitreal dexamethasone (n = 18) or oral ivermectin at a dose of 1.5 mg daily (n = 24). The primary endpoint was change in VA from baseline to 12 months. Secondary endpoints included change in VA at 6 months, rate of visual improvement, and adverse events.

Results: Patients in the intravitreal dexamethasone group had a significant improvement in VA at 12 months compared to baseline (P = 0.002). The oral ivermectin group showed a non-significant trend towards improvement (P = 0.10). The rate of visual improvement was higher in the intravitreal dexamethasone group compared to the oral ivermectin group (P = 0.04).

Safety: There were no serious adverse events reported in either group. The most common side effect was transient oral flu-like symptoms in the oral ivermectin group.

Conclusion: Our study suggests that intravitreal dexamethasone may be more effective than oral ivermectin in improving visual outcome in patients with active anterior uveitis. Further studies are needed to confirm these findings.
| Design | OS   | OD   |
|--------|------|------|
|        | Q1   | Q2   | Q3   | Q4   | Q1   | Q2   | Q3   | Q4   |
| Control| G1   | G1   | G1   | G1   | G1   | G1   | G1   | G1   |
| EAE    | G2   | G2   | G2   | G2   | G2   | G2   | G2   | G2   |
| EAE + treatment | G3 | G3 | G3 | G3 | G3 | G3 | G3 | G3 |

**ANOVA Table**

| Degrees of Freedom | OS | OD |
|--------------------|----|----|
|                     |    |    |
| Between subject     |    |    |
| Treatment           | 2  | 2297.86 | 1148.93 | 12.25 | 0.000 |
| Subject             | 30 | 2814.15 | 93.80 | 7.41 | 0.000 |
| Within subject      |    |    |    |    |    |
| Eye                 | 1  | 5.40 | 5.40 | 0.43 | 0.514 |
| Treatment × eye     | 2  | 2.38 | 1.19 | 0.09 | 0.910 |
| Quadrant            | 3  | 36.97 | 12.32 | 0.97 | 0.406 |
| Treatment × quadrant| 6  | 68.45 | 11.41 | 0.90 | 0.495 |
| Eye × quadrant      | 3  | 17.51 | 5.84 | 0.46 | 0.710 |
| Treatment × eye × quadrant | 6 | 92.61 | 15.43 | 1.22 | 0.298 |
| Residual            | 210| 2659.15 | 12.66 | —   | —   |
| Total               | 263| 8001.26 | —   | —   | —   |

The residual sum of squares pools the interaction sums of squares between subjects and the effects of eye, quadrant, and eye by quadrant interaction. The three-factor ANOVA in GraphPad Prism8 is quite limited (two of the three factors can only have two levels) and could not be used.

A standard two-way ANOVA on treatment (with three levels) and eye (with two levels) that does not account for repeated measurements also leads to incorrect results, as such analysis assumes that observations in the six groups are independent. This is not so, as observations in different groups come from the same subject.

**More Complicated Repeated Measures Designs**

Extensions of repeated measures designs are certainly possible. Here are two different illustrations for a potential third factor.

In the first model, the third factor is the (spatial) quadrant of the retina in which the measurement is taken. Measurements on the superior, inferior, nasal, and temporal quadrants are taken on each eye. The model includes random subject effects for the different mice in each of the three treatment groups (G1, G2, G3), with each mouse studied under all eight eye/quadrant combinations. The design layout is shown in Table 6.

Data for the 15, 12, and six mice from the three treatment groups are analyzed. A total of 33 subjects × 8 regions (four quadrants for the right eye and four for the left eye) = 264 measurements is used to estimate this repeated measures model. Results are shown in Table 6. MS(Subject) = 93.80 is used for testing the treatment effect, \( F(\text{Treatment}) = 1148.93/93.80 = 12.25 \). MS(Residual) = 12.66 is used in all other tests (subject effects, main and interaction effects of eye and quadrant, and all of their interactions with treatment). Treatment and subject effects are highly significant, but all effects of eye and quadrant are insignificant, meaning that eyes and quadrants had no effect on retinal thickness.

In the second model, a third factor, type, represents two different genetic mouse strains. The experiment studies the effect of treatment on mice from either of two genetic strains (type 1 and type 2 below). Treatment and strain are crossed fixed effects, as every level of one factor is combined with every level of the other. Each mouse taken from one of the six groups has a measurement made at four different quadrants in one eye. This is a different repeated measures design, as now the mice are nested within the treatment–strain combinations. The design looks as follows:

| Quadrant | Q1 | Q2 | Q3 | Q4 |
|----------|----|----|----|----|
| Control type 1 | G1 | G1 | G1 | G1 |
| Control type 2 | G2 | G2 | G2 | G2 |
| EAE type 1 | G3 | G3 | G3 | G3 |
| EAE type 2 | G4 | G4 | G4 | G4 |
| EAE + treatment type 1 | G5 | G5 | G5 | G5 |
| EAE + treatment type 2 | G6 | G6 | G6 | G6 |

The variability between subjects is used for testing main and interaction effects of treatment and strain. The measurement (residual) variability is used in the test for subject effects and the tests for the main effect of quadrant and its interactions with treatment and strain.

**Conclusions**

This tutorial outlines parametric inference tests for comparing means of two or more groups and how to interpret the output from statistical software packages. Critical assumptions made by the tests and ways of checking these assumptions are discussed.

Efficient study designs increase the likelihood of detecting differences among groups if such differences exist. Situations commonly encountered by vision scientists involve repeated measures from the same subject over time, on both right and left eyes from the same subject, and from different locations within the same eye. Repeated measures are usually correlated, and the statistical analysis must account
for the correlation. Doing this the right way helps to ensure rigor so that the results can be repeated and validated with time. The data used in this review (in both Excel and Prism 8 format) are available in the Supplementary Materials.

Two Excel data files can be found under the Supplementary Materials: Supplementary Data S1 contains measurements on each eye as well as on each subject, whereas Supplementary Data S2 contains measurements for each quadrant of the retina. The two GraphPad Prism8 files under the Supplementary Materials illustrate the data analysis: Supplementary Material S3 on the analysis of subject averages, and Supplementary Material S4 on the analysis of individual eyes.

**Acknowledgments**

Supported by a VA merit grant (C2978-R); by the Center for the Prevention and Treatment of Visual Loss, Iowa City VA Health Care Center (RR&D C9251-C, RX003002); and by an endowment from the Pomerantz Family Chair in Ophthalmology (RHK).

Disclosure: J. Ledolter, None; O.W. Gramlich, None; R.H. Kardon, None

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