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APPLICATION OF LINEAR MIXED-EFFECTS MODELS
TO Crossover DESIGNS

By

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M.D., Shandong University, China P. R., 2007

A Thesis
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University of Louisville
Louisville, Kentucky

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A Thesis Approved On

November 29, 2012

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ABSTRACT

APPLICATION OF LINEAR MIXED-EFFECTS MODELS TO CROSSOVER DESIGNS

Lei Zhou

November 29, 2012

Crossover design is a type of longitudinal study with each subject receiving different treatments in different time periods. It has been used frequently in the pharmaceutical industry and other medical fields to investigate the safety and efficacy of new drugs or new treatments. For crossover studies, the treatment effects from the earlier period may be carried over to the later period, which is called the carry-over effect, the response may naturally change over different periods. How to assess treatment effect with accounting for all these features deserves further investigation.

Linear mixed-effects (LME) model has been widely applied to analyze data resulted from longitudinal studies. In this project, we model treatment effects, period effects, and carryover effects using the LME for 2×2 crossover studies, where all subjects are randomly assigned to two sequences, and each subject is treated subsequently with two treatments, the order of the treatments depends on its sequence. We first investigated the simple 2×2 design, where each subject has only one response measurement under each treatment. Extensive simulations were carried out to compare the performance between LME and the traditional Grizzle's method. We extend the LME to the general 2×2 crossover studies, where the response under each treatment is taking over different time

point. We applied our model to an endothelial progenitor cell (EPC) study, where each patient was exposed to either air (placebo) first then airborne particular matters (PM) or PM first then air. In each exposure condition, the EPC cells were measured right before the exposure (Pre), right after the exposure (Post), and the second day follow-up (FU). The results obtained from LME and traditional methods are compared.

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CHAPTER 1

INTRODUCTION

1.1 Crossover trial

There are two commonly used study designs in clinical research: parallel design and crossover design. In a parallel study design, each subject is randomly assigned to one and only one treatment. A crossover design study is a longitudinal study in which each subject receives a sequence of different treatments, and there is a “washout” period between two treatments. Crossover designs are common for experiments in many scientific disciplines such as psychology, education, pharmaceutical science, and healthcare, especially medicine [1]. If the disease is chronic and the effect of treatment is reversible, a crossover trial may be an attractive option [2].

We first focus on the simple 2×2 crossover design, in which there are two treatments, traditionally labeled A and B, and two periods. Each subject receives the treatments in either of the two possible sequences, AB or BA. This kind of design is shown in Figure 1[3].

Figure 1. Illustration of 2×2 crossover design

The treatment effect is estimated using the differences of the pairs of observations from each subject. Usually, equal numbers of subjects are randomly assigned to the two groups. A crossover trial has two advantages over a non-crossover trial. First, the influence of confounding covariates is reduced because each subject serves as his or her own control [4]. In a non-crossover trial, such as randomized clinical trials, different treatment groups are often found to be unbalanced on some covariates. Second, optimal crossover designs are statistically efficient and require few subjects than do non-crossover designs. The so-called “optimal design” refers to such kind of experimental design which allows parameters to be estimated without bias and with minimum variance [4]. By contrast, a non-optimal design requires a greater number of experimental runs to estimate the parameters with the same precision as an optimal design. In practice, optimal designs can reduce the costs of experiments. An obvious deficiency of this design is the possible existence of “carry-over”, which means that the data from the second period may reflect not only the effect of the treatment given in that period but also the residual effect of treatment given in the previous period [5]. The presence of a differential carry-over effect, if ignored, may cause biased estimate for the treatment effects. In general, the carryover effect is first examined before testing treatment effects. The test for the presence of carry-over effect is usually carried out using the unpaired t test for the two groups based on the sums of within-subjects observations. However, this test could be less powerful [4]. In that case, we may have to increase sample size, which will exactly offset the advantage of efficiency provided by the crossover designs.

1.2 Baseline measurements

The statistical power of crossover trial may be increased by taking “baseline” measurements of the outcome variable at the start of each treatment period. If changes from baselines are to be analyzed, the between-subject variation will be removed and the power of tests will be increased [6]. Analysis of covariance (ANCOVA) has been recommended [6] by taking best advantage of baseline measures, as this method explicitly estimates the association between baseline and post-treatment measurements.

1.3 Mixed-effect models

A mixed-effect model is a statistical model containing both fixed effects and random effects, where random effects are often used to describe the subject-specific effect, while fixed effects are used to describe population-level effect. Mixed effect models are particularly useful in settings where repeated measurements are taken on the same statistical units, or where measurements are made on clusters of related statistical units [7]. The correlation between the repeated measurements is captured by the random effects and their distribution assumption [4]. Furthermore, the mixed-effects model can handle missing and unbalanced data, which are common in practice, especially for longitudinal data analysis [7].

In this thesis, we applied linear mixed-effects models to our recent 2×2 crossover experimental data on examining whether exposure to airborne particulate matter (PM) affects the circulating levels of endothelial progenitor cell (EPC) populations. Subjects are randomly assigned to two exposure sequences: one with AIR first then followed by PM, the other with PM first then followed by AIR. Under each exposure, the blood

sample was taken before exposure (Pre), two-hours after exposure (Post), and the second morning for follow-ups (FU). In an exposure sequence, the second exposure was carried out one week after the first exposure. Flow cytometry was applied to measure the cell counts for CD31+CD34+ /50K lymph.

CHAPTER 2

STATISTICAL METHODS FOR 2×2 CROSSOVER STUDIES WITH A SINGLE OBSERVATION PER PERIOD

2.1 2×2 crossover design studies

In 2×2 crossover design studies, subjects are randomly assigned either to sequence 1, where each subject receives treatment A in the first period followed by treatment B in the second period; or to sequence 2, where each subject receives the two treatments in the reverse order. (See Table 1):

Table 1. Description of crossover design trials

Sequence	Period 1	Period 2
1 (Group 1)	A	B
2 (Group 2)	B	A

The two periods should be the same length of time and usually the same numbers of subjects are allocated to both groups. The run-in measurement, which is prior to the first given treatment, is always obtained as baseline measurement to deal with the recruitment variance. The washout period is set between two periods, which is expected to be long enough to eliminate the extra effect brought to the second period by the first treatment in the first period, (i.e., carry-over effect). The variables are denoted as below: y_{ikj} is the observation for i^{th} sequence ($i=1,2$) j^{th} subject ($j=1,2,\dots,n_i$) k^{th} period ($k=1,2$); μ is the overall mean; τ is the treatment effect; π is the period effect; λ is the carry-over effect; s is random subject effect, which is assumed to be a random variable with mean zero

and variance σ_s^2 ; e is a within-subject error, which is assumed to be random variable with mean zero and variance σ^2 .

In this chapter, we first introduce the procedure of traditional Grizzle's method for 2×2 crossover design studies, then introduce the linear mixed-effect (LME) model. A case study was carried out by using both methods. At last, simulations are carried out to compare the performance of both methods.

2.2 Grizzle's method

In the traditional 2×2 crossover design, the following parameters are usually introduced: τ_1 and τ_2 are treatment parameters for treatment A and B, respectively; π_1 and π_2 are period parameters for period 1 and 2; λ_1 and λ_2 are carry-over parameters. The expected effect in each group and each period are displayed in Table 2:

Table 2. The fixed effects in the full model

Sequence	Period 1	Period 2
1 (AB)	$\mu + \pi_1 + \tau_1$	$\mu + \pi_2 + \tau_2 + \lambda_1$
2 (BA)	$\mu + \pi_1 + \tau_2$	$\mu + \pi_2 + \tau_1 + \lambda_2$

This parameterization can be obtained by the following model:

$$y_{ijk} = \mu + \pi_k + \tau_u + \lambda_v + s_{ij} + \varepsilon_{ijk} \quad (1)$$

Where y_{ijk} is the observation for i^{th} sequence ($i=1,2$) j^{th} subject ($j=1,2,\dots,n_i$) k^{th} period ($k=1,2$); μ is the overall mean; π_k is the effect of the k th period ($k=1,2$); τ_u represents the effect of the u th treatment ($u=A, B$); λ_v represents the residual effect of the v th treatment in the first period on the response in the second period ($v=A, B$); s_{ij} is the effect for the j th subject in the i th group ($i=1,2; j=1,2,\dots,n$); ε_{ijk} is the within-subject error for j th subject in the i th group and the k th period.

Here we assume that μ , π_k , τ_u , and λ_v are fixed effect, s_{ij} is an random effect with mean zero and variance σ_s^2 , and ε_{ijk} is a random error with means zero and variances σ^2 , and s_{ij} and ε_{ijk} are mutually independent. Thus, the between-subject variance is σ_s^2 and the within-subject variance is σ^2 . Assume that the within-subject correlation is ρ , the variance-covariance matrix is given as:

$$\text{var} \begin{pmatrix} y_{ij1} \\ y_{ij2} \end{pmatrix} = \begin{pmatrix} \sigma^2 + \sigma_s^2 & \sigma_s^2 \\ \sigma_s^2 & \sigma^2 + \sigma_s^2 \end{pmatrix}$$

This variance structure is called uniform or compound symmetry structure.

In a crossover design, the measured effect from the second period may reflect not only the treatment given in that period but also the residual effect of treatments given in the first period. This phenomenon is referred to as “carry-over” effect [4].

When the carry-over effect exists, the evaluation for treatment effect without considering the carry-over effect may result in a biased estimate. Grizzle (1965) proposed first test carry-over effect using the sums of the pairs of observations from each subject:

$$t_{1j} = y_{1j1} + y_{1j2} \quad \text{for the } j\text{th subject in sequence (group) 1}$$

$$t_{2j} = y_{2j1} + y_{2j2} \quad \text{for the } j\text{th subject in sequence (group) 2}$$

$$E[t_{1j}] = 2\mu + \pi_1 + \tau_1 + \pi_2 + \tau_2 + \lambda_1$$

$$E[t_{2j}] = 2\mu + \pi_1 + \tau_1 + \pi_2 + \tau_2 + \lambda_2$$

To test if $\lambda_1 = \lambda_2$, we can use two-sample t test to test whether the summations in sequence 1 is significantly different from the summation in sequence 2. That is:

$$\lambda_d = \lambda_1 - \lambda_2, \text{ is estimated by } \hat{\lambda}_d = \bar{t}_1 - \bar{t}_2. \quad (2)$$

$$\text{VAR}(\hat{\lambda}_d) = (4\sigma_s^2 + 2\sigma^2) \left(\frac{1}{n_1} + \frac{1}{n_2} \right) \quad (3)$$

The pooled sample variance is [4]:

$$\widehat{\text{var}}(\lambda_d) = \left[\sum_{i=1}^2 \sum_{j=1}^{n_i} (t_{ij} - \bar{t}_i)^2 / (n_1 + n_2 - 2) \right] \times \left(\frac{1}{n_1} + \frac{1}{n_2} \right)$$

The test statistic for carry-over effect is:

$$T_\lambda = \hat{\lambda}_d / (\text{Var} [\hat{\lambda}_d])^{0.5} \sim t_{(n_1+n_2-2)} \quad (4)$$

This test is criticized as lacking power to detect sizable carry-over effect because of the usage of between-subject comparison. Grizzle (1965) recommended that the test be performed at a significance level greater than the traditional value of 0.05, for example 0.1 or even 0.15 [4]. Since the low power, we should be careful and cannot assert that the lack of significance implies lack of carry-over effect [8].

The treatment effect is estimated using the differences of the pairs of observations from each subject, which is a relatively powerful test (CROS) [4]. The unbiasedness of the resulting estimate rests on the assumption of equality of the carry-over effects. If the test of carry-over effects is significant, Grizzle (1965) suggested that only the data from period 1 should be used to test the treatment effects, as in a parallel groups design (PAR) [4].

Grizzle proposed the following strategy to analyze 2×2 crossover design studies [9]. First, test whether there is a carry-over effect at type I error level of $\alpha = 0.1$. If the test is not rejected, one then tests the treatment effects using CROS at type I error level of $\alpha = 0.5$; if the test for carry-over effect is rejected, test treatment effects using PAR at type I error level of $\alpha = 0.5$. This has come to be known as “the two-stage procedure” (TS). The reasons can be verified by the following mathematical derivations.

If we can assume that there is no carry-over effect (i.e. $\lambda_1 = \lambda_2$), then the period differences are:

$d_{1j} = y_{1j1} - y_{1j2}$ for the j th subject in sequence (group) 1

$d_{2j} = y_{2j1} - y_{2j2}$ for the j th subject in sequence (group) 2

We have:

$$E[d_{1j}] = \pi_1 - \pi_2 + \tau_1 - \tau_2$$

$$E[d_{2j}] = \pi_1 - \pi_2 + \tau_2 - \tau_1$$

We use the two-sample t test to test treatment effect by using the period differences:

$$\hat{\tau}_d = \frac{1}{2} (\bar{d}_1 - \bar{d}_2), \text{ where } \bar{d}_i = 1/n_i (\sum_{j=1}^{n_i} d_{ij}), (i=1, 2) \quad (5)$$

One can easily see that:

$$E(\hat{\tau}_d) = \tau_d = \tau_1 - \tau_2, \text{ and } \text{Var}(\hat{\tau}_d) = (\sigma^2/2) \times \left(\frac{1}{n_1} + \frac{1}{n_2} \right) \quad (6)$$

The pooled sample variance is [4]:

$$\widehat{\text{var}}(\lambda_d) = \left[\sum_{i=1}^2 \sum_{j=1}^{n_i} (d_{ij} - \bar{d}_i)^2 / (n_1 + n_2 - 2) \right] \times \left(\frac{1}{4n_1} + \frac{1}{4n_2} \right)$$

The test statistic for treatment effect is constructed as:

$$T_\tau = \hat{\tau}_d / (\text{Var} [\hat{\tau}_d])^{0.5} \sim t_{(n_1+n_2-2)} \quad (7)$$

If we cannot assume that there is no carry-over effect (i.e. $\lambda_1 \neq \lambda_2$), then:

$$E(\hat{\tau}_d) = E\left[\frac{1}{2} (\bar{d}_1 - \bar{d}_2)\right] = \tau_d - (\lambda_d/2).$$

That is, $\hat{\tau}_d$ is no longer an unbiased estimator of τ_d if $\lambda_d = \lambda_1 - \lambda_2 \neq 0$ [4]

Since $\hat{\lambda}_d = \bar{y}_{11} + \bar{y}_{12} - \bar{y}_{21} - \bar{y}_{22}$, and $\hat{\tau}_d = \frac{1}{2} [\bar{y}_{11} - \bar{y}_{12} - \bar{y}_{21} + \bar{y}_{22}]$, we have

$$\hat{\tau}_d | \lambda_d = \frac{1}{2} [\bar{y}_{11} - \bar{y}_{12} - \bar{y}_{21} + \bar{y}_{22}] + \frac{1}{2} [\bar{y}_{11} + \bar{y}_{12} - \bar{y}_{21} - \bar{y}_{22}] = \bar{y}_{11} - \bar{y}_{21}.$$

That is the difference between the groups in terms of their first period means. In other words, if $\lambda_d \neq 0$ then the estimator of τ_d is based on between-subject information and is the estimator we would have obtained if the trial has been designed as a parallel study.

2.3 Linear mixed-effects models for 2×2 crossover design

Since the main purpose of the crossover study is to investigate treatment effects, we consider a linear mixed effect model which directly models the treatment effect. Meanwhile, we introduce period effect and carry-over effect. Instead of using two-step procedure, we directly estimate the parameters involved, and test whether there is treatment effect based on the results obtained from LME model. The model can be described by:

$$y_{ijk} = \beta_0 + \beta_1 X_{\text{treat}} + \beta_2 X_{\text{period}} + \beta_{12} X_{\text{treat}} X_{\text{period}} + s_{ij} + \varepsilon_{ijk}$$

s_{ij} is a random subject effect term and s_{ij} is assumed to be independently and identically distributed with $N(0, \sigma_s^2)$

ε_{ijk} is error term and is assumed to be independently and identically distributed with $N(0, \sigma^2)$

Two dummy variables are introduced as:

$X_{\text{treat}} = 1$ if the subject is in treatment B, and $X_{\text{treat}} = 0$ otherwise;

$X_{\text{period}} = 1$ if the subject is in period 2, and $X_{\text{period}} = 0$ otherwise.

Introducing random subject effects in the regression model can capture the within-correlation of the subject observations. In addition, random subject effects could recover the information in the subject totals [4]. However, if there is little variation in the subject totals, the between-subject variation will be small and the random effect may not need to be included. The variation can often be described by the intraclass correlation, which is defined as $\sigma_s^2 / (\sigma_s^2 + \sigma^2)$ [4], meaning the size of the between-subject variance (σ_s^2) relative to the within-subject variance (σ^2). In many crossover trials, we expect the

between-subject correlation is large and the remaining within-period correlation is small and in many cases quite close to zero [10].

2.4 Case studies

2.4.1 Case study 1: PEFR data

We apply Grizzle’s method and LME to analyze PEFR data which were from a randomized, placebo-controlled, double-blind study to evaluate the efficacy and safety of an inhaled drug (A) given twice daily via an inhaler in patients with chronic obstructive pulmonary disease (COPD). The eligible patients were randomized to receive either Drug (A) or Placebo (B) twice daily for 4 weeks. The patients then switched over to the alternative treatment for an additional 4 weeks. Peak Expiratory Flow Rate (PEFR) was measured as the response variable.

The raw data for sequence AB and BA are illustrated in Figure 2. From Figure 2, we expect that A is higher than B.

Figure 2. Subject-level profiles under group AB and BA for PEFR data

First, we apply Grizzle's method to examine the treatment effect for PEFR data. To do that, we first test the carry-over effects.

$$\hat{\lambda}_d = \bar{t}_{1.} - \bar{t}_{2.} = 38.89, \widehat{var}(\hat{\lambda}_d) = 1681.68, \text{ and}$$

$$T_{\lambda} = 38.89 / (1681.68)^{0.5} = 0.9483$$

P-value = $2 * P(T_{\lambda} > t_{(0.975, 54)}) = 0.347$, therefore, we conclude that there is no carry-over effect.

We come to test the treatment effects under the assumption of equality of carry-over effects (i.e. no carry-over effect).

$$\hat{\tau}_d = \frac{1}{2} (\bar{d}_{1.} - \bar{d}_{2.}) = 10.4, \widehat{Var}(\hat{\tau}_d) = 11.666, \text{ and}$$

$$T_{\tau} = 10.4 / (11.666)^{0.5} = 3.045$$

$$P\text{-value} = 2 * P(T_{\tau} > t_{(0.975, 54)}) = 0.004$$

Therefore, we conclude that there is a significant treatment effect.

We apply LME method to PEFR data.

The LME model is as follows:

$$y_{ijk} = \beta_0 + \beta_1 X_{\text{treat}} + \beta_2 X_{\text{period}} + \beta_{12} X_{\text{treat}} X_{\text{period}} + S_{ij} + \epsilon_{ijk}$$

$X_{\text{treat}} = 1$ when given drug (A), 0 otherwise; $X_{\text{period}} = 1$ for second period, 0 otherwise.

The results based on LME are summarized in Table 3. The results are similar to those based on Grizzle's method.

Table 3. Estimations of fixed effects components for linear mixed-effects model for PEFR data

	Estimate	SE	DF	t-value	p-value
B_0	235.769	10.527	55	22.396	0
$\beta_{1(\text{treatment})}$	10.402	3.416	54	-3.046	0.004
$\beta_{2(\text{period})}$	3.767	3.416	54	1.103	0.275
$\beta_{12(\text{interaction})}$	38.888	41.008	53	0.948	0.347

2.4.2 Case study 2: FEV1 data

We also applied Grizzle's method and LME model to FEV1 data by Patel (1983). FEV1 data was reported as being taken from the results of a trial involving subjects with mild to acute bronchial asthma [4]. The treatments were single doses of two active drugs, say A and B. The response of interest was the forced expired volume in one second (FEV₁). The baseline FEV₁ measurement was taken during the run-in period immediately prior to giving the first treatment. FEV₁ measurements were taken again, 2 and 3 hours after treatment, the average of the two measurements is the observed measurement for this period. A suitable period of time was then left before a second treatment was given. The measurements from the second treatment were then taken at 2 and 3 hours to give the average value for period 2. A general treatment profile is shown in Figure 3,

Figure 3. Treatment A versus Treatment B for FEV1 data and the subject profiles in each group are shown in Figure 4

Figure 4. Subject-level profiles under group AB and BA for FEV1 data

According to treatment profile and the subject profile, treatment B may have higher responses than treatment A.

We first analyzed the data using Grizzle's method:

We first tested the carry-over effect (Type I error is set as 0.1):

$$\hat{\lambda}_d = \bar{t}_{1.} - \bar{t}_{2.} = -1.005$$

And the pooled sample variance is:

$$\widehat{\text{Var}}(\hat{\lambda}_d) = \left[\sum_{i=1}^2 \sum_{k=1}^{n_i} (t_{ik} - \bar{t}_{i.})^2 / (n_1 + n_2 - 2) \right] * \left(\frac{1}{n_1} + \frac{1}{n_2} \right) = 0.451$$

$$\text{Therefore, } T_{\lambda} = \hat{\lambda}_d / (\widehat{\text{Var}}[\hat{\lambda}_d])^{0.5} = -1.496 \sim t_{(14)}$$

The p-value is 0.157, which is greater than 0.1, therefore, we are not able to reject the null hypothesis on carry-over effect. We concluded that there is not carry-over effect.

Therefore we use CROS to test the treatment effects.

$$\hat{\tau}_d = \frac{1}{2}(\bar{d}_{1.} - \bar{d}_{2.}) = -0.176, \widehat{\text{Var}}(\hat{\tau}_d) = 0.008, \text{ and}$$

$T_\tau = -0.176/(0.008)^{0.5} = -2.011$. We get P-value= 0.064. Therefore, there is not significant difference between the two treatments. We also analyzed the FEV1 data using LME model, the results are shown in Table 4. Based on Table 4, there is still not significant difference for the period effects and treatment. Again, the results based on Grizzle's method and LME are similar.

Table 4. Estimations of fixed effects based on LME for FEV1 data

	Estimate	SE	DF	t-value	p-value
β_0	1.742	0.245	15	9.174	0
β_1	0.678	0.347	13	-1.956	0.0723
β_2	0.428	0.347	13	-1.617	0.1298
β_{12}	1.005	0.672	13	1.496	0.1584

2.5 Simulations

We generated the simulation data according to the following model:

$$Y_{ijk} = \beta_0 + \beta_1 X_{\text{treat}} + \beta_2 X_{\text{period}} + \beta_{12} X_{\text{treat} \times \text{period}} + S_{ij} + \varepsilon_{ijk}$$

X_{treat} and X_{period} are dummy-coded variables as specified in Section 2.3, and the underlying regression coefficients are those obtained in the case study in Section 2.4 (Table 4.). The random subject effects are assumed to be normal distributed with mean zero, variances $\hat{\sigma}_s^2=0.428$ and the within-subject is assumed to be normal with mean zero, and variance $\hat{\sigma}^2=0.06$. In simulations, each group was generated with 10 and 30 subjects, respectively. First, we set $\beta_1=0$, which means no treatment effect. We examined the rejection rate for $H_0: \beta_1=0$ over 1,000 times of simulations. Simultaneously, we also use the Grizzle's method to analyze these randomly generated data and calculate the rejection rate for carry-over effects and the rejection rate of tests for treatment effects. We repeated the same process for $\beta_{12}=0$ (i.e. no carry-over effect), $\beta_{12}=0.5$ (i.e. small carry-over effect) and $\beta_{12}=1$ (i.e. large carry-over effect), and $\beta_1=-0.6, -0.3, 0.3$ and 0.6 treatment effects.

The simulations results are summarized in Table 5 to 7, and in Figures 5.

In each table, the three columns under LME method are the fraction of rejection rate (power), the mean of 1,000 estimated parameter β_1 , and the average standard deviation (s.d.) for β_1 . The four columns under “Grizzle’s method” are the rejection rate of carry-over effects, the rejection rate of treatment effect being zero based on the Grizzle’s method (power), the average estimated treatment effect, and the average of standard deviation (s.d.).

Table 5. Simulations results for LME and Grizzle’s method without carryover effects ($\beta_{12}=0$)

		LME			Grizzle’s method			
		Power	est.	s.e.	Carryover*	power	est.	s.e.
Group size=10	-0.60	0.811	-0.586	0.195	0.119	0.940	-0.593	0.130
	-0.30	0.308	-0.302	0.199	0.118	0.948	-0.307	0.136
	0.00	0.045	0.007	0.187	0.085	0.086	-0.002	0.113
	0.30	0.305	0.299	0.196	0.115	0.943	0.302	0.133
	0.60	0.831	0.603	0.191	0.083	0.972	0.602	0.113
Group size=30	-0.60	0.999	-0.598	0.111	0.086	0.999	-0.603	0.069
	-0.30	0.755	-0.297	0.108	0.096	0.943	-0.296	0.072
	0.00	0.044	0.002	0.113	0.104	0.103	0.003	0.073
	0.30	0.768	0.302	0.112	0.102	0.948	0.299	0.076
	0.60	0.999	0.600	0.113	0.099	0.999	0.602	0.073

*The values in this column is rejection rate for carryover effects tests.

Table 6. Simulations results for LME and Grizzle’s method with small carryover effects ($\beta_{12}=0.5$)

		LME			Grizzle’s method			
		Power	est.	s.e.	Carryover*	power	est.	s.e.
Group size=10	-0.60	0.826	-0.601	0.202	0.333	0.998	-0.503	0.232
	-0.30	0.299	-0.299	0.197	0.349	0.762	-0.200	0.227
	0.00	0.054	0.007	0.197	0.335	0.287	0.099	0.222
	0.30	0.324	0.309	0.192	0.311	0.698	0.411	0.219
	0.60	0.858	0.610	0.189	0.321	0.866	0.708	0.217
Group size=30	-0.60	0.999	-0.602	0.110	0.727	0.999	-0.570	0.151
	-0.30	0.739	-0.297	0.111	0.705	0.996	-0.262	0.153
	0.00	0.051	0.009	0.112	0.685	0.340	0.047	0.154
	0.30	0.741	0.300	0.114	0.688	0.742	0.338	0.158
	0.60	0.999	0.602	0.111	0.707	0.999	0.638	0.153

*The values in this column is rejection rate for carryover effects test

Table 7. Simulations results for LME and Grizzle's method with large carryover effects ($\beta_{12}=1.0$)

β_1		LME			Grizzle's method			
		Power	est.	s.e.	Carryover*	power	est.	s.e.
Group size=10	-0.60	0.824	-0.588	0.193	0.796	0.997	-0.541	0.259
	-0.30	0.301	-0.304	0.189	0.814	0.488	-0.256	0.259
	0.00	0.047	0.010	0.192	0.791	0.228	0.059	0.263
	0.30	0.312	0.303	0.195	0.804	0.346	0.349	0.262
	0.60	0.823	0.604	0.200	0.795	0.820	0.650	0.267
Group size=30	-0.60	0.999	-0.597	0.108	0.997	0.999	-0.597	0.110
	-0.30	0.753	-0.298	0.112	0.996	0.754	-0.297	0.114
	0.00	0.056	0.000	0.113	0.996	0.157	0.000	0.116
	0.30	0.774	0.306	0.109	0.999	0.705	0.306	0.109
	0.60	0.999	0.597	0.113	0.996	0.999	0.597	0.115

*The values in this column is rejection rate for carryover effects tests

Figure 5. Comparisons of powers of the two methods for different group sizes and different carry-over effects. (— LME model - - - - Grizzle's method).

2.6 Results and conclusion

According to the above simulation results, we conclude that the significant level based on LME method is maintained in the nominal level (i.e. 0.05) when β_1 is assumed to be zero, no matter the different settings on group size or carryover effects.

While the Grizzle's method cannot maintain the significance level of 0.05 when there is no treatment effect. The powers of the tests for both methods increase as β_1 increases.

We conclude that the linear mixed-effects model is more appropriate for the data analysis of this kind of 2×2 crossover designs.

CHAPTER 3

STATISTICAL METHODS FOR 2×2 CROSSOVER DESIGNS WITH MULTIPLE OBSERVATIONS PER PERIOD

3.1 Introduction

In this chapter we focus on the analysis of 2×2 crossover design trials with repeated measurements per period, that is, a sequence of observations are collected within the same treatment period. The multiple observations over different time points on the same subject could provide more information of response profile than a single measurement [11]. The observations of each subject in 2×2 crossover designs in chapter 2 can also be regarded as repeated measurements [4], as presented in Chapter 2.

It is known that the test of carry-over effects will generally be less powerful than that of treatment effects because of the usage of between-subject comparison. One way towards a solution of this problem is to include a “run-in” period and a “washout” period, and to take measurements during these periods [8]. The run-in period precedes administration of the first treatment and the wash-out follows the first treatment period. If we assume that a measurement of the response is taken at the end of the run-in period and at the end of the wash-out period, then these baseline measurements can be used to provide a within-subject test of carry-over effects [8]. However, sometimes for ethical reasons, a washout period is not possible and only the measurement of run-in period can be taken. In this case, the first baseline measurement can be treated as a genuine covariate because it cannot be affected by treatments. Thus, there are two options to make use of

baseline measurements. One is to analyze the differences from the baseline measurements, the other is to use the baseline as a covariates. The conventional analysis of the change from baseline will provide unbiased least squares estimator of the direct treatment effects [10]. We should realize that the carry-over effect of the first treatment on second baseline measurement may not be equal to that on the measurement after receiving the second treatment [12]. Ignoring it may cause the overestimation of the direct treatment effects. In the case that there is a second baseline measurement, we should be very careful for the two different orders of carry-over effects [4]. Baseline measurements can also be used as covariates. It often happens in clinical trials that additional information is available for each subject, such as age, sex, or weight [6]. These additional variables are usually called covariates. One may wish to know if a treatment effect is related to the covariate. It is possible that some of the between-subject variation can be accounted for by the covariate value. Thus, by introducing the baseline as covariate, the between-subject residual variance may be reduced [6].

3.2 LME method

In order to make the best use of baseline measurements, we first calculated differences between after-treatment measurements and baseline measurements. The values of differences are used to fit the LME model. It is possible that there may be different order of carry-over effects for different time points within the same period. For the design with two time points without counting for baseline measurement, the full model is:

$$y_{ijk} = \beta_0 + \beta_1 X_{\text{treat}} + \beta_2 X_{\text{time}} + \beta_{12} X_{\text{treat}} * X_{\text{time}} + \delta_0 X_{\text{period}} + \delta_1 X_{\text{period}} * X_{\text{treat}} \\ + \delta_2 X_{\text{period}} * X_{\text{time}} + \delta_{12} X_{\text{period}} * X_{\text{time}} * X_{\text{treat}} + s_{ij} + \varepsilon_{ijk}$$

where y represents the difference of the measurement after treatment from its baseline measurement, s_{ij} is random subject-effect, and s_{ij} is independently and identically distributed with $N(0, \sigma_s^2)$, ε_{ijk} is a random error and is assumed to be independently and identically distributed with $N(0, \sigma^2)$.

3.3 Byron Jones's analysis method [4]

We come to examine the design with two after-treatment measurements plus baseline measurement. The expectations of the responses in each group are summarized in the following Table 8:

Table 8. Expectations of responses for each sequence with baseline	
Sequence 1 (PM/AIR)	Sequence 2 (AIR/PM)
$E(y_{110}) = \mu - \gamma + \pi_1$	$E(y_{210}) = \mu + \gamma + \pi_1$
$E(y_{111}) = \mu - \gamma + \pi_2 - \tau_1$	$E(y_{211}) = \mu + \gamma + \pi_2 + \tau_1$
$E(y_{112}) = \mu - \gamma + \pi_3 - \tau_2$	$E(y_{212}) = \mu + \gamma + \pi_3 + \tau_2$
$E(y_{120}) = \mu - \gamma + \pi_4 - \theta$	$E(y_{220}) = \mu + \gamma + \pi_4 + \theta$
$E(y_{121}) = \mu - \gamma + \pi_5 + \tau_1 - \lambda_1$	$E(y_{221}) = \mu + \gamma + \pi_5 - \tau_1 + \lambda_1$
$E(y_{122}) = \mu - \gamma + \pi_6 + \tau_2 - \lambda_2$	$E(y_{222}) = \mu + \gamma + \pi_6 - \tau_2 + \lambda_2$

The parameter μ represents overall mean; the parameter π represents period effect, where $\pi_1 = \pi_2 = \pi_3$, $\pi_4 = \pi_5 = \pi_6$; the parameter γ represents group effect; the parameter τ represents treatment effect, let us denote τ_1 as the first-order treatment effect and τ_2 as the second-order treatment effect; the parameter θ represents first-order carry-over effect; the parameter λ_1 and λ_2 represent second-order carry-over effect at two different time points.

Based on Jones's method, we carried out the following series of tests:

(i) test whether $\lambda_1 = \lambda_2$; (ii) test whether λ_1 or λ_2 equals zero; (iii) test whether θ equals zero; (iv) test whether $\tau_1 = \tau_2$. We first obtain the least squares estimator for each parameter of λ , θ , and τ , all of which take the form of $\hat{c}_1 - \hat{c}_2$, where c_i is a contrast

among the six means from group i ($i=1, 2$). The estimators are defined by the following contrasts:

$$(\lambda_1 - \lambda_2)|\tau, \theta: 0.5*(0, -1, 1, 0, -1, 1), \lambda|\tau, \theta: 0.5*(2, -1, 0, 0, -1, 0),$$

$$\theta|\tau, \lambda: 0.5*(1, 0, 0, -1, 0, 0), \tau|\theta, \lambda: 0.5*(1, -1, 0, 0, 0, 0).$$

The two sample t tests are applied to test the carry-over effect and treatment effect.

3.4 Case study

We applied linear mixed-effects models to analyze the endothelial progenitor cell (EPC) study. In this experiment, subjects were randomly assigned to two exposure sequences: one with AIR first, and then followed by airborne particulate matter (PM); the other sequence with PM first, and then followed by AIR. Under each exposure, the blood sample was taken before exposure (Pre), two-hours after exposure (Post), and the second morning for follow-ups (FU). In an exposure sequence, the second exposure was carried out one week after the first exposure. Flow cytometry was applied to measure the cell counts for CD31+CD34+ /50K lymph. We plotted the response profile during the three different time points of “Pre”, “Post”, “FU” to examine whether the response profile are associated with different treatment (see Figure 6-8).

This experiment was crossover design because each subject received both treatments, either in the sequence of PM/AIR or reverse order. There are three measurements at three different time points, namely baseline (“Pre”), tow-hour after treatment (“Post”), and the second day morning follow-ups (“FU”). We adopted the analysis method of crossover design to handle this experiment design. According to the definition of these time points, the measurement of “Pre” was to be regarded as baseline measurement.

Figure 6. Subject-level profiles for "CD31+CD34+/50K lymph"

Figure 7. Treatment profile for all subjects at every time points (exclude "Pre") for PM/AIR data

Figure 8. Comparison between PM and AIR in different sequences

The difference between the measurement of “Post” and “Pre” (denoted by d.post), and the difference between the measurement of “FU” and “Pre” (denoted by d.fu), were the change score from baseline, which was considered as the responses at two different time points. With the changes from baselines, we may be able to eliminate the possible between-subject effect and the first-order carry-over effect, make use of more within-subjects information to improve statistical power of test.

When we applied the LME to analyze the experiment data, we introduce the following dummy variables in our model proposed in Section 3.2:

$$X_{\text{treat}} = \begin{cases} 1 & \text{when given treatment of PM} \\ 0 & \text{when given treatment of AIR} \end{cases}$$

$$X_{\text{time}} = \begin{cases} 1 & \text{when time point is Post} \\ 0 & \text{when time point is FU} \end{cases}$$

$$X_{\text{period}} = \begin{cases} 1 & \text{when given treatment in period 1} \\ 0 & \text{when given treatment in period 2} \end{cases}$$

The results based on LME are summarized in the following Table 9.

Table 9. LME model regression results summary for AIR/PM data

Parameter	Estimate	S.E.	D.F.	t-value	p-value
β_0	-38.23	52.25	28	-0.73	0.47
β_1 (treat)	-59.82	78.30	28	-0.76	0.45
β_2 (time)	33.40	57.02	28	0.59	0.56
β_{12} (trt*time)	82.86	85.93	28	0.96	0.34
δ_0 (period)	-224.65	78.30	28	-2.87	0.01
δ_1 (period*trt)	281.37	128.62	28	2.19	0.04
δ_2 (period*time)	115.57	85.93	28	1.35	0.19
δ_{12} (per*trt*time)	-179.05	120.28	28	-1.49	0.15
σ_s^2 (between-sub)	81.725 ²				
σ^2 (within-sub)	101.636 ²				

When we applied Jones's traditional method, we used the raw data and constructed contrasts using the observations in the three time points, "Pre", "Post", and "FU". Seven subjects were included in PM/AIR sequence and five subjects were included in AIR/PM sequence. We calculated the contrasts in section 3.3 for each subject, and then obtained the parameter estimates and pooled variance. Using the two-sample t test, we tested whether there is carry-over effect and treatment effect. To test for the second-order carry-over effect (i.e. $H_0: \lambda_1 - \lambda_2 = 0$), the estimate of $\lambda_1 - \lambda_2$ is 90.45, with a standard error of 38.90 on 12 degrees of freedom. The p-value of the t test is 0.042, indicating that the second-order carry-over effect is significantly different between the two sequences. Then we examined whether the first-order carry-over effects between the two sequences are significantly different by testing whether $\theta = 0$. We have $\hat{\theta} = -262.2$, with a standard error of 64.8 on 12 degrees of freedom. The p-value of the test is 0.002, indicating that the

first-order carry-over effects are significantly different. That is, the first-order and second-order carry-over effects are significantly different between the two sequences. Therefore, we could only use the data in the first period (y_{i1}) to test the treatment effect. To test the treatment effect (i.e. $\tau=0$), we have $\hat{t}=-62.68$, with a standard error of 41.12 on 12 degrees of freedom. The p-value of t test is 0.158, indicating that there is no significant difference between the two treatments.

3.5 Discussion and conclusion

For this case study, we got similar results using both LME model and Jones's method. We conclude that the carry-over effects are extremely significant and the treatment effects are no significant different. However, LME models use all collected data and model the treatment effect, period effect and different order carry-over effects simultaneously. While Jones's method uses two-stage tests: first test carry-over effect, then test treatment effect based on whether carry-over effect is significant. We expect LME will be more power for testing treatment effect.

CHAPTER 4

DISCUSSION AND FUTURE WORK

A linear mixed-effects model (LME) includes both containing both fixed effects and random effects. It is particularly useful in analyzing repeated measurements, where the measurements between within-subject observations can be captured by random effect. The mixed-effects models can use all available data, no matter whether the subject has completed observations. LME has performed better than the traditional methods.

In this thesis, we first analyzed the classic 2×2 crossover data using LME model and traditional Grizzle's method. We carried out simulations for both methods to compare their performances. The LME model performs better than Grizzle's method in terms of maintaining the type I error rate. We extend LME to crossover data with multiple measurements within each period. The LME model is easy to implement. In addition, we applied the method suggested by Byron Jones to the 2×2 crossover design with multiple observations within each period. The required t tests are based on the constructed contrasts. After carrying out different t tests, we obtained the similar results as LME models. LME models are easy to implement, preserve the type I error rate. LME models are recommended for the crossover designs.

It is also noticed that we have not carried out simulations to compare the LME and Jones's method, we are still working on it and will present it in someplace else.

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APPENDIX: R code for the thesis

```
*****
Plot for PEFr data
*****
PEFR<-read.table("c:/PEFR.txt", header=T)
par(mfrow=c(1,2))
for(i in 1:27){
plot(PEFR$Per1[i]:PEFR$Per2[i], type="l",
ylim=c(100, 500), xlab="", ylab="", main="sequence AB")
par(new=TRUE)
}
plot(mean(PEFR$Per1[1:27]):mean(PEFR$Per2[1:27]),
type="l", col=2, lwd=4, ylim=c(100, 500))
for(i in 28:56){
plot(PEFR$Per1[i]:PEFR$Per2[i], type="l", ylim=c(100, 500),
xlab="", ylab="", main="sequence BA")
par(new=TRUE)
}
plot(mean(PEFR$Per1[28:56]):mean(PEFR$Per2[28:56]),
type="l", col=3, lwd=4, ylim=c(100, 500))
*****
Plot and analysis for FEV data using LME
*****
library(nlme)
rr<-read.table("c:/rr.txt", header=T)
rr
attach(rr)
value<-rep(0, 32)
for(i in 1:16){
value[2*i-1]<-period1[i]
value[2*i]<-period2[i]
}
period
subject<-rep(c(1:16), each=2)
subject
period<-rep(c(0, 1), 16)
period
trt<-c(rep(c(0, 1), 8), rep(c(1, 0), 8))
trt
FEV<-data.frame(subject, trt, period, runin, value)
```



```

FEV
FEV.ANA<-lme(value~runin+trt*period, random=~1|subject, data=FEV)
summary(FEV.ANA)
plot(FEV$value[FEV$trt==0], type="l", lty=1)
par(new=TRUE)
plot(FEV$value[FEV$trt==1], type="l", lty=2, col=2)
legend(0, 3.5, legend=c("treatment A", "treatment B"), lty=c(1, 2))
par(mfrow=c(1, 2))
for(i in 1:8) {
plot(FEV$value[(2*i-1):(2*i)], type="l", col=i, ylim=c(0.5, 3), ylab="")
par(new=TRUE)
}
par(new=FALSE)
for(i in 9:16) {
plot(FEV$value[(2*i-1):(2*i)], type="l", col=i, ylim=c(0.5, 3), ylab="")
par(new=TRUE)
}
*****
Analysis of FEV data using Grizzle's method
*****
sum<-rep(NA, 16)
for(i in 1:16) {
sum[i]<-FEV$value[2*i-1]+FEV$value[2*i]
}
sum
mean.t<-mean()-mean(sum[9:16])
mean.t
var.t<-sqrt((var(sum[1:8])*7+var(sum[9:16])*7)/14*0.25)
var.t
T<-mean.t/var.t
T
pvalue<-2*pt(-abs(T), 14)
pvalue
diff<-rep(NA, 16)
for(i in 1:16) {
diff[i]<-FEV$value[2*i-1]-FEV$value[2*i]
}
diff
mean.diff<-(mean(diff[1:8])-mean(diff[9:16]))*0.5
mean.diff
var.diff<-sqrt((var(diff[1:8])*7+var(diff[9:16])*7)/14*0.25/4)
var.diff^2
T.diff<-mean.diff/var.diff
T.diff
pvalue<-2*pt(-abs(T.diff), 14)
pvalue

```

Simulations

```
library(nlme)
beta.treat.lme<-beta.treat.tra<-reject.lme<-reject.tra<-c()
beta0<-1.7416; beta1<-0; beta2<-0.4275; beta12<-1.005
TC<-Period<-Sub<-y<- meandiff.sum<-var.sd<-yy.mean<-beta1.lme<-c()
n1<-n2<-10
aa<-rep(NA,1000)
## iter<-1
Count.carry<-Count.trad<-0
for(iter in 1:1000){
  TC<-Period<-Sub<-y<-c()
  for (j in 1:n1)
    {Sub<-c(Sub, rep(j, 2))
      TC<-c(TC, "T", "C")
      Period<-c(Period, 1,2)
      s1<-rnorm(1,0,0.428)
      y<-c(y, beta0+beta1+s1+rnorm(1,0,0.06),beta0+beta2+s1+rnorm(1,0,0.06))
    }
  for (j in (n1+1):(n1+n2))
    {Sub<-c(Sub, rep(j, 2))
      TC<-c(TC, "C", "T")
      Period<-c(Period, 1,2)
      s1<-rnorm(1,0,0.428)
      y<-c(y, beta0+s1+rnorm(1,0,0.06),beta0+beta1+beta2+beta12+s1+rnorm(1,0,0.06))
    }
  Period<-factor(Period); TC<-factor(TC)
  temp<-summary(lme(y~Period*TC,random=~1|Sub))
  beta1.lme[iter]<-temp$tTable[3,1]
  reject.lme[iter]<-ifelse(temp$tTable[3,5]<0.05, 1,0)
  y11<-y[2*(1:n1)-1]; y12<-y[2*(1:n1)]
  y21<-y[2*((n1+1):(n1+n2))-1]; y22<-y[2*((n1+1):(n1+n2))]
  sum1<-y11+y12
  sum2<-y21+y22
  meandiff.sum[iter]<-mean(sum1)-mean(sum2)
  var.sd[iter]<-sqrt((var(sum1)*(n1-1)+var(sum2)*(n1-1))/(2*n1-2)*(2/n1))
  stat.sum<-meandiff.sum[iter]/var.sd[iter]
  aa<-2*pt(-abs(stat.sum),2*n1-2)
  if(aa>0.1){
    diff1<-y11-y12
    diff2<-y21-y22
    yy<-c(diff1,diff2)
    yy.mean[iter]<-(mean(diff1)-mean(diff2))*0.5
    yy.sd<-sqrt((var(diff1)*(n1-1)+var(diff2)*(n1-1))/(2*n1-2)/(2*n1))
    t.statistic<-(yy.mean[iter])/yy.sd
    if(2*pt(-abs(t.statistic), (2*n1-2))<0.05){Count.trad<-Count.trad+1}
```

```

}
if(aa<0.1){
  Count.carry<-Count.carry+1
  yy.mean[iter]<-mean(y11)-mean(y21)
  p.value<-t.test(y11, y21)$p.value
  if(p.value<0.05){Count.trad<-Count.trad+1}
}
}
sum(reject.lme)/1000
mean(beta1.lme); sd(beta1.lme)
Count.carry/1000
Count.trad/1000
mean(yy.mean); sd(yy.mean)
par(mfrow=c(3,2), mai=c(0.4, 0.4, 0.4, 0.1) )
y<-c(0.811, 0.308, 0.045, 0.305, 0.831)
x<-c(-0.6, -0.3, 0, 0.3, 0.6)
plot(y~x, xaxt="n", main="A1:                                     n=10,
      beta12=0", ylab="power", xlab="beta1", ylim=c(0, 1), type="l")
axis(side=1, at=c(-0.6, -0.3, 0, 0.3, 0.6), labels=c(-0.6, -0.3, 0, 0.3, 0.6))
par(new=TRUE)
b<-c(0.94, 0.948, 0.086, 0.943, 0.972)
plot(b~x, xaxt="n", type="l", lty=2, ylim=c(0, 1), ylab="power", xlab="beta1")
axis(side=1, at=c(-0.6, -0.3, 0, 0.3, 0.6), labels=c(-0.6, -0.3, 0, 0.3, 0.6))
m<-c(0.999, 0.755, 0.044, 0.768, 0.999)
n<-c(0.999, 0.943, 0.103, 0.948, 0.999)
plot(m~x, xaxt="n", main="A2:                                     n=30,
      beta12=0", ylab="power", xlab="beta1", ylim=c(0, 1), type="l")
axis(side=1, at=c(-0.6, -0.3, 0, 0.3, 0.6), labels=c(-0.6, -0.3, 0, 0.3, 0.6))
par(new=TRUE)
plot(n~x, xaxt="n", type="l", lty=2, ylim=c(0, 1), ylab="power", xlab="beta1")
axis(side=1, at=c(-0.6, -0.3, 0, 0.3, 0.6), labels=c(-0.6, -0.3, 0, 0.3, 0.6))
p<-c(0.826, 0.299, 0.054, 0.324, 0.858)
q<-c(0.998, 0.762, 0.287, 0.698, 0.866)
plot(p~x, xaxt="n", main="B1:                                     n=10,
      beta12=0.5", ylab="power", xlab="beta1", ylim=c(0, 1), type="l")
axis(side=1, at=c(-0.6, -0.3, 0, 0.3, 0.6), labels=c(-0.6, -0.3, 0, 0.3, 0.6))
par(new=TRUE)
plot(q~x, xaxt="n", type="l", lty=2, ylim=c(0, 1), ylab="power", xlab="beta1")
e<-c(0.999, 0.739, 0.051, 0.741, 0.999)
f<-c(0.999, 0.996, 0.34, 0.742, 0.999)
plot(e~x, xaxt="n", main="B2:                                     n=30,
      beta12=0.5", ylab="power", xlab="beta1", ylim=c(0, 1), type="l")
axis(side=1, at=c(-0.6, -0.3, 0, 0.3, 0.6), labels=c(-0.6, -0.3, 0, 0.3, 0.6))
par(new=TRUE)
plot(f~x, xaxt="n", type="l", lty=2, ylim=c(0, 1), ylab="power", xlab="beta1")
u<-c(0.824, 0.301, 0.047, 0.312, 0.823)
v<-c(0.997, 0.488, 0.228, 0.346, 0.82)

```

```

plot(u~x, xaxt="n", main="C1:                                     n=10,
      β 12=1.0", ylab="power", xlab="β 1", ylim=c(0, 1), type="l")
axis(side=1, at=c(-0.6, -0.3, 0, 0.3, 0.6), labels=c(-0.6, -0.3, 0, 0.3, 0.6))
par(new=TRUE)
plot(v~x, xaxt="n", type="l", lty=2, ylim=c(0, 1), ylab="power", xlab="β 1")
j<-c(0.999, 0.753, 0.056, 0.774, 0.999)
k<-c(0.999, 0.754, 0.157, 0.705, 0.999)
plot(j~x, xaxt="n", main="C2:                                     n=30,
      β 12=1.0", ylab="power", xlab="β 1", ylim=c(0, 1), type="l")
axis(side=1, at=c(-0.6, -0.3, 0, 0.3, 0.6), labels=c(-0.6, -0.3, 0, 0.3, 0.6))
par(new=TRUE)
plot(k~x, xaxt="n", type="l", lty=2, ylim=c(0, 1), ylab="power", xlab="β 1")
*****
Analysis for PM/AIR data
*****
library(nlme)
x<-read.csv(file="c:/r.csv")
a<-as.data.frame(x[, c("subject", "Time", "ExposureAtmosphere", "Period", "X4th")])
attach(a)
par(mfrow=c(4, 4))
for(i in 1:13) {
  plot(X4th[(6*i-5):(6*i-3)], type="b", col="red")
  par(new=TRUE)
  plot(X4th[(6*i-2):(6*i)], type="b", col="blue", lty=2)
}
par(mfrow=c(1, 1))
plot(a$X4th[a$Time!="Pre"&a$ExposureAtmosphere=="PM"],
      type="l", col="red", ylim=c(0, 400), xlim=c(0, 30), xlab="", ylab="CD31+CD34+/50K lymph")
par(new=TRUE)
plot(a$X4th[a$Time!="Pre"&a$ExposureAtmosphere=="AIR"],
      type="l", col="blue", lty=2, ylim=c(0, 400), xlim=c(0, 30), xlab="", ylab="CD31+CD34+/50K lymph")
legend("topleft", legend=c("PM", "AIR"), lty=c(1, 2), col=c("red", "blue"))
aa<-a[1:42,]
attach(aa)
par(mfrow=c(1, 2))
for(i in 1:7) {
  plot(X4th[(6*i-5):(6*i-3)], type="l", lty=(i+1), ylim=c(20, 300), ylab="CD31+CD34+/50K lymph", xlab="", main="PM in sequence PM/AIR")
  par(new=TRUE)
}
m1<-mean(X4th[Period==1&Time=="Pre"])
m2<-mean(X4th[Period==1&Time=="Post"])
m3<-mean(X4th[Period==1&Time=="FU"])
plot(c(m1, m2, m3), type="l", ylim=c(20, 300), col="red", ylab="CD31+CD34+/50K lymph", xlab="", lwd=4)
for(i in 1:7) {

```

```

plot(X4th[(6*i-2):(6*i)], type="l", lty=(i+1), ylim=c(20, 300), ylab="CD31+CD34+/50K
lymph", xlab=" ", main="AIR in sequence PM/AIR")
par(new=TRUE)
}
m4<-mean(aaa$X4th[Period==2&Time=="Pre"])
m5<-mean(aaa$X4th[Period==2&Time=="Post"])
m6<-83.8328
plot(c(m4,m5,m6), type="l", ylim=c(20, 300), col="green", ylab="CD31+CD34+/50K
lymph", xlab=" ", lwd=4)
par(mfrow=c(1,2))
a.back<-a[43:72,]
for(i in 1:5){
plot(a.back$X4th[(6*i-
2):(6*i)], type="l", lty=(i+1), ylim=c(20, 800), ylab="CD31+CD34+/50K      lymph", xlab="
", main="PM in sequence AIR/PM")
par(new=TRUE)
}
m11<-na.omit(a.back$X4th[Period==2&Time=="Pre"])
mean(m11)
m22<-na.omit(a.back$X4th[Period==2&Time=="Post"])
mean(m22)
m33<-na.omit(a.back$X4th[Period==2&Time=="FU"])
mean(m33)
plot(c(mean(m11), mean(m22), mean(m33)), type="l", ylim=c(20, 800), col="red", ylab="CD31
+CD34+/50K lymph", xlab=" ", lwd=4)
for(i in 1:5){
plot(a.back$X4th[(6*i-5):(6*i-
3)], type="l", lty=(i+1), ylim=c(20, 800), ylab="CD31+CD34+/50Klymph", xlab="
", main="AIR in sequence AIR/PM")
par(new=TRUE)
}
m44<-na.omit(a.back$X4th[Period==1&Time=="Pre"])
m66<-na.omit(a.back$X4th[Period==1&Time=="FU"])
m55<-na.omit(a.back$X4th[Period==1&Time=="Post"])
plot(c(mean(m44), mean(m55), mean(m66)), type="l", ylim=c(20, 800), col="green", ylab="CD
31+CD34+/50K lymph", xlab="", lwd=4)
for(i in 1:27){
z[2*i-1]<-a$X4th[3*i-1]-a$X4th[3*i-2]
z[2*i]<-a$X4th[3*i]-a$X4th[3*i-2]
}
y1.omit<-which(a$Time=="Pre")
y1.reduced<-a[-y1.omit,]
y.full<-data.frame(y1.reduced,z)
z.treat<-c(rep(c(1,1,0,0),7), rep(c(0,0,1,1),5), c(0,0,1,1,1,1))
z.time<-rep(c(0,1),27)
z.period<-c(rep(c(0,0,1,1),13), c(1,1))
y.final<-data.frame(y.full,z.treat,z.time,z.period)

```

```
library(nlme)
abc<-lme(z~z.treat*z.time*z.period, random=~1|subject, data=y.final, ra.action=
ra.exclude" )
summary(abc)
```

CURRICULUM VITAE

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