Semiparametric generalized estimating equations for repeated measurements in cross-over designs

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Abstract
A model for cross-over designs with repeated measures within each period was developed. It was obtained using an extension of generalized estimating equations that includes a parametric component to model treatment effects and a non-parametric component to model time and carry-over effects; the estimation approach for the non-parametric component is based on splines. A simulation study was carried out to explore the model properties. Thus, when there is a carry-over effect or a functional temporal effect, the proposed model presents better results than the standard models. Among the theoretical properties, the solution is found to be analogous to weighted least squares. Therefore, model diagnostics can be made by adapting the results from a multiple regression. The proposed methodology was implemented in the data sets of the cross-over experiments that motivated the approach of this work: systolic blood pressure and insulin in rabbits.

Keywords
Carry-over effect, cross-over design, experimental design, splines estimation

1 Introduction
In the context of cross-over experimental designs, each experimental unit receives a sequence of treatments, and each treatment is applied over a period of time.¹ These designs are very useful in medical experimentation, since they require fewer experimental units to obtain the same results as cross-sectional studies and longitudinal studies. The disadvantage is given by the possible appearance of carry-over effects, which are defined as the residual effects that remain in the response of the individual and that are caused by the treatments applied in the previous periods.²,³

The most recent works on the analysis of cross-over designs assume the non-existence of carry-over effects, due to the presence of a washout period between the successive applications of treatments.⁴ This assumption is common in works based on classical generalized linear models,⁵ Bayesian models,⁶ or generalized estimating equation (GEE) models.⁴ However, in some cross-over designs the length of the washout period is very short and does not guarantee the elimination of the residual effects of each of the treatments, such as the one presented in Jones and Kenward.⁷(p.204) In this design, three treatments for blood pressure control are used and treatment C is a placebo. In this experiment, if there is a carry-over effect of the placebo, it is not cleaned in the washout period. The doctors tried to control the hypertension in patients, and so, treatments can be stopped for a very short time due to the characteristics of the disease.

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Table 1. Structure of the blood pressure cross-over design, with three period, six sequences and ten measurements (that were taken $-30$, $-15$, 15, 30, 45, 60, 75, 90, 120, and 240 min from application) per period.

| Sequence | Period 1          | Period 2          | Period 3          |
|----------|-------------------|-------------------|-------------------|
| (1) ABC  | Ind 1             | 10 observations   | 10 observations   |
|          | Ind 2             | 10 observations   | 10 observations   |
|          | ⋮                 | ⋮                 | ⋮                 |
| (6) CBA  | Ind 11            | 10 observations   | 10 observations   |
|          | Ind 12            | 10 observations   | 10 observations   |

Furthermore, in the design presented in Jones and Kenward, $^7$ p. 204 the systolic blood pressure is observed 10 times within each period: 30 and 15 min before the application, and 15, 30, 45, 60, 75, 90, 120, and 240 min after the application, as shown in Table 1, which generates a repeated measurement structure for each application period. This type of design is known as a repeated measures cross-over design. $^9,8$ Dubois et al., $^9$ Diaz et al., $^{10}$ and Forbes et al. $^{11}$ used Gaussian linear mixed models to study cross-over repeated measures designs. However, those studies considered one observation per period by calculating the area under the curve, and they did not include the carry-over effects, or used mixed models with various measurements per period and normality assumption. $^{12}$ Additionally, this modeling approach does not allow to observe the temporal behavior of the response variable within the period, nor the presence of carry-over effects that fluctuate over time.

On the other hand, when the response variable of the cross-over experiment shown by Kenward and Roger $^{13}$ is analyzed, it is observed that it does not fit a normal distribution. Since the response in this experiment is blood sugar levels, which are skewed and always positive, a gamma distribution, lognormal distribution, or some other skewed distribution seems more suitable for analysis. In both experiments, we have a response that can be assumed to be in the exponential family and the responses of the same experimental unit are correlated. Therefore, in this article, we propose an extension of the GEE to model the effects of main interest in the design (treatments, period) with a parametric component and the temporary effects through smoothing splines.

As for the carry-over effects, the most common is that they are simple, that is, it is assumed that if a treatment leaves a carry-over, this is the same regardless of the treatment sequence. Fleiss $^{14}$ and Senn, $^{15}$ discuss the problems of this assumption. In addition, they provide examples where a simple carry-over does not improve, but lowers the performance of the estimators. For this reason, in this work we model complex carry-over effects will, that is, the carry-over effect depends on both, the preceding treatment and the affected treatment.

This methodology makes it possible to unbiasedly isolate the temporal behavior of the carry-over effect from the period and treatment effects, which is demonstrated theoretically and through a simulation exercise. Subsequently, when applying the methodology in the blood pressure design, a significant carry-over effect of the placebo treatment is obtained, corroborating the importance of taking it into account in the analysis.

This article is structured as follows: In Section 2, the semiparametric model with GEE is described, and the estimation equations are derived. In Section 3, asymptotic consistency and unbiasedness of estimators are established. In Section 4, a simulation study is carried out to display the advantages of the proposed model over those models often found in the literature, and to compute some diagnostics measures for its residuals. In Section 5, an application of to blood pressure data is performed out to illustrate the model properties and to carry out an overall analysis of this dataset. Finally, some conclusions are presented in Section 6.

2 Repeated measures cross-over design

A cross-over design entails four components $^7$: (i) sequences which are randomly assigned combinations of the treatments applied to the experimental units, (ii) treatments, that are applied to each experimental unit as a part of a sequence in a given time, (iii) periods, that represent the application lapse for the treatments which are part of a sequence, and (iv) experimental units, which are the elements to which a treatment is applied. Further, it is frequent that each period has the same length for all sequences; therefore, the number of observation periods equals the length of each sequence.

For the structure of cross-over designs, the carry-over effects constitute part of them. Vegas et al. $^{16}$ defined the carry-over as a treatment’s effect persistence over those treatments applied later. That is, if a treatment is applied on a given period, then there exists the possibility of a residual or carry-over effect that persists in the following periods when other treatments are applied. When the carry-over effect of a treatment affects the one applied in the next period, it is known as a first-order carry over effect.
In the modeling of carry-over effects, these are divided into two types: (i) simple, where the residual effect of a treatment affects equally each of the treatments that are preceded by it, and (ii) complex, where the residual effect of the treatment affects each of the other treatments differently. Fleiss and Senn demonstrated that if the complex carry-over effect is an unrealistic scenario in pharmacological studies, but if it does occur, the model under the assumption of simple carry-over effects performs better than the model not assuming the carry-over effect. Therefore, from now on complex carry-over effects will be assumed. However, the methodology can also be adapted to modeling simple carry-over effects.

In a cross-over design with \( S \) sequences of length \( P \), let \( n_{ij} \) be the number of observations on the \( i \)-th experimental unit at the \( j \)-th period, then \( Y_{ij} \) is a vector defined as

\[
Y_{ij} = (Y_{i1}, \ldots, Y_{in_{ij}})^T
\]

Moreover, we define \( Y_i \) that contains all the observations on the \( i \)-th experimental unit

\[
Y_i = (Y_{i1}, \ldots, Y_{iP})^T
\]

and its size is \( \sum_{j=1}^{P} n_{ij} \). Regarding the use of smoothing functions, Wild and Yee proposed a kernel smoothing to select explanatory variables in GEE models. Lin and Carroll derived a semiparametric estimation equation for repeated measures data and presented some asymptotic properties without including the correlation matrix. On the other hand, He et al. presented a semiparametric model with correlated normal data and explored the properties of symmetric kernels. Stoklosa discussed a GEE model with two semiparametric functions for normally distributed responses and some kernel smoothing functions.

Accordingly, GEE will be used because \( Y_{ijk} \) (the response variable) has a distribution that belongs to the exponential family, and also a semiparametric model with B-splines for the time and carry-over effects as follows:

\[
E(Y_{ijk}) = \mu_{ijk}, \quad \text{Var}(Y_{ijk}) = \phi V(\mu_{ijk})
\]

\[
g(\mu_{ijk}) = x_{ijk}^T \beta + f(Z_{ijk}) + \sum_{c=1}^{C} f_c(Z_{ijk})
\]

\[
V(\mu_{ij}) = [D(\mu_{ijk})^T]R(\alpha)D(\mu_{ijk})^{1/2}
\]

where \( g(\cdot) \) is the link function associated to the exponential family, \( x_{ijk} \) is the vector of the design matrix associated to the \( k \)-th response of the \( i \)-th experimental unit in the \( j \)-th period, \( \beta \) represents the parametric effects, \( \phi \) is the dispersion parameter, and \( Z_{ijk} \) is the time at which the \( k \)-th observation of the \( i \)-th experimental unit was measured in the \( j \)-th period. For example, in the blood pressure data set described in Table 1, \( Z_{i11} = -30 \), \( Z_{i12} = -15 \), \( Z_{i110} = 240 \), that is, the minutes of measurement within each period, \( f \) is a function describing the time’s effect, \( f_c \) is a function describing the previous treatment carry-over effect on the current period (with \( f_c(Z_{ijk}) = 0 \) because it is assumed that there are no carry-over effects in period 1). \( C \) is the total number of complex carry-over effects, for example, in the design described in Table 1, \( C = 6 \), that is, \( f_1 \) is the carry-over effect of A on B, \( f_2 \) is the carry-over effect of A on C, \( f_3 \) is the carry-over effect of B on A, \( f_4 \) is the carry-over effect of B on C, \( f_5 \) is the carry-over effect of C on A and \( f_6 \) is the carry-over effect of C on B, \( V(\mu_{ijk}) \) is the variance function related to the exponential family and \( R(\alpha) \) is the associated correlation matrix.

Let \( \{s_1(t), \ldots, s_m(t)\} \) be the basis splines, then the \( f \) and \( f_c \) functions can be approximated through the following equations, respectively

\[
\hat{f}(t) = \sum_{h=1}^{m} \hat{a}_{1h} s_h(t)
\]

\[
\hat{f}_c(t) = \sum_{h=1}^{m} \hat{a}_{2h} s_h(t)
\]
where \( m = \max(n_i) \). Adapting the estimation equations given by He et al.,\(^{19}\) the following GEEs are proposed for \( \alpha_1 = \{\alpha_{11}, \ldots, \alpha_{1m}\}, \alpha_2 = \{\alpha_{21}, \ldots, \alpha_{2m}\} \) and \( \beta \):

- For the time effect

\[
U_i(\alpha_1, \beta, \alpha_2, \alpha) = \sum_{i=1}^{n} \left\{ \text{diag} \left( \frac{\partial \mu_{ijk}}{\partial \alpha_1} \right) \right\} V_{1i}^{-1} \left( \frac{1}{\phi} \left( y_i - \mu_i \left[ X_i \beta, \sum_{b=1}^{m} \alpha_1 s_b(t), \hat{f}_c(Z_i) \right] \right) \right) \tag{7}
\]

where

\[
V_{1i} = \left\{ \text{diag} \left( V \left( \mu_i \left[ X_i \beta, \sum_{b=1}^{m} \alpha_1 s_b(t), \hat{f}_c(Z_i) \right] \right) \right) \right\}^{\frac{1}{2}} \times R(\alpha)
\]

\[
\times \left\{ \text{diag} \left( \frac{\partial \mu_{i}}{\partial \alpha_1} \right) \right\}_{i} = \text{diag} \left( \frac{\partial \mu_{i}}{\partial \alpha_1} \right)
\]

and \( V(\cdot) \) is the variance function of the exponential family applied to each of the \( i \)-th individual’s expected values.

- For the carry-over effects

\[
U_2(\alpha_1, \beta, \alpha_1, \alpha) = \sum_{i=1}^{n} \left\{ \text{diag} \left( \frac{\partial \mu_{ijk}}{\partial \alpha_2} \right) \right\} V_{2i}^{-1} \left( \frac{1}{\phi} \left( y_i - \mu_i \left[ X_i \beta, \sum_{b=1}^{m} \alpha_2 s_b(t), \hat{f}(Z_{1i}) \right] \right) \right) \tag{8}
\]

where

\[
V_{2i} = \left\{ \text{diag} \left( V \left( \mu_i \left[ X_i \beta, \sum_{b=1}^{m} \alpha_2 s_b(t), \hat{f}(Z_{1i}) \right] \right) \right) \right\}^{\frac{1}{2}} \times R(\alpha)
\]

\[
\times \left\{ \text{diag} \left( \frac{\partial \mu_{i}}{\partial \alpha_1} \right) \right\}_{i} = \text{diag} \left( \frac{\partial \mu_{i}}{\partial \alpha_1} \right)
\]

- For the fixed effects, that is, treatment, sequence, period, or other covariates

\[
U_3(\beta | \alpha_1, \alpha_2, \alpha) = \sum_{i=1}^{n} \left\{ \text{diag} \left( \frac{\partial \mu_{ijk}}{\partial \beta} \right) \right\} V_{3i}^{-1} \left( \frac{1}{\phi} \left( y_i - \mu_i \left[ X_i \beta, \hat{f}(Z_i), \hat{f}(Z_i) \right] \right) \right) \tag{9}
\]

where

\[
V_{3i} = \left\{ \text{diag} \left( V \left( \mu_i \left[ X_i \beta, \hat{f}(Z_i), \hat{f}(Z_i) \right] \right) \right) \right\}^{\frac{1}{2}} \times R(\alpha)
\]

\[
\times \left\{ \text{diag} \left( \frac{\partial \mu_{i}}{\partial \alpha_1} \right) \right\}_{i} = \text{diag} \left( \frac{\partial \mu_{i}}{\partial \alpha_1} \right)
\]
2. Find the value $\alpha$

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To solve equation (9), the Fisher scoring algorithm is used, that is, in the $6. \text{Repeat steps (2) to (5) until convergence.}$

For the estimation of $\phi$, the following equation is used:

$$\hat{\phi} = \frac{1}{n} \sum_{i=1}^{n} \sum_{j=1}^{P} \sum_{k=1}^{n_i} r_{ijk}^2$$

To get the estimators of $\alpha_1, \alpha_2, \beta$, and $\alpha$ the following steps are performed:

1. Set initial values $\alpha_1^{(0)}, \alpha_2^{(0)},$ and $\alpha^{(0)}$
2. Find the value $\alpha_1^{(1)}$ that solves the equation

$$U_1(\alpha_1^{(1)}, t|\beta^{(0)}, \alpha_2^{(0)}, \alpha^{(0)}) = 0$$

3. Find the value $\alpha_2^{(1)}$ that solves the equation

$$U_2(\alpha_2^{(1)}, t|\beta^{(0)}, \alpha_1^{(1)}, \alpha^{(0)}) = 0$$

4. Find the value $\beta^{(1)}$ that solves the equation

$$U_3(\beta^{(1)}|\alpha_1^{(1)}, \alpha_2^{(1)}, \alpha^{(0)}) = 0$$

5. Find the value $\alpha^{(1)}$ that solves the equation

$$U_4(\alpha^{(1)}|\beta^{(1)}, \alpha_1^{(1)}, \alpha_2^{(1)}, \alpha^{(0)}) = 0$$

6. Repeat steps (2) to (5) until convergence.

To solve equation (9), the Fisher scoring algorithm is used, that is, in the $m$-th step, the estimator of $\beta$ is given by

$$\beta^{(m+1)} = \beta^{(m)} - \left[ E \left\{ U_3(\beta^{(m)}|\alpha_1^{(m)}, \alpha_2^{(m)}, \alpha^{(m)}) \right\} \right]^{-1} U_3(\beta^{(m)}|\alpha_1^{(m)}, \alpha_2^{(m)}, \alpha^{(m)})$$

$$= \beta^{(m)} - \left\{ \sum_{i=1}^{n} X_i^{T} W_i^{(m)} X_i \right\}^{-1} \left\{ \sum_{i=1}^{n} X_i^{T} W_i^{(m)} (N_i^{(m)})^{-1} u_i^{(m)} \right\}$$

where

$$E \left\{ U_3(\beta^{(m)}|\alpha_1^{(m)}, \alpha_2^{(m)}, \alpha^{(m)}) \right\} = E \left\{ \frac{\partial U_3(\beta^{(m)}|\alpha_1^{(m)}, \alpha_2^{(m)}, \alpha^{(m)})}{\partial \beta^{(m)}} \right\} = \sum_{i=1}^{n} X_i^{T} W_i^{(m)} X_i$$

$$W_i^{(m)} = \text{diag} \left\{ \frac{\partial \mu_{ijk}^{(m)}}{\partial \beta^{(m)}} \right\} \mu_{ijk}^{(m)} \text{diag} \left\{ \frac{\partial \mu_{ijk}^{(m)}}{\partial \beta^{(m)}} \right\}^{-1}.$$
\[ N_i^{(m)} = \begin{pmatrix} \text{diag} & \begin{pmatrix} \frac{\partial \mu_{ij}^{(m)}}{\partial \beta^{(m)}} \end{pmatrix} \end{pmatrix}_i \]

\[ u_i^{(m)} = (y_i - \mu_i^{(m)}) X_i \beta^{(m)} + \hat{f}_i (Z_i, f_c (Z_i)) \]

Carrying out a procedure similar to the one in Tsuyuguchi et al., \(^{23}\) equation (11) can be written as

\[
\beta^{(m+1)} = \left( \sum_{i=1}^{n} X_i^T u_i^{(m)} X_i \right)^{-1} \left( \sum_{i=1}^{n} X_i^T u_i^{(m)} z_i^{(m)} \right)
\]

where

\[
z_i^{(m)} = X_i \beta^{(m)} - N_i^{-1(m)} u_i^{(m)}, \quad i = 1, \ldots, n
\]

Therefore, \( \hat{\beta}^{(m)} \) is obtained analogously to a weighted least squares solution on the transformed response variable \( z_i^{(m)} \), where the effects of the variables associated to time and the carry-over effect have been removed. With these considerations, the asymptotic theory of estimators is developed obtaining the following theorem:

**Theorem 2.1.** Under the assumption that the \( r \)-th derivative of \( f \) and \( f_c \) is bounded for some \( r \geq 2 \) and that the number of knots \( m = m_n \to \infty \), but \( \frac{m_m}{n} \to 0 \) then \( \hat{\beta} - \beta \to 0 \). Also, if \( m = O(n^{1/4}) \) then

\[
\frac{1}{n} \sum_{i=1}^{n} \sum_{j=1}^{n} \left( \sum_{h=1}^{m} \hat{\alpha}_{ijh} (Z_{ijk}) - f (Z_{ijk}) \right)^2 = O \left( n^{-2/5} \right)
\]

\[
\frac{1}{n} \sum_{i=1}^{n} \sum_{j=1}^{n} \left( \sum_{h=1}^{m} \hat{\alpha}_{ijh} (Z_{ijk}) - f_c (Z_{ijk}) \right)^2 = O \left( n^{-2/5} \right)
\]

\[
\sqrt{n} (\hat{\beta} - \beta) \to N(0, A^{-1}BA^{-1})
\]

where

\[
A = \sum_{i=1}^{n} N_i V_{3i}^{-1} N_i^T
\]

\[
B = \sum_{i=1}^{n} N_i V_{3i}^{-1} (y_i - \hat{\mu}_i) (y_i - \hat{\mu}_i)^T V_{3i}^{-1} N_i^T
\]

**Proof.** See Appendix 1.

### 3 Model diagnostics

#### 3.1 Selection

In order to compare the fit of the proposed model (equations (3) and (4)) with the fit of conventional models, the quasi-likelihood criterion \( QIC \) defined by Pan\(^ {24}\) is used

\[
QIC = -2QL(\hat{\mu}; I) + 2 \text{trace}(\hat{\Omega}_{\hat{\beta}}^{-1} \hat{V}_R)
\]

where \( \hat{\mu} = \hat{\eta} = g^{-1}(x \hat{\beta}) \) is the estimated expected value for the observation with the model assuming the correlation matrix \( R \) and the estimates are obtained using equation (12), \( \hat{\Omega}_\hat{\beta} \) is the estimated variance matrix for vector \( \hat{\beta} \) under a correlation matrix \( R(\alpha) = I_n \), and \( \hat{V}_R \) is the covariance matrix estimated for the vector \( \hat{\beta} \) assuming the correlation matrix \( R(\alpha) \) as in equation (15). After fitting several models, the model with the lowest \( QIC \) is selected because is the one featuring the best balance between goodness of fit and complexity.
3.2 Residuals

Due to the similarity of the proposed estimator of $\beta$ and the weighted least squares, with weights given by matrix $W_i$, Pearson standardized residuals are proposed to assess model validity

$$r_{ijk} = -\frac{e^{T}_j \hat{W}_i^{-1}(e_i - X_i \hat{\beta})}{\sqrt{1 - h_{ijk}}} \tag{17}$$

where $e_{ijk}$ is a vector of $n_{ij}$ zeros, except at position $k$, $\hat{W}_i^{-1}$ is the square root of the matrix $\hat{W}_i$, and $h_{ijk}$ is the $ijk$ element of the diagonal of the projection matrix $H$, which is

$$H = \text{diag}\{H_1, \ldots, H_s\} \tag{18}$$

with

$$H_i = W_i^{-1}X_i(X_i^TW_iX_i)^{-1}X_i^TW_i \tag{19}$$

According to Tsuyuguchi et al.,\textsuperscript{23} the residuals defined in (17) are asymptotically normal with zero mean and standard deviation close to 1. Therefore, these can be used to validate the fitted model and the conditional distribution assumption of $Y$.

4 Simulation study

For the simulation study, the example given by Senn\textsuperscript{15} was adapted and consists of a cross-over design with Williams array as shown in Table 2.

Suppose we have a dose-finding experiment using the Williams square, with the doses and responses shown in Table 3. Furthermore, we assume that the drug concentration follows an exponential decay, that is, if at time 1 a dose $X_i$ is applied, at time $k$, the dose ($X_i$) in the body will be given by the following equation:

$$X_i = X_i e^{-r(k-1)}$$

Assuming a washout time period between periods given by $\Delta$ and $L$ observations on the experimental unit at the period $j$-th, then the drug dose present in experimental unit $i$-th at time $k$-th in period $j$-th is given by following expression:

$$X_{ijk} = X_{ij} e^{-r(k-1)} + X_{ij-1} e^{-r(k-1+\Delta+L)} I_{j>3} + X_{ij-2} e^{-r(k-1+2\Delta+2L)} I_{j>3} + X_{ij-2} e^{-r(k-1+3\Delta+3L)} I_{j=4} \tag{20}$$

In equation (20), the value $X_{ij} e^{-r(k-1)}$ is the effect of the drug applied in that period, $X_{ij-1} e^{-r(k-1+\Delta+L)} I_{j>3}$ is the amount of drug left over from the previous dose (it is associated to the first-order carry-over effect), $X_{ij-2} e^{-r(k-1+2\Delta+2L)} I_{j>3}$ is the dose associated to the carry-over effect of the second order and $X_{ij-2} e^{-r(k-1+3\Delta+3L)} I_{j=4}$ is the dose associated to the carry-over effect of third order.

For the simulation study, the following values of each of the parameters of the model described above will be tested: (i) $r \in \{0, 0.05, 0.1, \ldots, 0.95, 1\}$, (ii) $\Delta \in \{0, 5, 10, 15, 50, 100\}$, (iii) $L \in \{2, 5, 10, 15\}$, (iv) the number of experimental units per sequence will be $\{2, 5, 10, 100\}$, and (v) $\alpha \in \{0, 0.1, 0.2, \ldots, 0.8\}$, the parameter for an autoregressive correlation structure between the measurements of the same experimental unit and the period effect were assumed linearly.

| Table 2. Cross-over design with Williams square. |
|-----------------|----------------|----------------|----------------|----------------|
| Sequence   | Period 1 | Period 2 | Period 3 | Period 4 |
| ABCD      | L observations | L observations | L observations | L observations |
| BDAC      | L observations | L observations | L observations | L observations |
| CADB      | L observations | L observations | L observations | L observations |
| DCBA      | L observations | L observations | L observations | L observations |
Table 3. Dose response of the treatment for cross-over design with Williams square. It is assumed that the dose response is given by the equation $Y = \alpha + \beta \log_2 \left( \frac{X}{\delta} \right) + \epsilon$. Table adapted from Senn.\\n
| Treatment | A  | B  | C  | D   |
|-----------|----|----|----|-----|
| Dose $X$  | $\delta$ | $2\delta$ | $4\delta$ | $8\delta$ |
| Response $Y$ | $\alpha$ | $\alpha + \beta$ | $\alpha + 2\beta$ | $\alpha + 3\beta$ |

Figure 1. Standardized bias for the estimator of the effect of treatment B without washout period ($\Delta = 0$); (a) two observations ($L = 2$) per period and five experimental units ($n = 5$) by sequence, (b) two observations ($L = 2$) per period and 10 experimental units ($n = 10$) by sequence, (c) five observations ($L = 5$) per period and five experimental units ($n = 5$) by sequence, and (d) five observations ($L = 5$) per period and 10 experimental units ($n = 10$) by sequence.

Each scenario was run 1000 times, then the following six models were fitted: (i) mod1: a model with only treatment and period effects, (ii) mod2: a model with period, treatment, and time effects, (iii) mod3: a model with effects of period, treatment, and simple carry-over, (iv) mod4: a model with effects of period, treatment, and simple carry-over with linear interaction with time, (v) mod5: a model with effects of period, treatment, and simple carry-over with linear interaction with time, and (vi) mod6: a model with effects of period, treatment, and complex carry-over as the one proposed in this work.

All were run with generalized estimating equations to account for correlation within each experimental unit. The simple carry-overs are three due to estimability restrictions, that of D is equal to the negative sum of the three previous ones. Regarding the complex carry-over effect, there are 12 effects ($C = 12$ value in equation (4)), these are: A over B, A over C, ..., D over C, and D over C. For each models, the following metrics were calculated: the standardized bias, $SB = \frac{|\hat{\theta} - \theta|}{SD(\hat{\theta})}$, and the square root mean square error, $RMSE = \sqrt{E(\hat{\theta} - \theta)^2}$. 
The effect of treatment B is $\beta$ according to Table 3. Figure 1 shows the standardized bias of the estimator of the treatment effect B under each of the six models. It is observed that when the number of observations per period is equal to 2, the proposed model has a lower bias than the other five. As for as $r$ is below 0.6, as soon as this value is exceeded, the models without carry-over are less biased.

When the number of observations per period increases to 5 or 10 (which is observed in the plots of the Supplemental Material), the complex carry-over model has a lower bias at all values of $r$. In addition, the model with only treatment, period and time effects behaves similarly to the model with only simple carry-over, which supports what is discussed by Senn\textsuperscript{15} and Fleiss.\textsuperscript{14} That is, if a complex carry-over effect is not assumed, it is better not to assume a simple carry-over. But the gain in the estimation with carry-over effects, begins to be noticed when there are five or more measurements per period, in which case the model proposed has a much lower bias than the other models; therefore, not assuming the carry-over effect will affect the estimate of treatment effects.

According to Figure 2, when the washout period is too long and it is assumed that there are no carry-over effects, the models without the estimation of the temporal effect have a large bias when the number of observations per period increases. The proposed model is able to detect the change throughout the observation period when there are more than five observations per period, and because of that, it has a smaller bias for all simulated values of $r$.

In the plots of the Supplemental Material it is observed that the model with complex carry-over effects proposed here is better in all scenarios where the number of measurements per period is greater than five. Behavior that is maintained regardless of the value of $r$ and the length of washout period, demonstrating the robustness of this model.

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**Figure 2.** Standardized bias for the estimator of the effect of treatment B with one hundred times of washout period ($\Delta = 100$): (a) two observations ($L = 2$) per period and five experimental units ($n = 5$) by sequence, (b) two observations ($L = 2$) per period and 10 experimental units ($n = 10$) by sequence, (c) five observations ($L = 5$) per period and five experimental units ($n = 5$) by sequence, and (d) five observations ($L = 5$) per period and 10 experimental units ($n = 10$) by sequence.
Figure 3. Blood systolic pressure (mm Hg) observed through time (minutes).

5 Application

Two studies where the model proposed in (3) is used are presented below. In both, the software R Core Team\textsuperscript{25} is used through adaptation of the package \texttt{geeM} built by McDaniel et al.\textsuperscript{26}

5.1 Systolic pressure data

Jones and Kenward\textsuperscript{(p.204)} described the following cross-over design: three treatments for blood pressure control are used; treatment A consists of a 20 mg dose of a test drug, treatment B is a 40 mg dose of the same drug, and treatment C is a placebo as shown in Table 1; the profile is shown in Figure 3.

Figure 4(a) shows the smoothed function corresponding to the effect of time on blood pressure; it is based on the moments of measurement for the design in Table 1. Additionally, Figure 4(a) shows the average function and its 95\% confidence bands obtained through cross-validation. A wide drop in pressure is observed from the time that the patient expects to receive treatment, then it rises a little and remains table. This behavior is widely studied in medical settings, see Stergiou et al.\textsuperscript{27} and Fanelli et al.\textsuperscript{28}

For the complex carry-over effects: in Figure 4(c), the carry-over effect of A on B is observed, which grows and then remains stable above 10 and in Figure 4(d), the carry-over effect of B on A, which decreases and then returns to zero. Figure 5(a) shows, the carry-over effect of A on C, which is close to zero except in the last measurements of each period, and in Figure 5(b) presents the carry-over effect of C on A, which behaves similarly to the carry-over effect of A on B. In Figure 5(c) shows the carry-over effect of B on C is observed, which grows and then remains stable close to five. Figure 5(d) shows the carry-over effect of C on B, which decreases and then returns to zero.

In summary, it is unrealistic to assume that carry-over effects are simple and it is better to model complex carry-overs.

Table 4 shows the parametric effects, their standard error and the Wald statistic built from matrices (14) and (15). It is worth highlighting the positive effect of the baseline, that is, people have the highest blood pressure before starting the study. The periods are not significant, that is, the conditions were similar across the study. Additionally, there is a significant effect of treatment C on blood pressure reduction.

Finally, Figure 4(b) shows the confidence bands for the quantiles of the standardized residuals defined in (17) compared to a standard normal distribution. These residuals seem to fit the asymptotic normal distribution assumption.
Figure 4. (a) Changes of blood systolic pressure through time using splines, (b) first-order carry-over effect of treatment A over treatment B through time using splines, (c) first-order carry-over effect of treatment B over treatment A through time using splines, and (d) residuals normal probability plot. All figures present 95% confidence intervals and are based on the cross-over design of Table 1.

Figure 5. (a) First-order carry-over effect of treatment A over treatment C through time using splines, (b) first-order carry-over effect of treatment C over treatment A through time using splines, (c) first-order carry-over effect of treatment B over treatment C through time using splines, and (d) first-order carry-over effect of treatment C over treatment B through time using splines. All figures present 95% confidence intervals and are based on the cross-over design of Table 1.
Table 4. Analysis of blood systolic pressure data using GEE-splines.

|                | Estimate | Std.err | Wald    | Pr(>|W|) |
|----------------|----------|---------|---------|----------|
| Intercept      | 107.2944 | 3.3474  | 1027.40 | 0.0000   |
| Base           | 3.8505   | 1.3484  | 8.1542  | 0.0043   |
| Period 2       | −1.0462  | 3.4939  | 0.0897  | 0.7646   |
| Period 3       | −1.8525  | 3.6337  | 0.2599  | 0.6102   |
| Treatment B    | 3.6068   | 2.9630  | 1.4818  | 0.2235   |
| Treatment C    | −5.8752  | 2.6404  | 1.1331  | 0.0261   |

GEE: generalized estimating equation.

Table 5. Structure of the cross-over design of blood sugar levels in rabbits.

| Sequence          | Period 1                | Period 2                | Period 3                | Period 4                |
|-------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| (1) ABAB          | Ind 1 5 observations    | 5 observations         | 5 observations         | 5 observations         |
|                   | 5 observations         | 5 observations         | 5 observations         | 5 observations         |
| (2) BABA          | Ind 11 5 observations   | 5 observations         | 5 observations         | 5 observations         |
|                   | 5 observations         | 5 observations         | 5 observations         | 5 observations         |
|                   | 5 observations         | 5 observations         | 5 observations         | 5 observations         |
|                   | 5 observations         | 5 observations         | 5 observations         | 5 observations         |

5.2 Blood sugar levels in rabbits

Jones and Kenward\(^7\) described the following cross-over experiment: two treatments for the control of diabetes A and B were used, two sequences of four periods (ABAB, BABA) are organized and each one was applied to 11 female rabbits; each period lasted one week. In each period, at the middle of the week, five successive measurements of the blood sugar level were taken: 0, 1.5, 3, 4.5, and 6 h after the application, as shown in Table 5 and Figure 6.

Assuming that the distribution of blood sugar levels is normal, a first analysis was run. When making the normal probability plot of the standardized residuals defined in (17), it is observed that they do not fit to a standard normal distribution, as shown in Figure 7, and so the assumption is rejected. A gamma distribution is then explored, with loglinear link. Figure 8(c) shows the confidence bands for the quantiles of the standardized residuals defined in (17) against a standard normal distribution, concluding that the gamma distribution assumption is adequate.

To compare the distributions, four models are fitted to analyze the response variable: (i) under the assumption of normality, (ii) under the assumption of normality and log-linear link, (iii) under the assumption of a gamma distribution and inverse link, and (iv) under the assumption of a gamma distribution and a loglinear link. In each one, the QIC is calculated and shown in Table 6.

According to these results, all the analysis was carried out with the gamma distribution assumption and the loglinear model given by:

\[
\ln(\mu_{ijk}) = x_{ijk}^T \beta + f(Z_{ijk}) + f_1(Z_{ijk}) + f_2(Z_{ijk})
\]

where \(x_{ijk}\) is the matrix design of period and treatment, \(Z_{ijk}\) contains the measurement time instants within each period, \(f\) is the function that describes the temporary effect, \(f_1\) is the function that describes the carry-over effect of A on B, and \(f_2\) is the carryover effect of B on A.

The spline-smoothed function for the time effect on the blood sugar level of female rabbits is shown in Figure 8(a), it is based on the moments for measurement for the design of Table 5; the average functions and their 95% confidence bands, estimated through cross validation, are presented. A marked decrease in blood sugar levels is observed until 2 h, and then an increase until hour 6. In Jones and Kenward\(^7\) it was stated that there was an effect of the hours, but its form is not explained. However, the proposed model permits to describe this effect. Figure 8(c) shows the carry-over effects of treatment A over treatment B and Figure 8(d) shows the carry-over effects of treatment B over treatment A. It decreases in the first hour and then increases over time in both scenarios, which would imply a similar carry-over from A over B and from B over A. The parametric effects, their standard error and the Wald statistic constructed with matrices (14) and (15) are presented in Table 7.

It is noteworthy that there are no significant effects of treatment, similar to that obtained by Jones and Kenward\(^7\) and Kenward and Roger\(^13\), but there is positive effect of period four. This behavior was not analyzed in previous studies and can be seen as increased insulin resistance by blood cells; similar to behaviors reported by Ning et al.\(^29\) and Da Silva et
Figure 6. Blood sugar levels (mg/dL) in rabbits through time (hours).

Figure 7. Residuals normal probability plot with 95% confidence intervals of the residuals obtained assuming a Gaussian distribution of the response in the cross-over design of Table 5: (a) with identity link and (b) with loglinear link.
Figure 8. (a) Change of blood sugar levels through time using splines, (b) first-order carry-over effect of treatment A over treatment B through time using splines, (c) first-order carry-over effect of treatment B over treatment A through time using splines, and (d) residuals normal probability plot. All figures present 95% confidence intervals and are based on the cross-over design of Table 5 with linear log link for the mean of an assumed gamma distribution.

| Table 6. QIC for the three fitted models to the response of blood sugar levels in rabbits. |
|---------------------------------------------------------------|
| **Model**          | **QIC** |
| Normal             | 987.13  |
| Normal (with ln(Yijk)) | 1017.4 |
| Gamma Inverse     | 971.2   |
| Gamma Log         | 962.2   |

QIC: quasi-likelihood criterion.

| Table 7. Analysis of blood sugar levels data using GEE-splines. |
|---------------------------------------------------------------|
| **Estimate** | **Std.err** | **Wald** | **Pr(|W|)** |
|-------------|-------------|----------|-------------|
| Intercept   | 4.4799      | 0.0458   | 9555.4740   | 0.0000       |
| Period 2    | -0.0294     | 0.0580   | 0.2572      | 0.6121       |
| Period 3    | -0.0357     | 0.0606   | 0.3473      | 0.5556       |
| Period 4    | 0.2167      | 0.0611   | 12.5928     | 0.0004       |
| Treatment B | -0.0014     | 0.0438   | 0.0010      | 0.9753       |

GEE: generalized estimating equation.

In the modeling of blood pressure, the normal distribution presents a better performance than the gamma distribution, while in blood sugar levels, the gamma distribution achieves a better fit than the normal distribution. Choosing the most suitable distribution is desirable because the standard errors of each estimator of the parametric effects of the model are smaller. In addition, the Pearson residuals show a behavior that conforms to a standard normal in both cases, suggesting that the model specification is adequate.
These applications show the usefulness of the semiparametric approach proposed in this paper to model time and carry-over effects in cross-over designs with repeated measures within periods. Also, it complements the simulation study, where the efficiency of the proposed model over conventional models was illustrated.

6 Conclusions

In the simulation study, it was noted that when there are very few observations per period (less than five), it is better to fit a model without complex carry-over effects. If there are more observations per period, the model with carry-over effects proposed in this paper is much more robust than other models without carry-over effects or with simple carry-over.

The proposed methodology provides highly desirable properties of the resulting estimators. It allows doing asymptotic inference and a better to modelling of temporal carry-over behaviors that would be intractable in parametric scenarios, as in the case of blood pressure data, where these effects do not follow the typical polynomial effects. In addition, detecting these carry-over effects of the placebo allows estimating treatment effects unbiasedly and with greater precision, which is basically the objective of any cross-over design.

In the insulin data in rabbits, the behavior of the estimated effect of time is similar to a quadratic function, which shows that this methodological proposal encompasses the classical parametric temporal models with linear or cubic polynomials. In addition, the GEEs allow modeling a large number of response variables, not only normal or continuous, but also counts or proportions. In the simulation, the inferential gain is evidenced in terms of coverage and control of the type I and II errors of the hypothesis tests associated to the parameters of interest; that is, treatment and period effects when the temporal behavior is sinusoidal. While linear or quadratic models lose efficiency and unbiasedness; estimation with splines is an useful tool for this type of design.

The asymptotic properties of the estimators allow an agile and fast verification of the model because of its similarity with weighted least squares. Therefore, the adaptation of widely used diagnostic tests in normal linear models can be used.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship and/or publication of this article.

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Supplemental material

Supplementary material for this article is available online.

- A PDF file with plots of the $SB$ and the $RMSE$ for different combinations of parameters in the simulation.
- An R code with the simulation exercise and the analyzes of the data of blood pressure and blood sugar levels.

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proof: theorem 2.1 Let 

$$\theta(\hat{\beta}, \hat{\alpha}_1, \hat{\alpha}_2) = \left( \frac{B_1(\hat{\beta} - \beta)}{\sqrt{n}H_{1n}(\hat{\alpha}_1 - \alpha_1)} + \frac{W_1^T X (\hat{\beta} - \beta)}{\sqrt{n}H_{2n}(\hat{\alpha}_2 - \alpha_2)} \right)$$

(21)

with $B$ as in equation (15), and

$$W_1 = (\pi_{11}, \ldots, \pi_{1n}), \quad \pi_{1i} = (\pi(Z_{i1}), \ldots, \pi(Z_{ijn}))$$

$$W_2 = (\pi_{21}, \ldots, \pi_{2n}), \quad \pi_{2i} = (\pi(Z_{i1}), \ldots, \pi(Z_{ijn}))$$
Following ideas presented by Speckman to guarantee that both \( X \) and \( Z_{ij} \) have finite second moments, we assume that there exists a random variable \( \delta_{ijk} \), with \( E(\delta_{ijk}) = 0 \) and \( \text{Var}(\delta_{ijk}) \leq \infty \) and continuous functions \( g_1, \ldots, g_m \) such that

\[
x_{ijkl} = g_l(Z_{ijk}) + \delta_{ijkl}, \quad 1 \leq i \leq n, 1 \leq j \leq P, 1 \leq k \leq n_j, 1 \leq l \leq \text{dim}(\beta)
\]  

(23)

These functions allow modeling the possible relationship between the vector of variables associated to the parametric effects and the measurement times within each period. Let \( X_{ij} = (x_{ij1}, \ldots, x_{ijn_j}) \) be the parametric effects design matrix, then the following properties hold:

(i) The succession \( \{n_{ij}\} \) is bounded for all \( 1 \leq i \leq n \) and \( 1 \leq j \leq P \), that is

\[
\max(n_{ij}) < \infty
\]

(ii) Since \( Y_{ijk} \) is a random variable that belongs to the exponential family and due to the definition of the generalized estimation equations by Liang and Zeger, and by Lemma 5.3 given by Lehmann and Casella, then

\[
E(Y_{ijk}) = \sum_{b=1}^{m} \alpha_b s_b(t_i, Z_{ij} \beta, f_c(Z_{ij})) = 0
\]

Therefore, the expected value of (7) is

\[
E(U_1) = E \left( \left[ Y_i - \mu_i, X_i \beta, \sum_{b=1}^{m} \alpha_b s_b(t_i, f_c(Z_{ij})) \right] \right) = 0
\]

Analogous results are obtained for equations (8) to (10). As \( E(Y_{ijk}^2) < \infty \) and the density function satisfies the regularity conditions then, by Theorems 1 and 2 of Pan and by theorem 2.6 of Lehmann and Casella, for equation (7), it follows that

\[
0 < E \left( U_1 U_1^T \right) < \infty
\]

Similarly, for equations (8) to (10), the following results are obtained
\[ E(U_2(e_i, t)) = 0, \quad 0 < E \left( U_2(e_i, t) U_2(e_i, t)^T \right) < \infty, \quad \forall t \in \mathbb{R} \]
\[ E(U_3(e_i, t)) = 0, \quad 0 < E \left( U_3(e_i, t) U_3(e_i, t)^T \right) < \infty, \quad \forall t \in \mathbb{R} \]
\[ E(U_4(e_i)) = 0, \quad 0 < E \left( U_4(e_i) U_4(e_i)^T \right) < \infty \]

where \( e_i = (e_{i1}, \ldots, e_{i\nu}) = z_i - \hat{z}_i \), with \( z_i \), defined equation (13).

(iii) According to theorem 2.6 of Lehmann and Casella, there hold, respectively

\[ \sup_{ijk} \left| E(U_1(e_{ijk} + s, t)) - b_{ijk}s \right| = O(s^2), \quad \forall t \in \mathbb{R} \]
\[ \sup_{ijk} \left| E(U_2(e_{ijk} + s, t)) - b_{2ijk}s \right| = O(s^2), \quad \forall t \in \mathbb{R} \]
\[ \sup_{ijk} \left| E(U_3(e_{ijk} + s)) - b_{3ijk}s \right| = O(s^2) \]  

(24)

Also, when \( s \to 0 \), exist constants \( c > 0 \) and, \( C < \infty \) such that

\[ \sup_{ijk} \left\{ E(U_1(e_{ijk} + s, t) - U_1(e_{ijk}, t))^2 \right\} \leq C[s], \quad \forall t \in \mathbb{R} \]
\[ \sup_{ijk} \left\{ E(U_2(e_{ijk} + s, t) - U_2(e_{ijk}, t))^2 \right\} \leq C[s], \quad \forall t \in \mathbb{R} \]
\[ \sup_{ijk} \left\{ E(U_3(e_{ijk} + s) - U_1(e_{ijk}))^2 \right\} \leq C[s] \]

Furthermore, \( |U_1(v+\eta, t) - U_1(v, t) - U_1(\eta, t)| \leq c, |U_2(v+\eta, t) - U_2(v, t) - U_2(\eta, t)| \leq c, \) and \( |U_3(v+\eta) - U_3(v) - U_3(\eta)| \leq c \) for any \( |\eta| \leq s \) and \( v, t \in \mathbb{R} \).

Let \( \Delta_n \) be a diagonal matrix with elements \( \delta_{ijd} \) defined in equation (23) and let \( \Lambda_n \) be a diagonal matrix with elements \( b_{3ijk} \) defined in equation (24), then by definition of the random variables \( \delta_{ijd} \) it follows that

\[ E(\Delta_n) = 0 \quad \text{and} \quad \sup_n \left\{ \frac{1}{n} E(||\Delta_n||^2) \right\} < \infty \]

Since, \( \Gamma_n \) is a block diagonal matrix with elements \( A_i = E(U_3(e_i) U_3(e_i)^T) \), then by theorem 1 of Pan,

\[ \frac{1}{n} \Delta_n^T \Gamma_n \Delta_n \xrightarrow{p} B \]
\[ \frac{1}{n} \Lambda_n^T \Lambda_n \xrightarrow{p} A \]  

(25)

(26)

The matrices \( H_{1n} \) and \( H_{2n} \) defined in (22) are symmetric and positive definite, so they have square root. Let \( H_{1n}^{-1} \) and \( H_{2n}^{-1} \) be those square roots, respectively. Also, as a kernel spline forms a linearly independent basis, and by Theorem 21.5.1 of Harville, \( H_{1n}^{-1} \) and \( H_{2n}^{-1} \) are non-singular matrices and their eigenvalues are bounded between zero and infinity. With the previous results and using Theorems 1 and 2 of He et al., the following results are obtained

\[ \frac{1}{n} \sum_{i=1}^{n} \sum_{j=1}^{n} \left\{ \sum_{b=1}^{m} \alpha_{b,i} s_b (Z_{ijk}) - f_s (Z_{ijk}) \right\}^2 = O \left( n^{-\frac{2}{(2+\delta)}} \right) \]
\[ \frac{1}{n} \sum_{i=1}^{n} \sum_{j=1}^{n} \left\{ \sum_{b=1}^{m} \alpha_{b,i} s_b (Z_{ijk}) - f_s (Z_{ijk}) \right\}^2 = O \left( n^{-\frac{2}{(2+\delta)}} \right) \]
\[ \sqrt{n}(\hat{\beta} - \beta) \to N(0, A^{-1} BA^{-1}) \]  

(27)

(28)

(29)

where the matrices \( A \) and \( B \) are defined in equations (14) and (15), respectively.