The statistical analysis of non-linear longitudinal data in biomedical research using generalized additive models

Beyond repeated measures ANOVA and Linear Mixed Models

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1 Abstract

In biomedical research, the outcome of a longitudinal study has been traditionally analyzed using the *repeated measures analysis of variance* (rm-ANOVA) or more recently, a *linear mixed model* (LMEM). Although LMEMs are less restrictive that rm-ANOVA in terms of correlation and missing observations, both methodologies share an assumption of linearity in the measured response, which results in biased estimates and unreliable inference when they are used to analyze data where the trends are non-linear. In contrast, generalized additive models (GAMs), are a class of models that relax the linearity assumption and allow the data to determine the fit of the model while permitting missing observations and different correlation structures. Although not commonly used in biomedical research, GAMs present an excellent choice to analyze non-linear longitudinal data. This paper summarizes the limitations of linear models (rm-ANOVA in particular), presents the basic theory of GAMs, and demonstrates their implementation in R via the package *mgcv* using simulated data that follows longitudinal trends reported in biomedical literature. To promote reproducibility in biomedical research, the code and data used to generate this paper are available at:_____.

2 Background

Longitudinal studies are designed to repeatedly measure a variable of interest in a group (or groups) of subjects, with the intention of observing the evolution of effect across time rather than analyzing a single time point (e.g., a cross-sectional study). Biomedical research frequently uses longitudinal studies to analyze the evolution of a "treatment" effect across multiple time points; and in such studies the subjects of analysis range from animals (mice, rats, rabbits), to human patients, cells, or blood samples, among many others. Tumor response [1–4], antibody expression [5,6], and cell metabolism [7,8] are examples of the different situations where researchers have used longitudinal designs to study some physiological response. Because the frequency of the measurements in a longitudinal study is dependent on the biological phenomena of interest and the experimental design of the study, the frequency of such measurements can range from minute intervals to study a short-term response such as anesthesia effects in animals[9], to weekly measurements to analyze a mid-term response like the evolution of dermatitis symptoms in breast cancer patients [10], to monthly measurements to study a long-term response such as mouth opening following radiotherapy (RT) in neck cancer patients [11].

Traditionally, a "frequentist" or "classical" statistical paradigm is used in biomedical research to derive inferences from a longitudinal study. The frequentist paradigm regards probability as the limit of the expected outcome when an experiment is repeated a large number of times [12], and such view is applied to the analysis of longitudinal data by assuming a null hypothesis under a statistical model that is often an *analysis of variance over repeated measures* (repeated measures ANOVA or rm-ANOVA). The rm-ANOVA model makes three key assumptions regarding longitudinal data: 1) linearity of the response across time, 2) constant correlation across same-subject measurements, and 3) observations from each subject are obtained at all time points through the study (a condition also known as *complete observations*) [13,14].

The expected linear behavior of the response through time is a key requisite in rm-ANOVA [15]. This "linearity assumption" in rm-ANOVA implies that the model is misspecified when the data does not follow a linear trend, which results in unreliable inference. In biomedical research, non-linear trends are the norm rather than the exception in longitudinal studies. A particular example of this non-linear behavior in longitudinal data arises in measurements of tumor response to chemo and/or radiotherapy in preclinical and clinical settings [1,8,16]. These studies have shown that the collected signal does not follow a linear trend over time, and presents extreme variability at different time points, making the fit of rm-ANOVA model inconsistent with the observed variation. Therefore, when rm-ANOVA is used to draw inference of such highly-variable data the estimates are inevitably biased, because the model is only able to accommodate linear trends that are far from adequately representing the biological phenomenon of interest.

A *post hoc* analysis is the statistical test used in conjunction with rm-ANOVA to perform repeated comparisons to estimate a *p-value*, which in turn is used as a measure of significance. Although it is possible that a *post hoc* analysis of rm-ANOVA is able to find "significant" *p-values*($p<0.05$) from non-linear data, the

validity of such metric is dependent on how adequate the model fits the data. In other words, *p-values* are valid only if the model and the data have good agreement; if that is not the case, a "Type III" error (known as "model misspecification") occurs[17]. For example, model misspecification will occur when a model that is only able to explain linear responses (such as rm-ANOVA) is fitted to data that follows a quadratic trend, thereby causing the resulting *p-values* and parameter estimates to be invalid [18].

Additionally, the *p-value* itself is highly variable, and multiple comparisons can inflate the false positivity rate (Type I error or α) [19,20], consequently biasing the conclusions of the study. Corrections exist to address the Type I error issue of multiple comparisons (such as Bonferroni [21]), but they in turn reduce statistical power (1-β)[22], and lead to increased Type II error (failing to reject the null hypothesis when the null hypothesis is false) [23,24]. Therefore, the tradeoff of *post hoc* comparisons in rm-ANOVA between Type I, II and III errors might be difficult to resolve in a biomedical longitudinal study where a delicate balance exists between statistical power and sample size.

On the other hand, the assumption of constant correlation in rm-ANOVA (often known as the *compound symmetry assumption*) is typically unreasonable because correlation between the measured responses often diminishes as the time interval between the observation increases [25]. Corrections can be made in rm-ANOVA in the absence of compound symmetry [26,27], but the effectiveness of the correction is limited by the size of the sample, the number of measurements[28], and group sizes [29]. In the case of biomedical research, where living subjects are frequently used, sample sizes are often not "large" due to ethical and budgetary reasons [30] which might cause the corrections for lack of compound symmetry to be ineffective.

Due to a variety of causes, the number of observations during a study can vary between all subjects. For example, in a clinical trial patients may voluntarily withdraw, whereas attrition due to injury or weight loss in preclinical animal studies is possible. It is even plausible that unexpected complications with equipment or supplies arise that prevent the researcher from collecting measurements at certain time points. In each of these missing data scenarios, the *complete observations* assumption of classical rm-ANOVA is violated. When incomplete observations occur, a rm-ANOVA model is fit by excluding all subjects with missing observations from the analysis [13]. This elimination of partially missing data from the analysis can result in increased costs if the desired statistical power is not met with the remaining observations, because it would be necessary to enroll more subjects. At the same time, if the excluded observations contain insightful information that is not used, their elimination from the analysis may limit the demonstration of significant differences between groups.

During the last decade, the biomedical community has started to recognize the limitations of rm-ANOVA in the analysis of longitudinal information. The recognition on the shortcomings of rm-ANOVA is exemplified by the use of linear mixed effects models (LMEMs) by certain groups to analyze longitudinal tumor response data [8,16]. Briefly, LMEMs incorporate *fixed effects*, which correspond to the levels of experimental factors in the study (e.g., the different drug regimens in a clinical trial), and *random effects*, which account for random variation within the population (e.g., the individual-level differences not due to treatment such as weight or age). When compared to the traditional rm-ANOVA, LMEMs are more flexible as they can accommodate missing observations for multiple subjects and allow different modeling strategies for the variability within each measure in every subject [15]. However, LMEMs impose restrictions in the distribution of the errors of the random effects, which need to be normally distributed and independent [13,31]. And even more importantly, LMEMs also expect a linear relationship between the response and time [15], making them unsuitable to analyze non-linear data.

As the rm-ANOVA and the more flexible LMEM approaches make overly restrictive assumptions regarding the linearity of the response, there is a need for biomedical researchers to explore the use of additional statistical tools that allow the data (and not an assumption in trend) to determine the trend of the fitted model, to enable appropriate inference.

In this regard, generalized additive models (GAMs) present an alternative approach to analyze longitudinal data. Although not frequently used by the biomedical community, these semi-parametric models are customarily used in other fields to analyze longitudinal data. Examples of the use of GAMs include the analysis of temporal variations in geochemical and palaeoecological data [32–34], health-environment interactions [35] and the dynamics of government in political science [36] . There are several advantages of GAMs over

LMEMs and rm-ANOVA models: 1) GAMs can fit a more flexible class of smooth responses that enable the data to dictate the trend in the fit of the model, 2) they can model non-constant correlation between repeated measurements [37] and 3) can easily accommodate missing observations. Therefore, GAMs can provide a more flexible statistical approach to analyze non-linear biomedical longitudinal data than LMEMs and rm-ANOVA.

The current advances in programming languages designed for statistical analysis (specifically R), have eased the computational implementation of traditional models such as rm-ANOVA and more complex approaches such as LMEMs and GAMs. In particular, R[38] has an extensive collection of documentation and functions to fit GAMs in the package *mgcv* [37,39] that not only speed up the initial stages of the analysis but also enable the use of advanced modeling structures (e.g. hierarchical models, confidence interval comparisons) without requiring advanced programming skills from the user. At the same time, R has many tools that simplify data simulation, an emerging strategy used to test statistical models [28]. Data simulation methods allow the researcher to create and explore different alternatives for analysis without collecting information in the field, reducing the time window between experiment design and its implementation, and simulation can be also used for power calculations and study design questions.

This work provides biomedical researchers with a clear understanding of the theory and the practice of using GAMs to analyze longitudinal data using by focusing on four areas. First, the limitations of LMEMs and rm-ANOVA regarding linearity of response, constant correlation structures and missing observations is explained in detail. Second, the key theoretical elements of GAMs are presented using clear and simple mathematical notation while explaining the context and interpretation of the equations. Third, using simulated data that reproduces patterns in previously reported studies [16] we illustrate the type of non-linear longitudinal data that often occurs in biomedical research. The simulated data experiments highlight the differences in inference between rm-ANOVA, LMEMs and GAMs on data similar to what is commonly observed in biomedical studies. Finally, reproducibility is emphasized by providing the code to generate the simulated data and the implementation of different models in R, in conjunction with a step-by-step guide demonstrating how to fit models of increasing complexity.

In summary, this work will allow biomedical researchers to identify when the use of GAMs instead of rm-ANOVA or LMEMs is appropriate to analyze longitudinal data, and provide guidance on the implementation of these models by improving the standards for reproducibility in biomedical research.

3 Challenges presented by longitudinal studies

3.1 The repeated measures ANOVA

The *repeated measures analysis of variance* (rm-ANOVA) is the standard statistical analysis for longitudinal data in biomedical research. This statistical methodology requires certain assumptions for the model to be valid. From a practical view, the assumptions can be divided in three areas: 1) linear relationship between covariates and response, 2) a constant correlation between measurements, and, 3) complete observations for all subjects. Each one of these assumptions is discussed below.

3.2 Linear relationship

3.2.1 The repeated measures ANOVA case

In a longitudinal biomedical study, two or more groups of subjects (e.g., human subject, mice, samples) are subject to different treatments (e.g., a "treatment" group receives a novel drug or intervention vs. a "control" group that receives a placebo), and measurements from each subject within each group are collected at specific time points. The collected response is modeled with *fixed* components. The *fixed* component can be understood as a constant value in the response which the researcher is interested in measuring, i.e., the average effect of the novel drug/intervention in the "treatment" group.

Mathematically speaking, a rm-ANOVA model with an interaction can be written as:

$$y_{ijt} = \beta_0 + \beta_1 \times time_t + \beta_2 \times treatment_j + \beta_3 \times time_t \times treatment_j + \varepsilon_{ijt} \tag{1}$$

In this model y_{ijt} is the response for subject i, in treatment group j at time t, which can be decomposed in a mean value β_0, *fixed effects* of time ($time_t$), treatment ($treatment_j$) and their interaction $time_t * treatment_j$ which have linear slopes given by β_1, β_2 and β_3, respectively. Independent errors ε_{tij} represent random variation not explained by the *fixed* effects, and are assumed to be $\sim N(0, \sigma^2)$ (independently normally distributed with mean zero and variance σ^2_μ). In a biomedical research context, suppose two treatments groups are used in a study (e.g., "placebo" vs. "novel drug" or "saline" vs. "chemotherapy"). Then, the group terms in Equation (1) can be written as below with $treatment_j = 0$ representing the first treatment group (Group A) and $treatment_j = 1$ representing the second treatment group (Group B). The linear models then can be expressed as

$$y_{ijt} = \begin{cases} \beta_0 + \beta_1 \times time_t + \varepsilon_{ijt} & \text{if Group A} \\ \beta_0 + \beta_2 + \beta_1 \times time_t + \beta_3 \times time_t + \varepsilon_{ijt} & \text{if Group B} \end{cases} \tag{2}$$

To further simplify the expression, substitute $\widetilde{\beta_0} = \beta_0 + \beta_2$ and $\widetilde{\beta_1} = \beta_1 + \beta_3$ in the equation for Group B. This substitution allows for a different intercept and slope for Groups A and B. The model is then written as

$$y_{ijt} = \begin{cases} \beta_0 + \beta_1 \times time_t + \varepsilon_{ijt} & \text{if Group A} \\ \widetilde{\beta_0} + \widetilde{\beta_1} \times time_t + \varepsilon_{ijt} & \text{if Group B} \end{cases} \tag{3}$$

Presenting the model in this manner makes clear that when treating different groups, an rm-ANOVA model is able to accommodate non-parallel lines in each case (different intercepts and slopes per group). In other words, the rm-ANOVA model "expects" a linear relationship between the covariates and the response, this means that either presented as Equation (1), Equation (2) or Equation (3), an rm-ANOVA model is only able to accommodate linear patterns in the data. If the data show non-linear behavior, the rm-ANOVA model will approximate this behavior with non-parallel lines.

3.2.2 The Linear Mixed Model Case

A linear mixed model (LMEM) is a class of statistical model that incorporates *fixed effects* to model the relationship between the covariates and the response, and *random effects* to model subject variability that is not the primary focus of the study but that might be important to distinguish [15,40]. A LMEM with interaction between time and treatment for a longitudinal study can be written as:

$$y_{ijt} = \beta_0 + \beta_1 \times time_t + \beta_2 \times treatment_j + \beta_3 \times time_t \times treatment_j + \mu_{ij} + \varepsilon_{ijt} \tag{4}$$

When Equation (1) and Equation (4) are compared, it is easily noticeable that LMEM and rm-ANOVA have the same construction regarding the *fixed effects* of time and treatment, but that the LMEM incorporates an additional source of variation (the term μ_{ij}). This term μ_{ij} is the one that corresponds to the *random effect*, accounting for variability in each subject within each group. The *random* component can also be understood as used to model some "noise" in the response, but that is intended to be analyzed and disentangled from the "global noise" term ε_{ijt} from Equation (1).

For example, if the blood concentration of the drug is measured in certain subjects in the early hours of the morning while other subjects are measured in the afternoon, it is possible that the difference in the collection time introduces some "noise" in the data. As the name suggests, this "random" variability needs to be modeled as a variable rather than as a constant value. The *random effect* μ_{ij} in Equation (4) is

assumed to be $\mu_{ij} \sim N(0, \sigma^2_\mu)$. In essence,the *random effect* in a LMEM enables to fit models with different slopes at the subject-level[15]. However,the expected linear relationship of the covariates and the response in Equation (1) and in Equation (4) is essentially the same, representing a major limitation of LMEMs to fit a non-linear response.

3.3 Covariance in rm-ANOVA and LMEMs

In a longitudinal study there is an expected *covariance* between repeated measurements on the same subject, and because repeated measures occur in the subjects within each group, there is a *covariance* between measurements at each time point within each group. The *covariance matrix* (also known as the variance-covariance matrix) is a matrix that captures the variation between and within subjects in a longitudinal study[41] (For an in-depth analysis of the covariance matrix see [40,42]).

In the case of an rm-ANOVA analysis, it is typically assumed that the covariance matrix has a specific construction known as *compound symmetry* (also known as "sphericity" or "circularity"). Under this assumption, the between-subject variance and within-subject correlation are constant across time [26,42,43]. However, it has been shown that this condition is frequently not justified because the correlation between measurements tends to change over time [44]; and it is higher between consecutive measurements [13,25]. Although corrections can be made (such as Huyhn-Feldt or Greenhouse-Geisser)[26,27] the effectiveness of each correction is limited because it depends on the size of the sample,the number of repeated measurements[28], and they are not robust if the group sizes are unbalanced [29]. Because biomedical longitudinal studies are often limited in sample size and can have an imbalanced design, the corrections required to use an rm-ANOVA model may not be able to provide a reasonable adjustment that makes the model valid.

In the case of LMEMs, one key advantage over rm-ANOVA is that they allow different structures for the variance-covariance matrix including exponential, autoregressive of order 1, rational quadratic and others [15]. Nevertheless, the analysis required to determine an appropriate variance-covariance structure for the data can be a challenging process by itself. Overall, the spherical assumption for rm-ANOVA may not capture the natural variations of the correlation in the data, and can bias the inferences from the analysis.

3.4 Missing observations

Missing observations are an issue that arises frequently in longitudinal studies. In biomedical research, this situation can be caused by reasons beyond the control of the investigator [45]. Dropout from patients and attrition or injury in animals are among the reasons for missing observations. Statistically, missing information can be classified as *missing at random* (MAR), *missing completely at random* (MCAR), and *missing not at random* (MNAR) [42]. In a MAR scenario, the pattern of the missing information is related to some variable in the data, but it is not related to the variable of interest [46]. If the data are MCAR, this means that the missingness is completely unrelated to the collected information [47], and in the case of MNAR the missing values are dependent on their value. An rm-ANOVA model assumes complete observations for all subjects, and therefore subjects with one or more missing observations are excluded from the analysis. This is inconvenient because the remaining subjects might not accurately represent the population, and statistical power is affected by this reduction in sample size [48].

In the case of LMEMs, inferences from the model are valid when missing observations in the data exist that are MAR or MCAR [40]. For example, if attrition occurs in all mice that had lower weights at the beginning of a chemotherapy response study, the missing data can be considered MAR because the missingness is unrelated to other variables of interest.

This section has presented the assumptions for analyzing longitudinal data using rm-ANOVA and LMEMs and compared their differences regarding linearity, the covariance matrix and missing data. In particular, LMEMs offer a more robust and flexible approach than rm-ANOVA and if the data follows a linear trend, they provide an excellent choice to derive inferences from a repeated measures study. However, when the data presents high a non-linear behavior, LMEMs and rm-ANOVA fail to capture the trend of the data. To

better convey the issues of linearity and correlation in linear models fitted to non-linear data, simulation is used in the next section.

3.5 What does an rm-ANOVA fit looks like? A visual representation using simulated data

To demonstrate the limitations of rm-ANOVA an LMEMs for non-linear longitudinal data, this section presents a simulation experiment of a normally distributed response of two groups of 10 subjects each. An rm-ANOVA model (Equation (1)) is fitted to each group, using R[38] and the package *nlme*[49]. Briefly, two cases for the mean responses for each group are considered: in the first case, the mean response in each group is a linear function with different intercepts and slopes; a negative slope is used for Group 1 and a positive slope is used for Group 2 (Figure 1, A). In the second case, a second-degree polynomial (quadratic) function is used for the mean response per group: the quadratic function is concave down for Group 1 and it is concave up for Group 2 (Figure 1, C). In both the linear and quadratic simulated data, the groups start with the same mean value at the first time point. This is intentional in order to simulate the expected temporal evolution of some physiological quantity.

Specifically, the rationale for the chosen linear and quadratic functions is the likelihood that a measured response in two treatment groups is similar in the initial phase of the study, but as treatment progresses a divergence in the trend of the response indicates an effect due to treatment. In other words, Group 1 can be thought as a "Control" group and Group 2 as a "Treatment" group. From the mean response per group (linear or quadratic), the variability or "error" of individual responses within each group is simulated using a covariance matrix with compound symmetry (constant variance across time). Thus, the response per subject in both the linear and quadratic simulation corresponds to the mean response per group plus the error (Figure 1 B,D). A more comprehensive exploration of the fit of rm-ANOVA for linear and non-linear longitudinal data is in Figure **??** and Figure **??** in the Appendix, where simulation with compound symmetry and independent errors (errors generated from a normal distribution that are not constant over time) and the plot of simulated errors, and fitted parameters in presented.

The simulation shows that the fit produced by the rm-ANOVA model is good for linear data, as the predictions for the mean response are reasonably close to the "truth" of the simulated data (Figure 1,B). When the linearity and compound symmetry assumptions are met, the model approximates well the individual trends and the mean trends by group.

However, consider the case when the data follows a non-linear trend, such as the simulated data in Figure 1, C. Here, the mean response per group was simulated using a quadratic function but errors, individual responses and the rm-ANOVA model were produced in the same manner as in (Figure 1 A and B) . The mean response in the simulated data with quadratic behavior is changing in each group through the timeline, and the mean value is the same as the initial value by the fifth time point for each group. Fitting an rm-ANOVA model (1) to this data produces the fit that appears in panel D in Figure 1.

A comparison of the fitted mean response of the rm-ANOVA model to the the simulated data in Figure ((1, D) indicates that the model is not capturing the changes within each group in a good way. Specifically, note that the fitted mean response of the rm-ANOVA model (panel D) shows that the change (increase for Treatment 1 or decrease for Treatment 2) in the response through time points 2 and 4 is not being captured by the model. Moreover, the rm-ANOVA model is not being able to capture the fact that the initial values are the same in each group, and instead fits non-parallel lines that have initial values that are markedly different from the "true" initial values in each case (compare panels C and D). If such change has important physiological implications,the rm-ANOVA model omits it from the fitted mean response. Thus, even though the model correctly detects a difference in treatment groups, the exact nature of this difference is not correctly identified, limiting valuable inferences from the data.

This section has used simulation to better convey the limitations of linearity and correlation in the response in non-linear data. Although the model fitted to the simulated data was an rm-ANOVA model, the main issue of an expected linear trend in the response is the same in the case of a LMEM. In the following section,

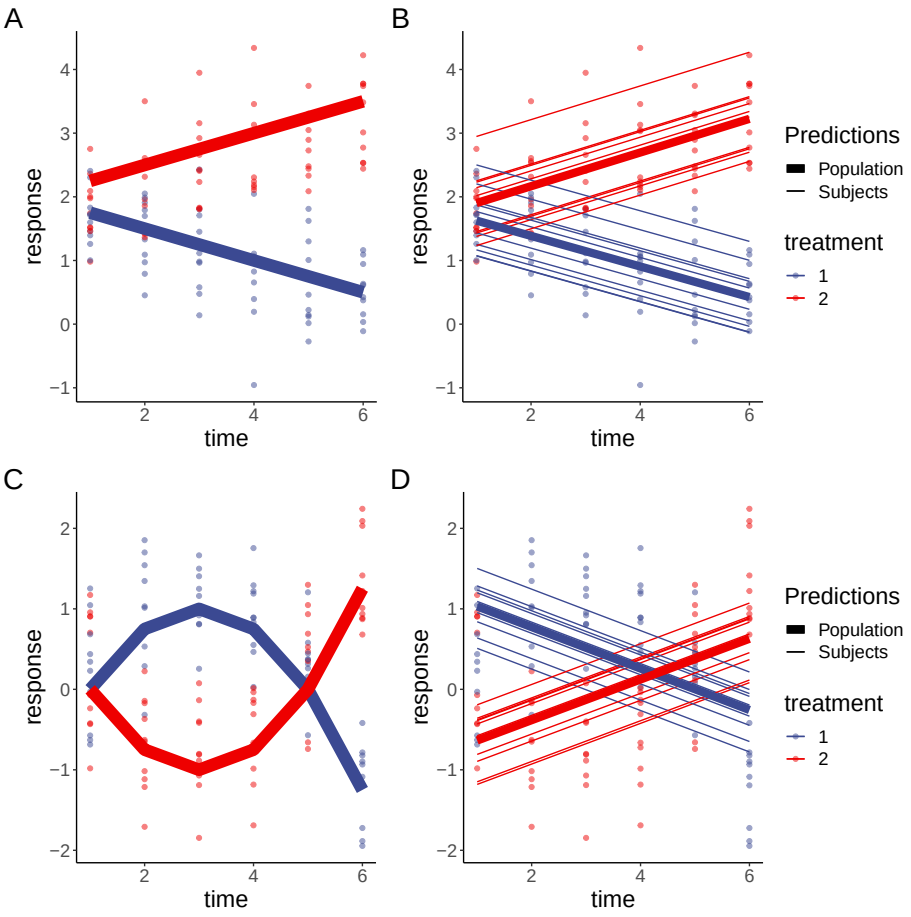

Figure 1: ref(l-q-response-caption)

we present generalized additive models (GAMs) as a data-driven alternative method to analyze longitudinal non-linear data.

4 GAMs as a special case of Generalized Linear Models

4.1 GAMs and Basis Functions

Generalized linear models (GLMs) are a family of models that fit a linear response function to data that do not have normally distributed errors[50]. In contrast, GAMs are a family of regression-based methods for estimating smoothly varying trends and are a broader class of models that contain the GLM family as a special case[34,37,51]. A GAM model can be written as:

$$y_{ijt} = \beta_0 + f(x_t \mid \beta_j) + \varepsilon_{ijt} \tag{5}$$

Where y_{ijt} is the response at time t of subject i in group j, β_0 is the expected value at time 0, the change of y_{ijt} over time is represented by the function $f(x_t \mid \beta_j)$ with inputs as the covariates x_t and parameters β_j, and ε_{ijt} represents the residual error.

In contrast to the linear functions used to model the relationship between the covariates and the response in rm-ANOVA or LMEM, GAMs use more flexible *smooth functions*. This approach is advantageous as it does

not restrict the model to a linear relationship, although a GAM will estimate a linear relationship if the data is consistent with a linear response. One possible set of functions for $f(x_t \mid \beta_j)$ that allow for non-linear responses are polynomials, but a major limitation is that polynomials create a "global" fit as they assume that the same relationship exists everywhere, which can cause problems with the fit [36]. In particular, polynomial fits are known to show boundary effects because as t goes to $\pm\infty$, $f(x_t \mid \beta_j)$ goes to $\pm\infty$ which is almost always unrealistic, and causes bias at the endpoints of the time period.

The smooth functional relationship between the covariates and the response in GAMs is specified using a semi-parametric relationship that can be fit within the GLM framework, by using *basis functions* expansions of the covariates and by estimating random coefficients for these basis functions. A *basis* is a set of functions that spans the space where the smooths that approximate $f(x_t \mid \beta_j)$ exist [34]. For the linear model in Equation (1), the basis coefficients are β_1, β_2 and β_3 and the basis vectors are $time_t$, $treatment_j$ and $time_t \times treatment_j$. The basis function then, is the combination of basis coefficients and basis vectors that map the possible relationship between the covariates and the response [52], which in the case of Equation (1) is restricted to a linear family of functions. In the case of Equation (5), the basis function is $f(x_t \mid \beta_j)$, which means that the model allows for non-linear relationships among the covariates.

A commonly used *basis function* is the cubic spline, which is a smooth curve constructed from cubic polynomials joined together in a manner that enforces smoothness [34,37]. Cubic splines have a long history in solving semi-parametric statistical problems and are often a default choice to fit GAMs as they are a simple, flexible and powerful option to obtain smoothness [53]. Therefore, this data-driven flexibility in GAMs overcomes the limitation that occurs in LMEMs and rm-ANOVA when the data is non linear.

To further clarify the concept of basis functions and smooth functions, consider the simulated response for Group 1 in Figure (1, C). The simplest GAM model that can be used to estimate such response is that of a single smooth term for the time effect; i.e., a model that fits a smooth to the trend of the group through time. The timeline can be divided in equally spaced *knots*, each knot being a region where a different basis function will be used. Because there are six timepoints for this group, five knots can be used. The model with five knots to construct the smooth term means that it will have four basis functions (plus one that corresponds to the intercept). The choice of basis functions is already optimized in the package *mgcv* depending on the number of knots. In Panel A of Figure 2, the four basis functions (and the intercept) are shown. Each of the basis functions is composed of six different points (because there are six points on the timeline). To control the "wigliness" of the fit, each of the basis functions of Panel A is penalized by multiplying it by a coefficient according to the penalty matrix of Panel B. The penalty reduces the "wigliness" of the smooth fit to prevent overfitting: A weak penalty estimate will result in wiggly functions whereas a strong penalty estimate provides evidence that a linear response is appropriate.

In other words, the six points of each basis are multiplied by the corresponding coefficient in panel B, thereby increasing or decreasing the original basis functions of Panel A. In Figure 2, Panel C shows the resulting penalized basis functions. Note that the penalization for basis 1 has resulted in a decrease of its overall value (because the coefficient for that basis function is negative and less than 1); on the other hand, basis 3 has roughly doubled its value. Finally, the penalized basis functions are added at each timepoint to produce the smooth term. The resulting smooth term for the effect of *time* is shown in Panel D (orange line) along the simulated values per group, which appear as points.

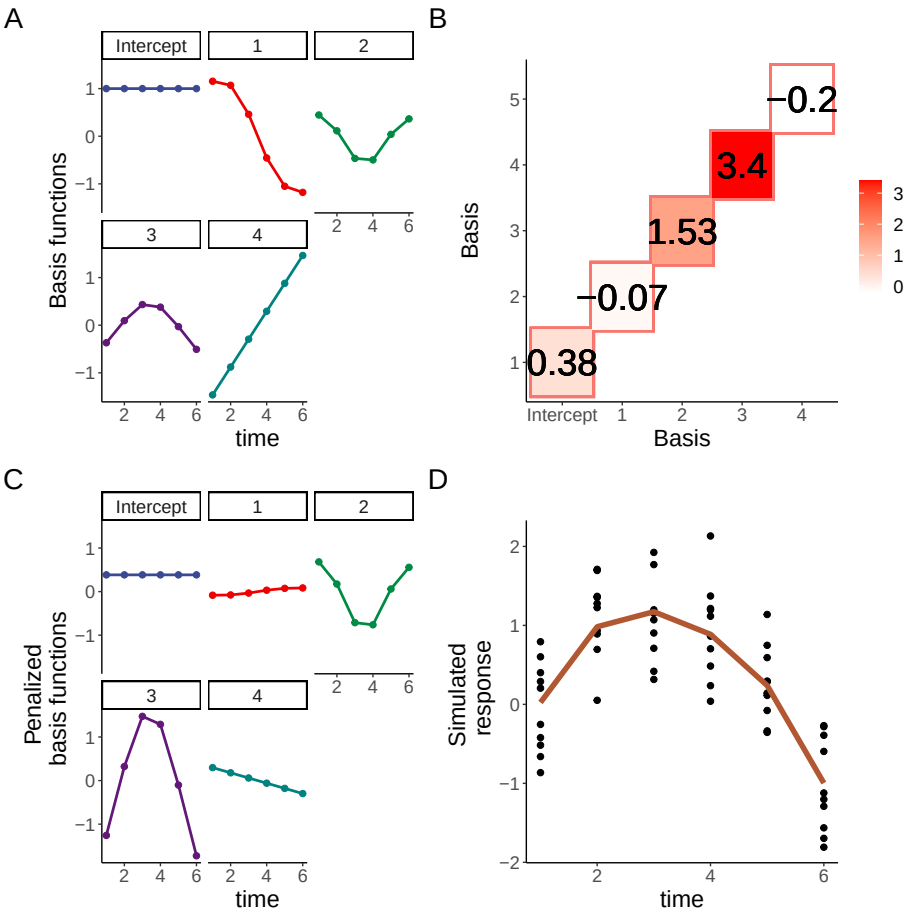

Figure 2: Basis functions for a single smoother for time with five knots. A: Basis functions for a single smoother for time for the simulated data of Group 1 from Figure 2, the intercept basis is not shown. B: Penalty matrix for the basis functions. Each basis function is penalized by a coefficient which can be positive or negative. The coefficient determines the overall effect of each basis in the final smoother. C: Penalized basis functions. Each of the four basis functions of panel A has been penalized by the corresponding coefficient shown in Panel B, note the corresponding increase (or decrease) of each basis. D: Smoother for time and original data points. The smoother (line) is the result of the sum of each penalized basis function at each time point, simulated values for the group appear as points.

5 The analyisis of longitudinal biomedical data using GAMs

The previous sections provided the basic theoretical framework to understand the GAM framework and how these models are more advantageous to analyze non-linear longitudinal data when compared to LMEMs or rm-ANOVA. This section will use simulation to present the practical implementation of GAMs for longitudinal biomedical data using R and the package `mgcv`. The code for the simulated data and figures, and a brief guide for model selection and diagnostics appear in the Appendix.

5.1 Simulated data

The simulated data is based on the reported longitudinal changes in oxygen saturation (StO_2) in subcutaneous tumors that appear in Figure 3(C) in [16]. In the paper, diffuse reflectance spectroscopy was used to quantify StO_2 changes in both groups at the same time points (days 0, 2, 5, 7 and 10). In the "Treatment" group (chemotherapy) an increase in StO_2 is observed through time, while a decrease is seen in the "Control" (saline) group. Following the reported trend, we simulated 10 normally distributed observations at each time

point with a standard deviation (SD) of 10% (matching the SD in the original paper). The simulated and real data appear in Figure 3, A and the inlet, respectively.

5.2 An interaction GAM for longitudinal data

An interaction model is typically the main interest in longitudinal biomedical data, as it takes into account the effect of treatment, time, and their combination. In a practical sense, when a GAM is implemented for longitudinal data, a smooth can be added to the model for the *time* effect to account for the repeated measures over time. Although specific methods of how GAMs model correlation structures is a topic beyond the scope of this paper, it suffices to say that GAMs are flexible and can handle correlation structures beyond compound symmetry. A detailed description on basis functions and correlations can be found in [52].

For the data in Figure 3,A it is of interest to know the effect that each treatment has in StO_2. The model then needs to incorporate independent smooths for *Group* and *Day*, respectively. The main thing to consider is that model syntax accounts for the fact that one of the variables is numeric (*Day*) and the other is a factor (*Group*). Because the smooths are centered at 0, the factor variable needs to be specified as a parametric term in order to identify any differences between the groups. Using `R` and the package `mgcv` the model syntax is:

```
m1<-gam(StO2_sim~Group+s(Day,by=Group,k=5,bs="gp"), method='REML',data=dat_sim
```

This syntax specifies that `m1` will store the model, and that the change in the simulated oxygen saturation (`StO2_sim`) is modeled using independent smooths for *Group* and *Day* (the parenthesis preceded by *s*) using 5 knots. The smooth is constructed using gaussian process smooths, which is indicated by `bs="gp"`. These splines are used to model temporal trends and might be particularly suited for long-term studies where the correlation between measurements changes as a function of the time intervals [34]. The parametric term *Group* is added to quantify differences in the effect of treatment between groups, and the `method` chosen to select the smoothing parameters is the restricted maximum likelihood (REML) [37]. When the smooths are plotted over the raw data, it is clear that the model has been able to capture the trend of the change of StO_2 for each group across time (Figure 3,B). Model diagnostics can be obtained using the `gam.check` function, and the function `appraise` from the package *gratia*[54]. A guide for model selection and diagnostics is in the Appendix, and an in-depth analysis can be found in [37] and [55].

One question that might arise at this point is "what is the fit that an rm-ANOVA model produces for the simulated data?". The rm-ANOVA model, which corresponds to Equation (1) is presented in Figure 3,C. This is a typical case of model misspecification: The slopes of each group are different, which would lead to a *p-value* indicating significance for the treatment and time effects, but the model is not capturing the changes that occur at days 2 and between days 5 and 7, whereas the GAM model is able to do so (Figure 3,B) .

Because GAMs do not require equally-spaced or complete observations for all subjects, they are advantageous to analyze longitudinal data where missingness exists. The rationale behind this is that GAMs are able to pick the trend in the data even when some observations are missing. However, this usually causes the resulting smooths to have wider confidence intervals and less ability to pick certain trends. Consider the simulated StO_2 values from Figure (3, B). If 40% of the total observations are randomly deleted and the same interaction GAM fitted for the complete dataset is used, the resulting smooths are still able to show a different trend for each group, but the "Treatment" smooth takes an overall more linear profile (3, D). Although the confidence intervals have increased for both smooths, the model still shows different trends with as little as 4 observations per group at certain time points.

5.3 Determination of significance in GAMs for longitudinal data

At the core of a biomedical longitudinal study lies the question of a significant difference between the effect of two or more treatments in different groups. Whereas in rm-ANOVA a *post-hoc* analysis is required to answer such question by calculating some *p-values* after multiple comparisons, GAMs can use a different

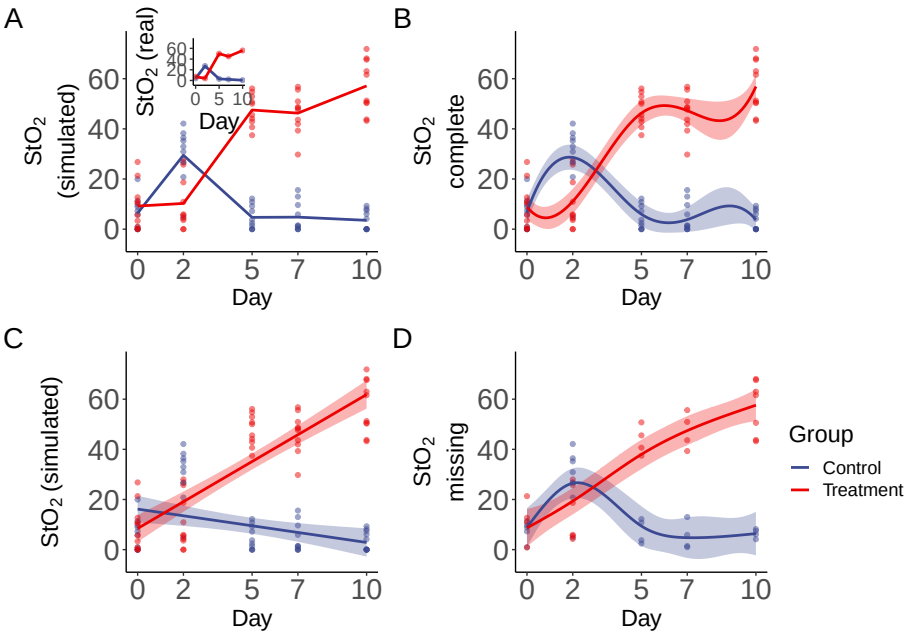

Figure 3: Simulated data and smooths for oxygen saturation in tumors. A: Simulated data that follows previously reported trends (inset) in tumors under chemotherapy (Treatment) or saline (Control) treatment. Simulated data is from a normal distribution with standard deviation of 10% with 10 observations per time point. Lines indicate mean oxygen saturation B: Smooths from the GAM model for the full simulated data with interaction of Group and Treatment. Lines represent trends for each group, shaded regions are 95% confidence intervals. C: rm-ANOVA model for the simulated data, the model does not capture the changes in each group over time. D: Smooths for the GAM model for the simulated data with 40% of its observations missing. Lines represent trends for each group, shaded regions are 95% confidence intervals.

approach to estimate significance. In essence, the idea behind the estimation of significance in GAMs across different treatment groups is that if the *difference* between the confidence intervals of the fitted smooths for such groups is non-zero, then a significant difference exists at that time point(s). The absence of a *p-value* in this case might seem odd, but the confidence interval comparison can be conceptualized in the following manner: Different trends in each group are an indication of an effect by the treatment. This is what happens for the simulated data in Figure 3, A where the chemotherapy causes StO_2 to increase over time.

With this expectation of different trends in each group, computing the difference between the trends will identify if the observed change is significant. The difference between groups with similar trends is likely to yield zero, which would indicate that the treatment is not causing a change in the response in one of the groups (assuming the other group is a Control or Reference group).

Consider the calculation of pairwise differences for the smooths in 3 B and D. Figure 4, shows the comparison between each treatment group.Here, the effect of the "Control" group is compared to that of the "Treatment" group. The shaded region under the curve highlights the interval where each group has a higher effect than the other. Notice that the shaded region between days 0 and 4 indicates that through that time, the "Control" group has higher StO_2, but as therapy progresses the effect is reversed and by day 5 it is the "Treatment" group the one that has greater contribution. This would suggest that the effect of chemotherapy is significant after day 5 for the model used; which is noticeable in the data with missing observations albeit the missing data model has a wider confidence interval and is not able to pick the change between days 7 and 10 that the full dataset model is able to detect.

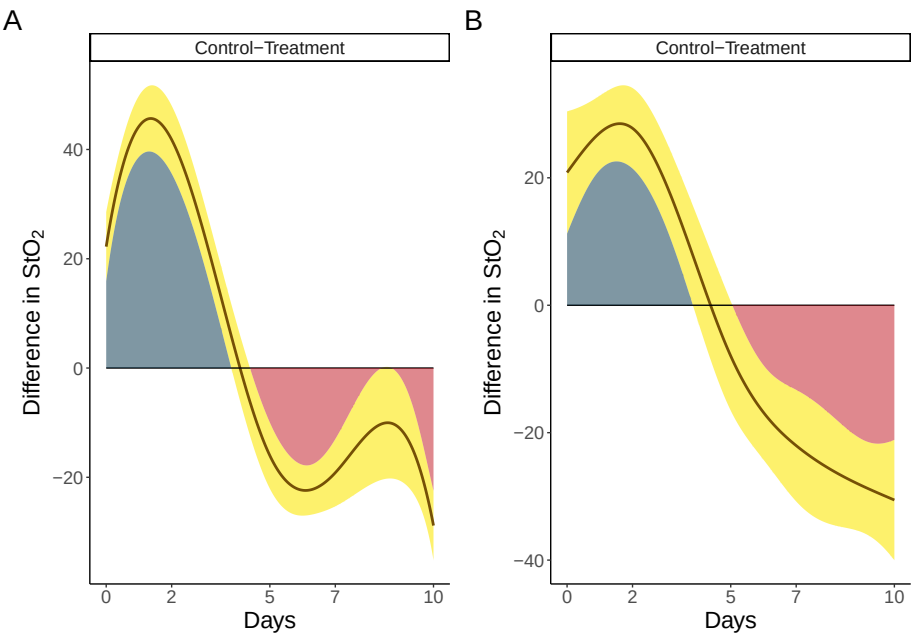

Figure 4: Pairwise comparisons for smooth terms. A: Pairwise comparisons for the full dataset. B: Pairwise comparisons for the dataset with missing observations. Significant differences exist where the interval does not cover 0. In both cases the effect of treatment is significant after day 5.

6 Conclusion

7 References

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A Simulation

A.1 Compound symmetry and independent errors in linear and quadratic responses

This section simulated linear and quadratic data in the same manner as in Section 3.5. The linear simulations using Figure **??** show in panels A and D the simulated mean responses and individual datapoints. Panels C and G show a visual interpretation of "correlation" in the responses: In panel C, subjects that have a value of the random error ε either above or below the mean group response are more likely to have other observations that follow the same trajectory, thereby demonstrating correlation in the response. In panel G,because the errors are independent, there is no expectation that responses are likely to follow a similar pattern. Panels D and H show the predictions from the rm-ANOVA model.

```
## Example with linear response
example <- function(n_time = 6,
                    fun_type = "linear",
                    error_type = "correlated") {

  if (!(fun_type %in% c("linear", "quadratic")))
    stop('fun_type must be either "linear", or "quadratic"')
  if (!(error_type %in% c("correlated", "independent")))
    stop('fun_type must be either "correlated", or "independent"')

  library(tidyverse)
  library(mvnfast)
  library(nlme)
  library(ggsci)

  x <- seq(1,6, length.out = n_time)
  mu <- matrix(0, length(x), 2)
  # linear response
  if (fun_type == "linear") {
    mu[, 1] <- - (0.25*x)+2
    mu[, 2] <- 0.25*x+2
  } else {
    # nonlinear response

    mu[, 1] <-  -(0.25 * x^2) +1.5*x-1.25
```

```
  mu[, 2] <- (0.25 * x^2) -1.5*x+1.25
}
# matplot(mu, type = 'l')

y <- array(0, dim = c(length(x), 2, 10))
errors <- array(0, dim = c(length(x), 2, 10))

if (error_type == "independent") {
  ## independent errors
  for (i in 1:2) {
    for (j in 1:10) {
      errors[, i, j] <- rnorm(6, 0, 0.25)
      y[, i, j] <- mu[, i] + errors[, i, j]
    }
  }
} else {
  for (i in 1:2) {     # number of treatments
    for (j in 1:10) {  # number of subjects
      # compound symmetry errors
      errors[, i, j] <- rmvn(1, rep(0, length(x)), 0.1 * diag(6) + 0.25 * matrix(1, 6, 6))
      y[, i, j] <- mu[, i] + errors[, i, j]
    }
  }
}


## subject random effects

## visualizing the difference between independent errors and compound symmetry
## why do we need to account for this -- overly confident inference


dimnames(y) <- list(time = x, treatment = 1:2, subject = 1:10)
dimnames(errors) <- list(time = x, treatment = 1:2, subject = 1:10)
dimnames(mu) <- list(time = x, treatment = 1:2)
dat <- as.data.frame.table(y, responseName = "y")
dat_errors <- as.data.frame.table(errors, responseName = "errors")
dat_mu <- as.data.frame.table(mu, responseName = "mu")
dat <- left_join(dat, dat_errors, by = c("time", "treatment", "subject"))
dat <- left_join(dat, dat_mu, by = c("time", "treatment"))
dat$time <- as.numeric(as.character(dat$time))
dat <- dat %>%
  mutate(subject = factor(paste(subject, treatment, sep = "-")))


## repeated measures ANOVA in R
fit_lm <- lm(y ~ time + treatment + time * treatment, data = dat)
dat$preds_lm <- predict(fit_lm)

fit_lme <- lme(y ~ treatment + time + treatment:time,
               data = dat,
               random = ~ 1 | subject,
               correlation = corCompSymm(form = ~ 1 | subject)
)
```

```
668
669
670   pred_dat <- expand.grid(
671     treatment = factor(1:2),
672     time = unique(dat$time)
673   )
674
675   dat$y_pred <- predict(fit_lme)
676
677
678   return(list(
679     dat = dat,
680     pred_dat = pred_dat,
681     fit_lm = fit_lm,
682     fit_lme = fit_lme
683
684   ))
685 }
686
687 plot_example <- function(sim_dat) {
688   library(patchwork)
689   ## Plot the simulated data
690   p1 <- sim_dat$dat %>%
691     ggplot(aes(x = time, y = y, group = treatment, color = treatment)) +
692     geom_point(show.legend=FALSE) +labs(y='response')+
693     geom_line(aes(x = time, y = mu, color = treatment),show.legend=FALSE) +
694     theme_classic() +
695     #ggtitle("Simulated data with true response function")+
696     theme(plot.title = element_text(size = 30,
697                                     face = "bold"),
698         text=element_text(size=30))+
699     scale_color_aaas()
700
701   p2 <- sim_dat$dat %>%
702     ggplot(aes(x = time, y = y, group = subject, color = treatment)) +
703     geom_line(aes(size = "Subjects"),
704               show.legend = FALSE) +
705     # facet_wrap(~ treatment) +
706     geom_line(aes(
707       x = time,
708       y = mu,
709       color = treatment,
710       size = "Simulated Truth"
711       ),
712       lty = 1,
713       show.legend = FALSE
714       ) +
715     labs(y='response')+
716     scale_size_manual(
717       name = "Type",
718       values=c("Subjects" = 0.5, "Simulated Truth" = 3)
719       ) +
720     #ggtitle("Simulated data\nIndividual responses with population mean") +
721     theme_classic()+
```

```
    theme(plot.title = element_text(size = 30,
                                    face = "bold"),
    text=element_text(size=30))+
   scale_color_aaas()

  p3 <- sim_dat$dat %>%
   ggplot(aes(x = time,
              y = errors,
              group = subject,
              color = treatment)) +
   geom_line(show.legend=FALSE) +
    labs(y='errors')+
    theme_classic()+
   # facet_wrap(~ treatment) +
   #ggtitle("Simulated errors") +
    theme(plot.title = element_text(size = 30,
                                    face = "bold"),
       text=element_text(size=30))+
   scale_color_aaas()

  p4 <- ggplot(sim_dat$dat,
               aes(x = time,
                   y = y,
                   color = treatment)) +
   geom_point()+
   labs(y='response')+
   geom_line(aes(
     y = predict(sim_dat$fit_lme),
     group = subject,
     size = "Subjects")
     ) +
   geom_line(data = sim_dat$pred_dat,
             aes(y = predict(sim_dat$fit_lme,
                             level = 0,
                             newdata = sim_dat$pred_dat),
                 size = "Population")) +
   scale_size_manual(name = "Predictions",
                     values=c("Subjects" = 0.5,
                              "Population" = 3)) +
   theme_classic() +
   #ggtitle("Fitted Model")+
   theme(plot.title = element_text(size = 30,
                                    face = "bold"),
       text=element_text(size=30))+
   scale_color_aaas()

  return((p1+p3+p2+p4)+plot_layout(nrow=1)+plot_annotation(tag_levels = 'A'))


}

txt<-18
A1<-plot_example(example(fun_type = "linear", error_type = "correlated"))
```

```
B1<-plot_example(example(fun_type = "linear", error_type = "independent"))

C1<-plot_example(example(fun_type = "quadratic", error_type = "correlated"))

D1<-plot_example(example(fun_type = "quadratic", error_type = "independent"))
```

```
## Error in eval(expr, envir, enclos): object 'A1' not found
```

For the quadratic response case, Figure **??** shows the simulated responses using compound symmetry and independent errors.

```
## Error in eval(expr, envir, enclos): object 'C1' not found
```