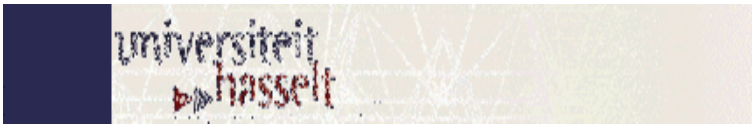


University Hasselt
Center for Statistics

The effect of physical training on maintenance and recovery of the functional muscular strength in rats injected with EAE

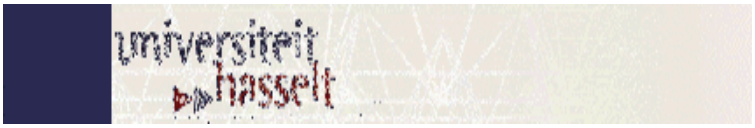
By
Ekwempe Ebwekoh Clifford

Internal Supervisor: Dr.Herbert Thijs

External Supervisor: Dr. Raf Meesen

Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Applied Statistics.

2005-2006



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ABSTRACT

Objective: The aim of this report was to study the effect of physical training on maintenance and recovery of the functional muscular strength in rats injected with Experimental Allergic Encephalomyelitis. For this effect 25 rats from different mothers and of the same gender were randomly selected. The 25 rats were again randomly assigned to two treatments groups. One group treated with EAE (13 rats) and the rest assigned to the control group (12 rats). Each treatment group was subdivided into two: swimmers and non swimmers.

The data revealed a longitudinal set up that is repeated measures were performed on each rat over time (25 days) and to have an idea of the trend of the average behavior of the population of rats, a mean profile was plotted considering the time spent on the Rotorot as the main response. The profile revealed a non constant pattern of the evolution trend. Further, to determine the co-variance structure, a variance function was plotted and it also revealed a non constant pattern suggesting and unstructured type.

Methodology: To capture the flexibility of the mean profile, a MACRO was implemented to generate fractional polynomial time variables. The obtained fractional time variables were used in PROC MIXED statement in SAS with the repeated option.

Conclusion: We found that on an average point of view, Experimental Allergic Encephalomyelitis (EAE) has an effect that is; rats that were subjected to EAE had a different evolution pattern over time as compared to the ones in the control group. We also discovered that physical (training) has an effect on the functional strength of the rats. According to the results, rats that were subjected to EAE and who were undergoing physical training spent more time on the Rotorot than rats that were not subjected to physical training.

Key Words: longitudinal data, Linear mixed model, covariance structure, Experimental Allergic Encephalomyelitis, variance plots and mean profile plots.

1. Introduction

1.1 Background

Experimental Allergic encephalomyelitis (EAE), and the related experimental autoimmune encephalomyelitis (EAE), are diseases of the brain and spinal cord, similar to MS (multiple Sclerosis), which are induced in laboratory animals. Animals with EAE serve as animal models for MS, allowing experimentation that would be impossible and unethical in humans who have the disease. Like MS (Multiple Sclerosis), EAE is a demyeleting disease. It destroys the myelin, the fatty sheath that protects and surrounds nerve fibers. EAE can be induced in rats, mice, guinea pigs, rabbits, and monkeys. Like MS, it takes several clinical forms, including relapsing-remitting and progressive-relapsing. EAE is induced in strains of lab animals that are susceptible to the disease injecting myelin or specific myelin proteins in combination with an immune-exciting agent, called an adjuvant (FCA).

Much of the current knowledge about the disease damage (pathology) and immune responses underlying MS has been gained from studies of animals with EAE. Information derived from microscopic and biochemical examination of the brain, blood and spinal cord of animals with EAE includes:

-Identifying sites in the central nervous system that are more likely to develop MS lesions-damaged areas also as plaques.

-Finding out which immune cell are involved in the formation of plaques and how they interact

-Developing experimental treatments or manipulations that can stop or reverse the demyelinating process.

At least two of the currently available MS therapies—mitoxantrone (Novantrone) and glatiramer acetate (Copaxone) were developed because of promising findings in mice with EAE. Ongoing studies of EAE in the laboratory will continue to be an important early step in the development of new therapies for MS. Muscle weakness and fatigue are important symptoms in patient with multiple sclerosis. During the disease damage and recovery occur both simultaneously and

serially. The therapeutic possibilities for MS are limited and new approaches to influence the balance between damage and recovery are needed. This is not only true for the demyelating effect but also true for the muscle weakness resulting from the pathological events in the central nervous system.

Like multiple sclerosis, EAE has a mode of action (damage and recovery) it reacts on the induced rats and after a period of time, if the dose administered is relatively not high, these rats recover progressively in the other hand if the dose is consistent the induced rats will have their limbs paralyzed then if the severity of the dose persist will have all of the body paralyzed and this might lead to the death of the rats. EAE (Experimental allergic encephalomyelitis) serves as an animal model for certain neuroinflamatory diseases of the central nervous system in particular multiple sclerosis. Multiple sclerosis is a dimyelinating disease of the central nervous system that can result in impaired muscle function leading to weakness, fatigue. Decreased ambulatory ability EAE in animals (rats) is like multiple sclerosis in human being accompanied by transient weakness or paralysis of hind limbs.

Regular physical activity provides enormous health benefits in human beings. It helps reduce heart disease, cancer and many other diseases and metabolic conditions. Regular fitness exercise is also highly beneficial for weight reduction and weight maintenance, and may improve brain chemistry to reduce depression. By contrast, health studies that have monitored the wellbeing of large groups of people over many years clearly show that inactivity significantly increases the risk of overweight, obesity and chronic diseases. Inactivity consistently influences the state of skeletal muscles in human being and animals. Non physical status leads to the decrease of performance and in case of any effort, the subject gets tired and is subject to body weakness.

1.2 Material and Methods

This study was carried out on 25 Lewis rats coming from different mothers each and all of them female and of the same age. They are raised and supported in the animalium of the University of Hasselt during a constant day/night (12h/12h). Accordingly after the induction of EAE, several measures were carried out during the recovery dual phase consisting of the paralytic and recovery episode of the induced rats and also at the same time those measures carried out on the control rats. After being administered on rats, EAE react on the skeletal muscles of the induced rats by

paralyzing them and has a length of reaction time on the skeletal muscles and if the induced rats succeed to support the substance, the EAE effects on the skeletal muscle disappears progressively. More also the study was interested on the effect of physical training on the treatment status (induced by EAE or not) of the rats.

Each experimental group was subdivided into two sub groups. In the first group the rats in this group were in a sort of sedentary state (no physical activity) and in the second group, the rats were subjected to physical training (swimming). Swimming activity was performed every day during one hour in water of 34°C with a weight on their tail of 2% of their body weight. Every day the body weight, the neurogical characteristics were recorded. Also each day all the rats were set on a Rotorot, this apparatus turns on a frequency of 15 turns/min.

1.3 Objective of the study

This study was to compare firstly on a general scale between two groups of rats. One group that was induced by EAE (treatment group) and the second group that was not induced by EAE (control group). On a specific note, this study also was to compare the effect of physical training on rats been induced by Experimental Allergic Encephalomyelitis that is the treatment group.

1.4 Structure of the report

The report begins with an introduction in which some information is given on Experimental Allergic Encephalomyelitis in the background section relating its relation to Multiple sclerosis in human being. This section also includes information on the contribution of physical training on skeletal muscles. This is then followed by the objective of the study. In the second section of the report, we give a brief description of the data set and of the experimental settings. The third section contains the methodology utilized to carry out investigation on the data set. The results of the investigation are shown in the fourth section of our report. The report ends with the fifth part with the discussions and conclusions and also some recommendations. Lastly we have the references follow by the appendix. We used S-plus and SAS software.

2. Data Description

2.1 Emplacement and experimental settings

The experiment took place in the laboratories of University of Hasselt. The rats (25 Lewis rats) were raised and supported in the annimalium of the University of Hasselt during a constant day/night (12h/12h) cycle and a temperature of 22° C. They are maintained in individual cages. The study is approved by aesthetic laboratory animals committee of the University of Hasselt.
The 25 rats were divided into two groups: treatment group and Control group. 13 rats that were injected by EAE were considered to belong to the treatment group while the remaining twelve rats formed the control group. Still in the experimental design each of the two groups (treatment group and control group) were subdivided into two subgroups, a sub group consisted of swimming rats and the other subgroup consisted of non swimmers. EAE was administered 7 days after the rats entered the study. Also for the sake of habituation, rats were subject to swim one week before entering the study.

Having put in place the above experimental setting, the body weight of each rat was measured each day during the entire study period. Also every day, for each rat, the time spent on the Rotorot was measured. They have to stay maximal 3 minutes on it. Each day rats are placed on the Rotorot 5 times with a maximum time of 3 minutes. The average time of the five daily measures is determined every day till the end of the study.

2.2 Variable description

Table 1: variable Description

Variables name	Description
ROT	Time spent on the Rotorot by each rat.
WEIGHT	Weight measured on each rat every day.
SCORES	Description of the state of paralysis of the rats.
TREATMENT	Designating if a rat was induced by EAE. If a rat was induced by EAE we assigned the value 1 if not we assigned the value 2.
SWIM	Designate if a rat was swimming or not. If a rat was swimming, we assigned the value 1 if not we assigned 0.
TIMEC	Time points in which each measurement was carried out.

2.3 Data set

The dataset results from a longitudinal observation because of repeated measures carried on each rat. It consists of 625 observations obtained from 25 Lewis rats. The time spent on Rotorot was measured on each of these rats during 25 days (time points) and was considered as the main response variable. Also the weight of each rat was measured during these 25 days. Other variables included swimming was coded as a binary indicator for being in swimming group as 1 and as being in none swimming group as 0. Another binary variable recorded was treatment coded as 1 for rats been induced by EAE and 2 for rats in the control group. We also have as variable the variable scores which is a categorical variable determining the state of paralysis of the rat. This categorical variable has values fluctuating between 0-3.5.

3. Methodology

Each rat is measured repeatedly every day over the entire period of the study (25 days). The principal variable of interest (time spent on the Rotorot), the response variable, is measured every day for the two groups of treatment (treatment and control). This is a typical setting of a longitudinal data.

A longitudinal study is defined as a study in which the response for each experimental unit in the study is observed on two or more occasions. The defining feature of a longitudinal data set is repeated observations on experimental units over time. Longitudinal data require special statistical methods because the set of observations on each subject tends to be correlated. These correlations must be taken into account to draw valid inferences. One of the statistical methods commonly known to handle this type of data, for normal responses, is the Linear Mixed-effects Models (LMM).

3.1 Exploratory Data Analysis (EDA)

It is usually very useful as a first step in analysis, to do some data exploration in order to get an insight of the data. In this step, various graphical plots were used to explore the mean, variance and correlation structures. This exploration of the data it should be noted was done through a series of graphical plots. The average evolution of the two groups of rats was plotted to have a view of how apparently they behave over time and by so doing comparing between the two groups of treatments over time and also to investigate if the average trend is linear or non linear. This exploratory data analysis was done to choose the variance structure of the model to handle the data.

Exploration data analysis was also done through plots like box plot, scatter plots to help have a knowledge on how the two treatment groups differ and to know the relation between variables. The latter was helpful to choose as response between weight and time spent on the Rotorot (Rot). A scatter plot reveals no apparently relation between Rot and weight and from this revelation; it was possible to choose Rot alone as the principal response of interest. Pearson correlation

coefficient helped us to have an idea of the relation between the principal response of interest and the covariates.

3.2 Linear Mixed Model

Many statistical techniques are encountered and are used in the statistical world to handle data set we can name techniques like; Analysis of Variance (Analysis of Variance), Regression, Principal components, Multivariate Analysis of Variance…The principal assumptions of these techniques is that the observations are independent; they don't take into account the correlated aspect of observations. The Linear Mixed Models (LMM) is an exceptional case in this direction. They consider the correlation among observations; there fore they appear to be recommendable to handle the present data set which is a longitudinal data set. In this model the 25 measures on the 25 time points were taken into account and the correlation between the observations as well.

The linear Mixed models (LMM) when modeling uses the response and all covariates in the data set.

The general formula of the linear Mixed models (LMM) has the following structure [3];

$$Y_i = X_i\beta + Z_i b_i + \varepsilon_i$$

Y_i is a vector of repeated measures

X_i is a matrix of covariates

β is a vector of regression parameters

$Z_i = n_i \times r$ design matrix for random effect

b_i is the vector of the subject specific regression parameters, $b_i \sim N(0,\mathrm{D})$

ε_i is vector of error components, $\varepsilon_i \sim N(0, \sum)$

$b_i,...,b_N, \varepsilon_1,...,\varepsilon_N$ are independent

Our main interest in this study is on the on the population level, we are interested on the evolution of the population over time. The individual evolution of each rat in the two groups of treatment is not of primary interest. The implication of this is that the above Linear Mixed Models (LMM) will be reduced to the following model [3];

$$Y_i = X_i\beta + \varepsilon_i \quad \text{(I)}$$

Y_i is a vector of repeated measures

X_i is a matrix of covariates

β is a vector of regression parameters

ε_i is vector of error components, $\varepsilon_i \sim N(0, \sum)$.

The relationship between a response variable and one or more continuous covariates is often curved. In this study the mean profile of the average time spent on the Rotorot by treatment over time yielded a non linear pattern (Fig.4). Also attempts to represent curvature in single or multiple regression models are usually made by means of polynomials of the covariates, typically quadratics. However, low order polynomials offer a limited family of shapes, and high order polynomials may fit poorly at the extreme values of the covariates. Fractional polynomial was proposed as a solution to this ambiguous situation by Royston and Altman (1994).

Fractional polynomial is a family of curves, whose power terms are restricted to a small predefined set of integer and non-integer values. The powers are selected so that conventional polynomials are a subset of the family. Regression models using fractional polynomials appear to show to have considerable flexibility and are straightforward to fit using standard methods.

Formally, Royston and Altman (1994) define a fractional polynomial as any function of the form [1].

$$f(u) = \phi_o + \sum_{k=1}^{m} \phi_{k x^{(p^k)}}$$

Where the degree m if a positive integers, where $p_{1<\ldots<}p_2$ are real prespecified powers, and where ϕ_o and $\phi_1, \ldots, \phi_1$ are real-valued regression coefficients. Finally, $x^{(pk)}$ is defined as:

$$x^{(pk)} = \begin{cases} x^{pk} & if\ pk \neq 0 \\ \ln(x)\, if\ pk = 0 \end{cases}$$

Thus, not only the conventional powers $x\, x^2 \ldots$ are allowable, also $\ln(x), \sqrt{x}$ (for $pk = 0.5$), $\frac{1}{x}$ (for $pk = -1$), etc.

A fractional polynomial approach was used to determine X_i in the above relation (I)

To generate pk represented here by X_i we used a macro of fractional polynomials then later, we fitted a mixed model with repeated statement with these fractional polynomials. This macro contains the procedure to estimate the parameters 'p1= X_i ' and 'p2= X_i ' for a fractional polynomial of order 2. The macro gives the possibility of using mixed effects model or fixed effect model. The macro is specified as follow:

%macro fp2(data=, resp=,indep=,subj=,rand,pi1=,pi2,step,n=,outfile=,var=,value=)

Where data option allows specifying the name of the dataset.

The option 'resp' permits to specify the name of the response variable.

The 'indep'macro variable offer the possibility of specifying the name of the time variable to use.

'subj' option is to specify in case usage of a mixed effect model: 'subj' contains the level associated to the random effects.

'pi1' and 'pi2' contain the lowest values to use for the data.

'Step' option allows to specify the step between 2 consecutive 'pi1' or 'pi2'.

'n' contains the maximum (pi1) – minimum ('pi1'))/'step'+1.

Setting rand=4 fits a mixed model with only repeated statement (no random effects) using unstructured covariance matrix.

3.3 Model Selection

To come out with a model, we first determine the covariance structure to know which structure to work with. For this effect the unstructured covariance structure was chosen. After determining the covariance structure, and based on the shape of the mean profile, fractional polynomials approach was performed to determine the minimum and maximum power (integers and fractional) to be used in the multiple regression model.

The time covariate with the minimum and maximum values were used in the Linear Mixed Models and a Likelihood ratio test was performed on this model which was implemented in the

SAS software by the PROC MIXED statement with the repeated option because of the objective of the study and the repeated measures over specific units and over time. The result of the Likelihood ratio test yields a parsimonious model, which was in fact the final model we considered for the subsequent analysis.

3.4 Covariance structure

The modeling of longitudinal data with repeated measures on specific units over time implies these measures are correlated and there exist statistical modeling techniques that take this aspect into account. Therefore fitting a model with the genuine correlation structure will yield valid results and acceptable inference. To come out with the correct covariance structure, tools like variance plot, correlation coefficients and likelihood ratio test were used.

These tools suggested the covariance structure to be unstructured. Unstructured implies that the variance and co-variances may be different for each time points, respectively.

More, the fact that our variance function yields a non constant plot suggest the co-variance structure to be unstructured.

3.5 Mean Structure

The fixed effects of our final model consist of the following covariates: treatment, swimming, the interaction treatment and time. This model was tested to investigate the difference between the two treatment groups. The general profile shows that at the beginning of the study, the two groups of rats have different starting point. Although at that particular time the rats belonging to the two groups of rats are similar in the sense that they were no induction at that particular time. At the beginning of the study, no rat is induced. At the 8^{th} day of the study (the induction day) the two groups have approximately the same behavior. We can separate this study in two parts: The part before induction and the part after induction.

4. Results

4.1 Exploratory data analysis

4.1.1 Correlation structure

Spearmen correlation was use to determine the possible linear relationship between the covariates of the data set (table2). It was found that the variable Rot (Time spent on the Rotorot) and weight had a moderate coefficient of correlation (0.407). While a relatively small correlation between weight and the time point variable (0.041) and lastly a negative relationship between the variable of the time spent on the Rotorot and the variable indicating the time point (-0.339).

Table 2 Spearman correlation coefficient

Variables	Rot	Weight	Timec
Rot	1.00		
Weight	0.407	1.00	
Timec	-0.339	0.041	1.00

The scatter plot between Rot (time spent on the Rotorot) and the Weight of each rat revealed an apparently not a clear relationship between the two variables (fig.1).

This is one of the motivations to choose weight as a covariate and not to consider it like a dependent variable as Rot (time spent on the Rototot) though the two variables were measured on the same animals and therefore could be correlated.

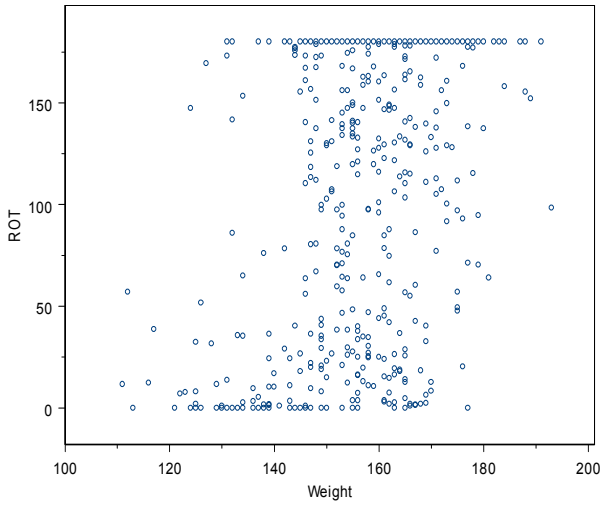

Figure 1: Scatter plot of Rot Weight

According to this figure (Fig.2) it appears to be a difference between the two treatments. Apparently, Control group which is not subject to any treatment presents a mean superior to the one of treatment group, Surprisingly Rats who were induced by EAE, have values from the minimum time to the maximum of time spent on the Rotorot.

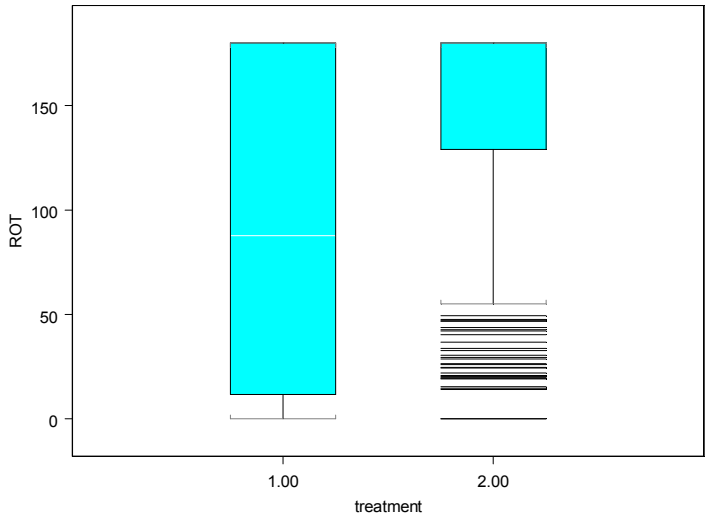

Figure 2: Box-plot of the time spent on the Rotorot by treatment (EAE group and control group)

4.1.2 Variance structure

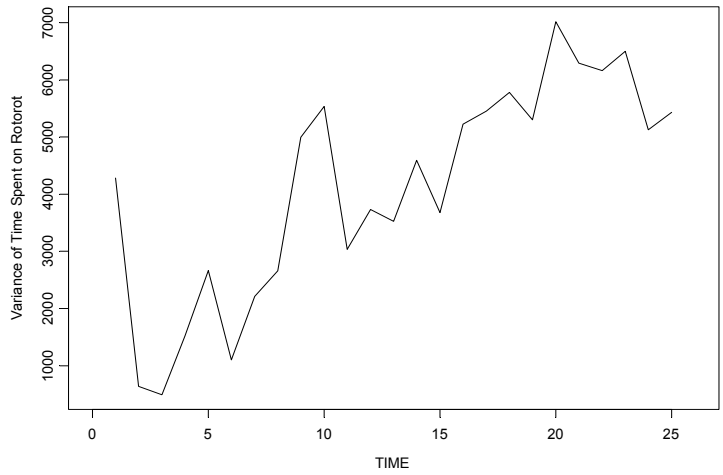

Figure 3: Variance function of the overall mean of Rot by time

The entire study period is 25 days (25 time points) and as mentioned in the mean structure, days before the induction are considered to be similar in both treatments groups. As from the 8th day (induction day) the two treatments groups presents some dissimilarities. But on an average scale we observe that the variance is not constant between time points. The variance plot between time points generally increases, and decreases in a non constant way.

Therefore these observations call for an unstructured co-variance structure because of the overall pattern of the variance structure which is not constant over the time of study.

4.1.3 Mean profiles

To gain insight of the average evolution over time of the two treatment groups (induced group by EAE and the control group), and to give an attempt of answer to the research question, different mean profiles were plotted. It is in this direction that the mean profile of the mean time spent on the Rotorot over the time by treatment (Fig.4) was plotted. Also the mean time spent on the Rotorot over the time of study by swimming (Fig.5) and non swimming groups was plotted. Finally to have an idea of the effect of physical training on the treatment status of the rat, the interaction swimming and treatment was considered and the mean profiles of the mean time spent on the Rotorot by this interaction over the time and by the four sub-groups formed from this interaction were plotted (Fig.6).

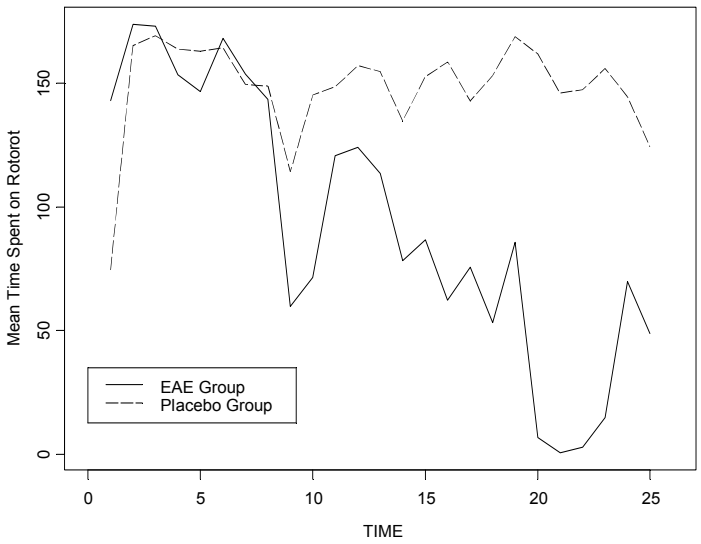

Figure 4: Mean profile of the mean of Rot over time by treatment

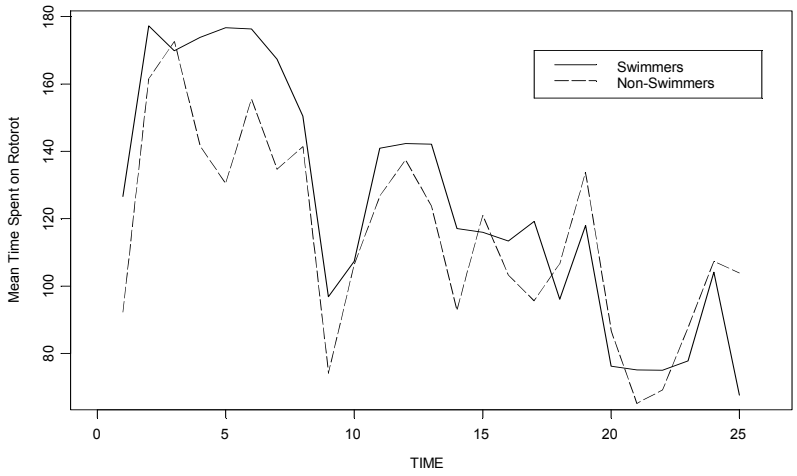

Figure 5: Mean profile of mean Rot over time by swimming group

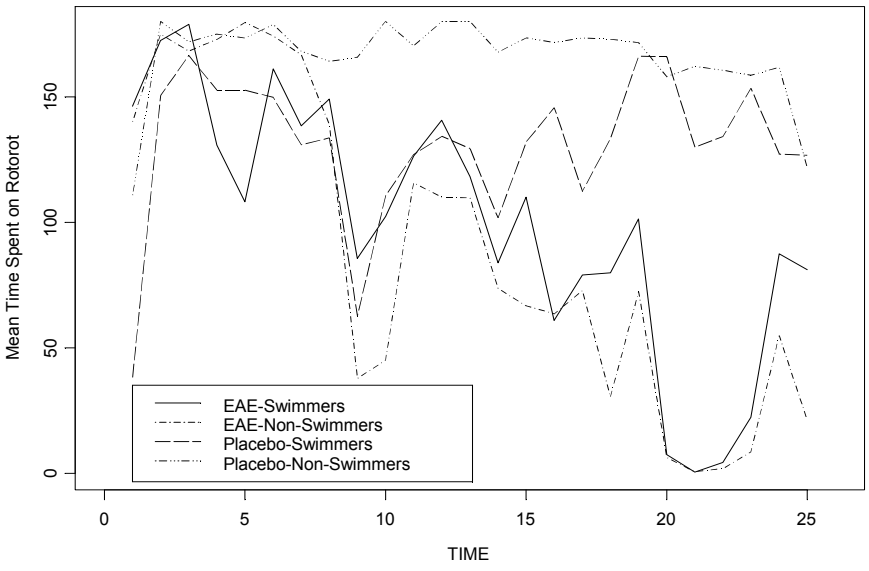

Figure 6: Mean profile of Mean Rot over time by treatment and swimming

According to these mean profiles it appears that if we consider from the induction day, treatment on an overall point of view has an effect. In other words, there is a difference in treatment between the group treated with experimental allergic encephalomyelitis and the control group. According to this figure (Fig.4), difference in treatment occurs few days after the induction and this seems to persist till the end of the study.

Considering the physical training (swimming) we see through the mean profile of the mean of Rot by swimming groups (swimmers and non swimmers) there seems to be a considerable slight difference between swimmers and non-swimmers. Further investigation to confirm these observations will be carried out in the statistical analysis section.

The main point of the research question was to investigate if physical training has effect on skeletal muscles been induced by Experimental Allergic Encephalomyelitis (EAE). We created the four following groups (EAE-swimmers, EAE-non swimmers, Placebo-swimmers and Placebo-non swimmers). And when taking EAE-swimmers and EAE-non swimmers into account, we observe they present a slight difference: they have an almost similar shape. Also the same observation goes with the Placebo-swimmers and Placebo-non swimmers. From this observations, we can say that swimming has no great impact on rats been injected by EAE and on rats in the control group. Though in the Placebo group, there seems to be an apparent swimming effect after the induction of EAE. But this relatively small difference disappears after some times. All these revelations have to be confirmed in our up coming statistical analysis.

4.1.4 Exploitation of the results of the exploratory data analysis

There is an important implication of the Explanatory data Analysis, it reveals that before the induction of EAE to the treatment group, the two groups were similar though they have on an average different starting point this could be due to problem of randomization during the study. After the treatment that is after the induction day, there is an apparent difference between the treatment groups (EAE and Control groups). There is also information that physical training seems to have no effect. There is no precise difference between Swimmers and non swimmers. This observation has to be proven statistically that is we need to investigate really if the variable swim is not significant.

The mean profile suggest that physical training don’t have a strong effect on skeletal muscles (though) they exist a slight difference between for example EAE-swimmers and EAE non swimmers. In the Control group, there is a difference between control-swimmers and control non

swimmers a couple of days after induction and these apparent differences persist till almost the end of the study.

From these observations, we can say that once a rat was induced by EAE and was not subjected to physical training, it spends averagely less time on the Rotorot than a rat induced with EAE and that was subjected to physical activity (swimming). The same observations hold for the control group. Rats that belong to the control group when swimming reveal to spend averagely more time on the Rotorot than those who were not swimming.

4.1.5 Motivations of the choice of the covariance structure

The mean profile revealed a non linear trend over time of the mean time spent on the Rotorot. More also the variance function was not constant over time these revelations suggest to choose a model which would account for these observations: the longitudinal aspect of the data called for the PROC MIXED statement with repeated option and the unstructured was chosen like the covariance structure: unstructured implies that the variances and co-variances may be different for each time point and pair of time points, respectively.

4.1.6 Determining the fractional polynomials

Knowing the co-variance structure to be unstructured, and given that the mean profile revealed a non linear trend over time, we had to come out with the ideal power of the time variable. Low order polynomials offer a limited family of shapes, and high order polynomials may fit poorly at the extreme values of the covariates. Fractional polynomial was therefore proposed as a solution to this ambiguous situation by Royston and Altman (1994). To this effect, we used a MACRO to generate the desire power of the time. We came out with two variables varfpp1 and varfpp2 representing respectively the minimum and the maximum fractional powers of time. These new variables were later used in the PROC MIXED model with the repeated statement option.

4.2 Modeling the results

We fitted the time spent Rotorot (Rot) as the main response of interest because all of the rats participating in the study had that characteristics and considering this variable as the main respond will permit us modeling taking into account measurements carried out on all rats. In other words

all rats had the time spent on the Rotorot. With this considerations, a univariate marginal-effects models were first fitted to the main response of interest (time spent on the Rotorot) with all covariates (fixed effect) including the fractional time powers gotten from the fractional polynomials in the model.

From this saturated model, we proceed to the reduction of the model and through the Likelihood Ratio Test, we compared each nested model and we arrived at a parsimonious model (reduced model) including all significant interactions.

The table below gives the parameters estimates of the final model together with their standard error and their significance level.

Table 3 : Parameters estimates(standard errors, p-value)

Effect	Estimate	StdErr	Probt
Intercept	167.740	34.416	<.0001
Varfp1	-40.649	441.509	<.0001
Varfp2	36.696	450.949	<.0001
Treatment	-80.016	46.951	<.0001
Swim	-26.529	47.194	<.0004
Varfp1*Treatment	-24.826	612.263	<.0001
Varfp2*Treatment	19.468	625.354	0.0020
Treatment*Swim	29.907	65.539	<.0001

According to table 3, the new time variables, varfp1 and varfp2 generated by the MACRO and then after fitted in the PROC MIXED statements without the repeating options are significant. But since these time variables are present in the interactions, our interpretations will be focused on the interactions. Also the other main effects here: treatment and swim will not really serve us in our interpretation. To gain a satisfactory interpretation, our analyses have to be focused in the interactions.

The interaction between varfp1 (time) and treatment is very significant (p<.0001); we can say there is a difference in treatment between the treatment groups (EAE group and Control group) over time and the estimate is negative which suggest that the mean time spent on the Rotorot is decreasing over time. For this group of rats over the period of study, treatment has an effect on the skeletal muscles: It is proven scientifically; rats that are induced with EAE after some time, show signs of paralysis of the limbs.
When comparing rats induced by EAE and control group the mean profile is revealing that the control group (group not administered with EAE) has its curve superior to the curve of the EAE group. Which means that Experimental Allergic has an effect on the skeletal muscles of rats?
The same observations and interpretations hold for the interaction between the time variable and treatment.

The last interaction that is the interaction between treatment and swim (physical training) and which is the main question of interest of the research question. This interaction is highly significant (<.0001).
From the mean profile (Fig.5) we observe that EAE-swimmers and EAE-non swimmers present on average slightly the same trend of evolution over time. We can divide this profile into two groups: first at the beginning of the study the EAE-non swimmers group spent more time on the Rotorot than EAE-swimmers. This is quite understandable because at the early stage of the study (first 8 days), none of the rat is subjected to a particular treatment. So in this interval it is possible that a group of rats present a time on the Rotorot higher than another group. The second part of the mean profile shows that EAE-non swimmers present less time spent on the Rotorot than EAE-swimmers. Which in other words means that physical training has an effect on rats induced by Experimental Allergic Encephalomyelitis? These observations could lead to the interpretation that induced rats through physical training are capable to fight against the paralytic effect of the EAE. Indeed physical training has an effect on Experimental Allergic Encephalomyelitis.

4.3 Predictions

To see if our model fits well, we plotted the predicted mean profile of the average time spent on the Rotorot over time by treatment on the same plot (Fig.7).

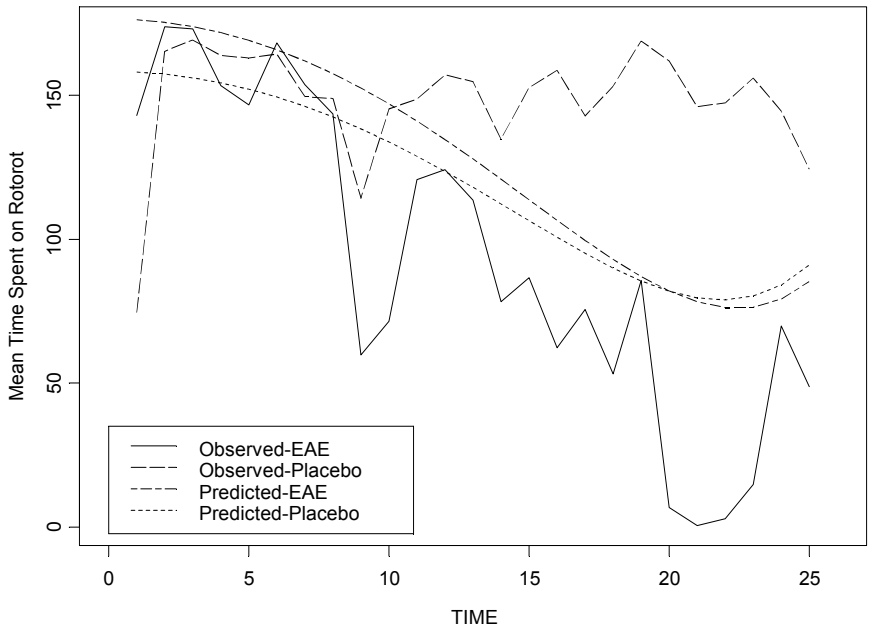

Figure 7: observed and predicted mean profile

Since there is no formal test to test if our final model fits well we opted for an alternative which was to compare the observed mean profile and the predicted mean profile. According to these profiles, there are no similarities between the EAE observed and EAE predicted. Also there exist a difference between a control (Placebo) observed and control predicted (Fig.6). These observations lead to the conclusion that our model did not fit well the data and there fore will not be a tool to carry out valid statistical inferences for subsequent analysis.

5. Discussions

In our attempt to answer the research question which was globally to state if there was difference between rats that were induced by Experimental Allergic Encephalomyetis and another group of rats been the control group and due to the repeated measures carried out on each during the entire period of the study. We implemented statistical methodology dealing with this type of data (longitudinal data) and with respect of the just mentioned characteristics; we used the PROC MIXED statements in SAS. One of the main characteristics of the PROC MIXED is: it assumes normality and it is robust against non normality and more also there were small number of rats participating in the experiment (25 rats) and will not be rewarding making inference base on the analysis of such data set.

For modeling, the need to specify the covariance structure of the model was crucial because of the correlated observations of the data set: to account for this a covariance structure was determined through the mean profiles and variance function. The latter profiles indicated a non constant pattern over time of the general trend which indicates that the co-variance matrix had different off diagonal and diagonal and the covariance structure with these characteristics is the unstructured co-variance.

The study was carried during 25 days and during this study we had a common time where in both treatments group they were no administration of Experimental Allergic Encephalomyelitis (EAE). Then after one week EAE was administered to one group then called the EAE group and the remaining group was then termed the control group. Since our interest was to determine the effect of physical training on maintenance and recovery of the functional muscular strength in rats injected with EAE. To determine the effect of EAE we focused our investigation from the day EAE was administered till the end of the period of study in both treatment groups (EAE group and control group).

Due to the fact that our time is positive, a transformation here will instead give an effect of shrinkage without really linearizing the mean profile. Also we observe that the mean profile of the average time spent on the Rotorot by treatment against time present a non linear trend for both treatments. We should however note that the control group presents an averagely linear evolution

over time. So to capture the flexibility revealed by the profiles of the two treatment groups, we had to generate fractional polynomials to take into account the average pattern offer by the mean profiles and more also will consider integers and non-integers powers.

To be able to determine the effect of EAE on skeletal muscles that are been induced by EAE, we took the entire data and modeled taking the time spent on the Rotorot as the main response and compare the time spent on the Rotorot for the group induced by Experimental Allergic Encephalomyelitis (EAE) and the time spent on the Rotorot by the non induced group

The research question was to investigate on the effect of physical training (variable swim) on skeletal muscle. This was done by investigating on a general note the effect of physical training considering swimmers and non swimmers. We found that according to the mean profile, there seems to be a difference between swimmers and non swimmers irrespective of the swimming group: these observations were confirmed by the p-value of the variable swim (<0.0004) which suggest there is difference between swimmers and non swimmers on a general scale. That irrespective of the treatment group (EAE or control group) there is an effect of physical training on the skeletal muscles of the rats on a general note over time.

When taken the treatment status into account, the mean profiles (Fig.6), averagely, there is a slight difference while considering the interaction treatment*swim. In fact there are four subgroups yielded by the just mentioned variables (EAE-swimmers, EAE-non swimmers, Control-swimmers, control-non swimmers). Here although the difference exist it was not really clear between the two sub groups composing each treatment group. That is a clear difference did not exist between for example EAE-swimmers and EAE-non-swimmers. They follow almost the same evolution over time. The same trend was noticed in the control group. It should be noted that the evolution of the combination of the treatment and swim was on a general note not really different but the p-value reveals that the combination treatment with the physical activity is very significant (<0.0001). As an interpretation of this, we can say that in both treatment groups, swimming has an effect: that is in the group of rats induced by Experimental Allergic Encephalomyelitis for example, there is a difference in the mean time spent on the Rotorot between swimmers and non-swimmers. The same interpretation holds for the control group (group none injected with the EAE substance).

6. Conclusion

The data set submitted to our investigation was observed from two groups of rats composed of a group with treatment and a group as control. The observations are recorded from rats from different mothers and all of the rats are of the same sex (female). Measures were recorded on a period of study of 25 days and the observations were correlated. The data set through the mean profile we can say suffered from certain problem such as the problem of randomization. We see through the mean profile that on an average scale the groups of rats of the treated and control group though at the beginning of the study (first week of the study) similar present different intercepts. This observation could be explained by the problem of randomization.

Because of the fact that the scores of the rats were only assigned to the rats been induced by EAE we fitted a general model where the time spent on the Rotorot was considered as the main response: this was done because we would like to carry out investigations basing our analysis on a common characteristics: the time spent on the Rotorot was measured in all the rats in the study which is not the case with the variable scores which was measured only on rats that were induced with EAE. So taking scores like the response will not take into account the other groups that were not assigned with Experimental Allergic Encephalomyelitis.

Due to the non linearity of the mean profile of the two treatment groups when considering the time spent on the Rotorot as the main response of interest, and to fit a model that will take into account the average trend of the population which was far from being linear. For that essence, a fractional polynomial was implemented to determine the precise power of the time variable. Fitting this model, it was not converging we came out with a macro (see appendix) which permitted us to estimate the parameters ‘p1’ and ‘p2’ representing respectively Varfp1 and Varfp2 in our case for a fractional polynomial of order 2. The macro gives the possibility of using mixed effects model or fixed effects model. The macro variables obtain from this macro ‘Varfp1’ and ‘Varfp2’ contained the lowest values used for our data (model). Using these variables in our PROC MIXED the model with the co-variance structure which was chosen with the repeated statement, our model converged and the macro variables were then used in the model and also interactions with the other main variables.

We came to the conclusion that according to this model that effectively there exist a difference between rats been induced by EAE and rats which served as control group. Also that the physical training has an effect on the skeletal muscles of rats. That is rats that were swimming and induced with Experimental Allergic Encephalomyelitis (EAE) spent much time on the Rotorot than rats that were not swimming and induced by EAE and found that the later group spent more time on the Rotorot than those that were induced by EAE. Further to investigate the effect of EAE on muscles we confronted the observations taken from EAE-swimmers and EAE-non swimmers. This investigation revealed that there was a difference between the two sub-groups induced by Experimental Allergic Encephalomyelitis. That is the sub-group of rats induced with EAE and who swam presented on an average scale more time on the Rotorot that the EAE-non swimmers.

Stating from these above observations that Experimental Allergic Encephalomyelitis hinders the Rats through their muscles also that even when induced with Experimental Allergic Encephalomyelitis, rats which performed physical exercise spent more time on the Rotorot than sedentary rats which were injected with EAE revealing to us that physical training could suppress the effect of EAE on the long run.

Also due to randomization, and treatment allocation, short comings in the experimental design; complete effective analysis of the data using the EAE scores could not be performed. The EAE scores were only allocated for EAE group and hence it was not validly feasible to compare the EAE scores for both groups (treated and control).

7. Reference

[1] Molenberghs. G. & Verbeke, G. (2005). *Models for Discrete Longitudinal Data.* Springer Series in statistics. New-York: Springer-verlag

[2]Verbeke, G. and Molenberghs, G. (2006) *Lecture Notes in Correlated and Multivariate data,* for Applied Statistics Uhasselt press.

[3] G. Verbeke, G. Molenberghs (2000) *Linear Mixed Model for Longitudinal Data Springer Series in Statistics*. New York: Springer-Verlag.

[4] http://www.nationalmssociety.org/Sourcebook-EAE.asphttp

[5] http://www.ncbi.nlm.nih.gov/entrez/query.fcgi

8. Appendix

```
%macro fp2(data=,resp=,indep=,subj=,rand=,pi1=,pi2=,step=,n=,outfile=,var=,value=);

data &outfile; set _null_;
run;
%do i=1 %to &n;
 %do j=&i %to &n;
  ods listing close;
  run;

  %let p1=(&pi1+&step*(&i-1));
  %let p2=(&pi2+&step*(&j-1));
  %put p1 = &p1 and p2 = &p2;

   data fracpol; set &data;
    hx1=&indep**&p1;
    if &p1=0 then hx1=log(&indep);
    varfp1=hx1;
    hx2=&indep**&p2;
    if &p2=0 then hx2=log(&indep);
    if &p1=&p2 then hx2=hx1*log(&indep);
    varfp2=hx2;
    pbmthcls=&indep;
            if &var=&value;
   run;
   PROC STANDARD DATA=fracpol MEAN=0 STD=1 OUT=zfracpol;
    VAR varfp1 varfp2 ;
   RUN;
   proc sort data=zfracpol;
   by &subj;
   run;

  %if &rand=0 %then %do;
   proc mixed data=zfracpol method=ml ic covtest update noinfo scoring=5 noclprint maxiter=200;
    id &subj &indep;
    class &subj pbmthcls;
    model &resp = varfp1 varfp2/ s outpm=predm;
            ods output fitstatistics=fit;
   run;
  %end;
 %if &rand=1 %then %do;
   proc mixed data=zfracpol method=ml ic covtest update noinfo scoring=5 noclprint maxiter=200;
    id &subj &indep;
    class &subj pbmthcls;
    model &resp = varfp1 varfp2/ s outpm=predm;
    random int  / sub=&subj type=un;
            ods output fitstatistics=fit pred=p;
   run;
  %end;
   %if &rand=2 %then %do;
   proc mixed data=zfracpol method=ml ic covtest update noinfo scoring=5 noclprint maxiter=200;
    id &subj &indep;
    class &subj pbmthcls;
```

```
 model &resp = varfp1 varfp2/ s outpm=predm;
 random int varfp1 / sub=&subj type=un;
        ods output fitstatistics=fit pred=p;
run;
%end;
 %if &rand=3 %then %do;
proc mixed data=zfracpol method=ml ic covtest update noinfo scoring=5 noclprint maxiter=200;
 id &subj &indep;
 class &subj pbmthcls;
 model &resp = varfp1 varfp2/ s outpm=predm;
 random int varfp1 varfp2 / sub=&subj type=un;
        ods output fitstatistics=fit pred=p;
run;
%end;
 %if &rand=4 %then %do;
proc mixed data=zfracpol method=ml ic covtest update noinfo scoring=5 noclprint maxiter=200;
 id &subj &indep;
 class &subj pbmthcls;
 model &resp = varfp1 varfp2/ s outpm=predm;
 repeated &indep / sub=&subj type=un;
        ods output fitstatistics=fit pred=p;
run;
%end;
   %if &rand=5 %then %do;
proc mixed data=zfracpol method=ml ic covtest update noinfo scoring=5 noclprint maxiter=200;
 id &subj &indep;
 class &subj pbmthcls;
 model &resp = varfp1 varfp2/ s outpm=predm;
 repeated &indep / sub=&subj type=cs;
        ods output fitstatistics=fit pred=p;
run;
%end;
proc transpose data=fit out=fit1;
run;
data fit; set fit1;
 keep p1 p2 Loglik AIC AICC BIC;
  p1=&p1;
  p2=&p2;
  LogLik=col1;
  AIC=col2;
  AICC=col3;
  BIC=col4;
run;
 data &outfile; set &outfile fit;
run;


proc sort data=&outfile;
by AIC;
run;
%end;
%end;
ods listing;
run;

proc iml;
```

```
 d=j(45,6,0);
 d1=j(1,2,0);
 use &outfile;
 read all  into d;
 d1=(d[1,1]||d[1,2]);
 names={'p1','p2'};
create trial123 from d1(|colname=names|) ;
                              append from d1;
            quit;


            proc sql;
             create table newdata as
             select * from &data, trial123;
             quit;

  data newdata; set newdata;
       hx1=&indep**p1;
   if p1=0 then hx1=log(&indep);
   varfp1=hx1;
   hx2=&indep**p2;
   if p2=0 then hx2=log(&indep);
   if p1=p2 then hx2=hx1*log(&indep);
   varfp2=hx2;
   pbmthcls=&indep;
            run;

            %mend;
```

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