LONGITUDINAL STUDIES: REPEATED MEASUREMENTS AND TRENDS ON BIOMEDICAL DATA

Summary of Ph.D. Thesis

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LIST OF PAPERS INCLUDED IN THE THESIS

I. **Laszlo AM**, Hulman A, Csicsman J, Bari F, Nyari TA: The use of regression methods for the investigation of trends in suicide rates in Hungary between 1963 and 2011. *Social Psychiatry and Psychiatric Epidemiology* 2015; DOI: 10.1007/s00127-014-0926-9

II. Bohár Z, Nagy-Grócz G, Fejes-Szabó A, Tar L, **László AM**, Büki A, Szabadi N, Vraukó V, Vécsei L, Párdutz Á: Diverse effects of Brilliant Blue G administration in models of trigeminal activation in the rat. *Journal of Neural Transmission* 2015; DOI: 10.1007/s00702-015-1445-x

III. Veres G, Fejes-Szabó A, Zádori D, Nagy-Grócz G, **László AM**, Bajtai A, Mándity I, Szentirmai M, Bohár Z, Laborc K, Szatmári I, Fülöp F, Vécsei L, Párdutz Á: A comparative assessment of two kynurenic acid analogs in the formalin model of trigeminal activation: a behavioral, immunohistochemical and pharmacokinetic study. *Journal of Neural Transmission* 2017; DOI: 10.1007/s00702-016-1615-5

IV. Molnár Z, Pálföldi R, **László A**, Radács M, László M, Hausinger P, Tiszlavicz L, Rázga Z, Valkusz Z, Gálfi M: The effects of hypokalaemia on the hormone exocytosis in adenohypophysis and prolactinoma cell culture model systems. *Experimental and Clinical Endocrinology and Diabetes* 2014; DOI: 10.1055/s-0034-1383580

V. Sepp K, **László A**, Radács M, Serester A, Valkusz Z, Gálfi M, Molnár Z: The Hormone Exocytosis in Prolactinoma and Normal Adenohypophysis Cell Cultures by the Effects of Hypocalcaemia. *Cell and Developmental Biology* 2017; DOI: 10.4172/2168-9296.1000182

VI. **Laszlo AM**, Ladanyi M, Boda K, Csicsman J, Bari F, Serester A, Molnar Z, Sepp K, Galfi M, Radacs M: Effects of Extremely Low Frequency Electromagnetic Fields on Turkeys. Accepted for publication at *Poultry Science* 2017; DOI: 10.3382/ps/pex304

1 INTRODUCTION

Applied biostatistical analyses need mathematical research to find the best analysis model for the scientific questions, based on the specific data structure. Data exploration is fundamental for the description of distributions and to identify errors, missing data and outliers. However, beyond descriptive statistics, statistical models can highlight differences and/or relationships in the data based on more than one variable at the same time. Application of more complex analysis methods needs cautious consideration, because the focus is always on the specific scientific field, and statistics is a tool to find answers for the scientific questions. But this tool has to be used properly; thus, appropriate model selection and result interpretation are important.

Data collection over time is common in biomedical studies. Although analysis methods for longitudinal data have been available for a long time, they are often misapplied. Several data structures can be the input of the analysis and many types of models can be used, such as trend analysis, time series analysis, repeated measures models, survival analysis. In this thesis I focus on trend and repeated measures analyses.

These analysis methods often occur in biological and medical research, requiring an interdisciplinary approach today, in which the analysis has equal emphasis within the research team. Finding suitable methods for this, requires biostatistical research. To investigate appropriate risk measures and trends are important in epidemiological studies, rather than using only descriptive statistics. Paired aspects are crucial to examine in the case of repeated measurements, to differentiate within-subject and between-subject effects. Repeated measurement aspects are usually examined in time or space. Selecting the suitable covariance structure is relevant in the analysis of dimensional distances from the paired aspect in repeated measurements. Appropriate data management techniques are needed to handle extreme outliers in a dataset, in order to find the best suitable model. In the case of joint linear trajectories, proper analysis models are essential.

In my research, negative binomial and joinpoint regression methods were used to find risk estimates and segmented trends on frequencies in an *epidemiological study*. In other *neurological* and *neuroendocrine* studies, repeated measurements were analyzed by repeated measures analysis of variance (RM-ANOVA) or by marginal models. In an *environmental study*, marginal and piecewise mixed models were applied on repeated measures data. That is, in all studies, time was an important effect to analyze. In the first case, the response was frequency data, then the dependent variable was continuous in the other studies. Therefore, it is important to deeply know the data we use for analysis, to find the best fitted model and to interpret the results precisely in detail referring to the original scientific questions.

In this thesis, six case studies of the above mentioned four areas are presented:

1. ***Epidemiological***: Trends in suicide rates in Hungary
2. ***Neurological***: Repeated measurements in a rat model of migraine
3. ***Neuroendocrine***: Repeated measurements of hormone release under hypoionic conditions
4. ***Environmental***: Repeated measurements in turkeys exposed to electromagnetic radiation

1.1 Epidemiological study

Suicide is an immense multifactorial problem throughout the world. Data have been reported from numerous countries (based on death registries), relating to various periods, with examinations of potential associations between suicide and aspects such as gender, age, ethnic origin, employment status, occupation, psychiatric disorders, physical characteristics, smoking, suicide methods and a history of self-harm or suicide attempts.

Suicide rates in Hungary have been investigated from many aspects in the past 50 years. The epidemiological and clinical perspectives were described relating to the period between 1961 and 2011. Studies have been published on the variation in annual suicide rates with gender and age, marital status, season, urban vs. rural living, regional distribution, alcohol and tobacco consumption, antidepressant prescription, unemployment rate, psychosocial factors, cultural, sociopolitical and economic features, and genetic and biological contributions (Finno-Ugrian suicide hypothesis).

Trends and risk estimates of annual suicide rates overall or broken down by risk factors have rarely been investigated for the whole of a country and not in a complex way in Hungary: only descriptive annual rates of suicide have been reported from Hungary, and publications were not found in our literature search on trends (especially segmented linear trends) and risk estimates of completed suicide by gender, age group or suicide method between 1963 and 2011.

1.2 Neurological studies

Longitudinal analytical problems may appear in the neurological field, such as in the examination of pain in brain research, as repeated measurements are common in animal experimental studies. The analysis of within-subject effects are of importance in the case of data with repeated measurements. Different substances have an effect on pain, such as the Brilliant Blue G (BBG), calcitonin gene-related peptide (CGRP) or kynurenic acid (KYNA). These effects had to be investigated by multiple factors, such as during time in a behavioral test, or how these spatially spread within the brain under different circumstances.

Activation of the trigeminal system in the brain has a pivotal role in the pathomechanism of headaches. BBG, as a P2X7 receptor antagonist, has proved effective in several models of pain, as have CGRP and KYNA, which show substantial effect in trigeminal nociceptive processing, thus in migraine-like headache.

1.3 Neuroendocrine studies

Hypophyseal hormones, such as adrenocorticotrophic hormone (ACTH) and prolactin (PRL), play an important role when biological systems adapt to their environment. The expressed ACTH is crucial for the functionality of the adrenal cortex and for the adaptation process of living organisms. PRL is a common mediator of the immune-neuroendocrine system, and its secretion can also be modified by stress. To investigate the hormone secretion mechanisms of endocrine cells, is essential to understand disorders like adenohypophyseal prolactinoma (PRLoma) since this is the most common (about 40% of) pituitary adenoma, which manifests itself in overexpression of PRL.

The cellular hormone secretion is greatly determined by the extracellular ionic milieu, namely, the concentration of potassium and calcium. The potassium ion (K^+) plays an important role in cell exocytosis e.g. hormone release through protein cascade activation, whose alteration can affect endocrine-related diseases and cell proliferation. The calcium ion (Ca^{2+}) is a messenger, which affects cellular functions.

Repeated measurements of ACTH and PRL hormone release needed to be analyzed on cell cultures from rats, examining different K^+ or Ca^{2+} treatment groups in normal adenohypophysis (AdH) and PRLoma groups over time.

1.4 Environmental study

Spontaneous electromagnetic radiation (20-30 μT) has an effect on the natural selection of living organisms on Earth. Natural electromagnetic background radiation has been altered by technological inventions and innovations of modern civilization (extensive use of electric devices), and has resulted in increased electromagnetic fields (EMF). There are several pieces of literature examining the potential biological effects of EMF (radiofrequency, microwave); however, very few of them deal with the issue of how extremely low frequency (ELF) radiation puts the welfare of humanity and/or the environment at any risk. ELF EMF are defined as frequencies between 0-300 Hz.

Norepinephrine (NE) is a β-adrenergic catecholamine, which is a common mediator of behavior and physiological functions. Dysregulation of the NE systems can be reflected in panic disorders, post-traumatic stress disorders, special phobias, social anxiety and generalized anxiety disorders. NE can enhance the intracellular 3’5’-cyclic-adenosine-monophosphate (cAMP) level of the erythrocytes. In our environmental study, a turkey model was used to detect the differences at the level of NE-activated β-adrenoceptor mechanisms and the biological effects of ELF EMF which represent commercially available electromagnetic devices.

Repeated measurements of cAMP levels from the blood had to be characterized by appropriate analysis models to compare ELF EMF treated group to the control over time considering inter-individual differences and intra-subject correlation. The reversibility of cell

functions after ELF EMF exposures in turkeys needed to be explained by analyzing the rate of change in time.

2 AIMS

The general objective of my research was to present known statistical methods that can be applied well in public health and biomedical studies to answer new biological questions. That is why we aimed to apply

- negative binomial and joinpoint regression methods to investigate trends and risk estimates in annual Hungarian suicide rates (*epidemiological study*)
- repeated measures ANOVA models to analyze the effects of different substances (BBG and KYNA analogs) in a rat model of migraine (*neurological studies*)
- repeated measures ANOVA and marginal models to study ACTH and PRL hormone release under hypoionic (low levels of potassium and calcium) conditions in adenohypophysis cell cultures (*neuroendocrine studies*)
- marginal and piecewise linear mixed models to examine the effects of extremely low frequency electromagnetic fields on turkeys (*environmental study*)

In connection with these studies, the following specific objectives were set:

Epidemiological study:

- Calculate risk estimates of suicide rates by gender, age group and suicide method in Hungary between 1963 and 2011
- Describe trends in annual Hungarian suicide rates overall, by gender and by age group
- Use proper reference population for the analysis of suicide rates
- Present detailed analyses applying negative binomial and joinpoint regression methods

Neurological studies:

- Analyze repeated measurements of trigeminal activation in the brain to investigate the effect of time and space considering related data in models of pain in the rat

Neuroendocrine studies:

- Investigate repeated measurements of ACTH and PRL hormone exocytosis over time at different, low extracellular K^+ and Ca^{2+} levels on normal adenohypophysis and adenohypophyseal prolactinoma cell populations using the appropriate statistical analysis methods, taking into account extreme outlier data and within-subject effect

Environmental study:

- Investigate repeated measurements in turkeys to explore the effect of ELF EMF on intracellular mechanisms to compare treated and untreated groups over time, considering inter-individual differences and intra-subject correlations
- Describe the pattern (rate of change) of the ELF EMF treated group over time
- Analyze trajectories of treated and untreated groups in the regeneration period

3 MATERIALS AND METHODS

3.1 Epidemiological study

The annual Hungarian suicide data were collected from tables published in the Demographic Yearbooks issued by the Hungarian Central Statistical Office between 1963 and 2011. The frequencies of suicides by gender (male or female), for four age groups (7-14, 15-39, 40-59 and 60- years) and eight suicide methods ("Poison", "Gas", "Hanging", "Drowning", "Fire-arms and explosives", "Cutting and stabbing instruments", "Jumping from a high place" and "Non-specified") were reported during the investigated period. The classification of suicide methods was based on the International Classification of Diseases (ICD; Tenth Revision: X60-X84, Y87.0 in 2010). Suicide codes of intentional self-harm were categorized by using the 7th – 10th revisions of the ICD during the examined 49 years.

Although the ICD coding underwent change, data on all the above suicide methods were published during the study period. The category "Run-over" was defined only in the first six years, and was then aggregated into "Non-specified" cases from 1969. The ICD-10 codes were applied to classify suicide methods as: "Poison", "Gas" (X60-X69), "Hanging" (X70), "Drowning" (X71), "Fire-arms and explosives" (X72-X75), "Cutting and stabbing instruments" (X78), "Jumping from a high place" (X80) and "Non-specified" (X76-X77, X79, X81-X84, Y87.0).

The Hungarian population data were published for age in five-year intervals (0-4 years, 5-9 years, 10-14 years, etc.) and for gender for each year in the Demographic Yearbooks of the Hungarian Central Statistical Office between 1963 and 2011. The live birth data on the 5-9-year age group were used to determine the 7-9-year population, and the counts for the 7-9 and 10-14-year groups were summed to obtain the data on the 7-14-year group. Overall, therefore, the suicide and the population dataset (>6 years) were considered from the aspect of six variables: year, gender, age group, suicide method, suicide frequency and population frequency.

When events occur over time, it is usually more relevant to model the rate at which they occur than the number of the events. The average annual Hungarian suicide rates per 100,000 population (>6 years) were calculated overall, by gender, age group and suicide method (ranges are given as minimum and maximum for annual suicide rates). Trends between 1963 and 2011 for suicide rates overall and in stratified analyses by factors such as gender (reference group: female), age group and suicide method, were investigated by using negative binomial regression models. The negative binomial regression model for total annual data contained only one independent variable year, all other negative binomial regression models included explanatory variables year and gender. The relative risk (RR) for factor gender (male relative

to female) and its 95% CI with p-value were calculated. Trends were also described by using joinpoint regression analyses, determining turning points for the examined 49-year period.

Crude rates were expressed in joinpoint regression analyses as the number of suicide cases per 100,000 population at risk, with the year as an independent factor. The assumption in the regression model was that the random errors followed Poisson distribution and the regression coefficients were estimated by weighted least squares. Further, multiple tests were performed to select the number of joinpoints. Annual changes in percentages were calculated for the fitted linear segments first getting the ratio of linearly fitted suicide rates at the end to the fitted suicide rates at the beginning of the segment then raised to the power of reciprocal of segment length in years, finally subtract one from this power and multiplied by 100.

We presented negative binomial regression models which had an acceptable goodness-of-fit chi-square test result. The type I error was reduced by using the Bonferroni correction for the p-values, multiplying them with the number of significance tests run in all negative binomial regression models and the number of models run separately in each strata in joinpoint regression models: total cases, by gender or by age groups. When a p-value was greater than 1 after the multiplication in Bonferroni correction, it was reduced to 1.00.

3.2 Neurological studies

Number of c-Fos immunoreactive cells from different levels of the trigeminal nucleus, and separately the sums of the areas covered by CGRP-immunoreactive fibres were analyzed by two-way RM-ANOVA models. A stimulation group with 8 levels (BBG or saline treatments, for electrically stimulated or sham controls, for control and stimulated sides: BStim, BSham, SStim and SSham in both sides of the rat) was used as the between-subject factor (separately for mild – 5 Hz, 5 minutes – and robust – 10 Hz, 30 minutes – electrical stimulation) and brain sections (distances from the bregma) with 9 levels (from -13.89 mm to -18.21 mm in 0.54 mm increments) as the within-subject factor for the analyses.

Nociceptive scores (time spent rubbing the injected (right) whisker pad measured in seconds) from the behavioral study were compared between 4 treatment groups (BBG or saline treatments before saline or formalin injections: BSal, SSal, BForm, SForm) during time (15 time blocks) by two-way RM-ANOVA.

The immunhistochemical parameters (the number of c-Fos immunoreactive cells and the area covered by CGRP-immunoreactive fibres) were analyzed as dependent variables over time by two-way RM-ANOVA.

To compare the means of jaw rubbing counts from the orofacial formalin tests in the different treatment groups on rats during time (n = 13-15 animals in each group), two-way RM-ANOVA was run. Treatment with 6 groups (saline or formalin injections to two KYNA analog

pretreatments compared to saline control) was used as between-subject factor and time with 15 time periods (3 minute long time blocks) as within-subject factor for the analysis.

The number of c-Fos immunoreactive cells from 15 adjacent measuring sites of the trigeminal nucleus were analyzed by three-way RM-ANOVA among sides (contralateral and ipsilateral) between pretreatment groups (control and two KYNA analogs) after formalin injection. Measuring sites and sides were used as within-subject factors, while pretreatment was the between-subject factor in the general linear model.

For both neurological studies, when Mauchly's test of sphericity was significant, the Greenhouse-Geisser correction was performed in the RM-ANOVA models. Pairwise comparisons of group means were performed on the basis of estimated marginal means with the Sidak adjustment.

3.3 Neuroendocrine studies

To compare the means of hormone release in adenohypophysis cell cultures between different concentrations of potassium ion treatment and control groups across time, two-way RM-ANOVA was used for each independent set of data: ACTH secretion of normal AdH or PRLoma, or PRL hormone secretion of normal AdH or PRLoma. Treatment with 6 levels ([K^+]: 0; 0.5; 1.0; 1.5; 2.0 mM and a control group; n=10 in each group) was considered as a between-subject factor and time with 5 time points (10, 20, 30, 60, 90 minutes) as a within-subject factor for the analysis. ACTH and PRL secretion of untreated AdH was compared with PRLoma group in hypokalaemia over time using RM-ANOVA.

When Mauchly's test of sphericity was significant, the Greenhouse-Geisser correction was performed. Estimated marginal means were calculated with Sidak adjustment for multiple comparisons.

Repeated measurements of ACTH and PRL hormone release in different treatment groups ([Ca^{2+}]: 0; 0.5; 1.0; 1.5 mM and a control group; n=12 in each group) on cell cultures in an 80 minute period (time points at 10, 20, 30, 60 and 90 minutes) were compared using marginal models. Disease with 2 groups (PRLoma and normal AdH) and treatment with 5 groups (calcium concentration levels) were used as between-subject fixed factors and time with 5 time points as within-subject fixed factor for the analysis. The reference group was the normal (healthy) control (no treatment) group at minute 10 (start) in the model. Restricted maximum likelihood (REML) estimation and Kenward-Roger method for adjusting the degrees of freedom were applied.

In the case of ACTH, unstructured covariance matrix, for PRL data, the heterogeneous first order autoregressive covariance matrix resulted the best fit among different structures (variance components, compound symmetry, first order autoregressive, toeplitz, unstructured

and their heterogeneous versions), based on Akaike's information criterion statistic. Pairwise comparisons were estimated by least squares means using Sidak p-value adjustment. Model residuals were displayed on quantile-quantile plots to check normality assumptions.

In case of extreme values, winsorization technique was applied by shifting the strongly outlying data toward the center to protect parameter estimation against the emergence of unexpectedly large errors.

3.4 Environmental study

The experiment was 9 weeks long: starting with a 1-week long adaptation period, followed by a 3-weeks long ELF-EMF treatment period and a 5-weeks long regeneration period at the end. The turkeys were exposed to intermittent (8 ms energy exposure – 2 ms energy free pause) ELF EMF treatment (50 Hz, 10 μT) for 20 minutes every 8 hours for the 3 weeks in the treatment period. NE-activated cAMP levels were determined from all blood samples taken weekly from the turkeys. All measurements performed 4-6 technical parallels.

Means were calculated from technical replicates for each subject (experimental unit: turkey), and used for the analyses of cAMP levels (as markers of β-adrenoceptor function). Descriptive statistics were calculated to identify distribution of cAMP levels by treatment groups and time points.

To estimate means in ELF EMF treated and untreated groups over time in the whole experimental period (weeks 1-9) considering between-subject differences and within-subject correlation, marginal model was applied. Differences of least squares means are calculated according to Sidak adjustment.

To characterize the reversible nature of NE-activated β-adrenoceptor function after ELF EMF treatment by the rate of change in time, piecewise linear mixed model (LMM) was used, which could describe the linear trajectories of cAMP levels in the treatment and in the regeneration periods. An intraclass correlation coefficient was calculated to describe variation.

In marginal and mixed models, the REML estimation method was used with unstructured covariance structure. Kenward-Roger method was applied for adjusting degrees of freedom. Model residuals were checked for normality.

4 RESULTS

4.1 Epidemiological study

Overall, 178,323 suicides were committed in Hungary during the period 1963-2011 (28.2% females and 71.8% males). The average suicide rates per 100,000 population (>6 years) varied between 26.03 and 51.31 and the overall average suicide rate was 38.44 in the population older than 6 years. The average suicide rate in males was 57.75 (range: 41.93-75.19) and in

females it was 20.78 (range: 11.11-32.12). The suicide rate was higher in males than in females overall, in each age group and in each suicide method. The average suicide rates increased with age (average suicide rates (range): 7-14 years: 1.13 (0.23-3.11), 15-39 years: 25.15 (13.17-35.81), 40-59 years: 52.56 (38.58-70.62), and 60- years: 67.09 (37.14-96.28)). Distribution of the Hungarian suicide frequencies overall and in the different subgroups (broken down by gender, age group and suicide method) are presented.

The most frequent suicide methods in the investigated period were “Hanging” (56.9%) and “Poison” (23.7%), the others remaining under 5%. The average suicide rates per 100,000 population (>6 years) for the various suicide methods during the 49 years were as follows: “Hanging”: 21.87, “Poison”: 9.11; “Jumping from a high place”: 1.83, “Non-specified”: 1.80, “Drowning”: 1.41, “Fire-arms and explosives”: 0.91, “Gas”: 0.80, “Cutting and stabbing instruments”: 0.72.

RRs of gender difference were calculated to estimate the risk of committing suicide overall, by age groups and suicide methods during the 49 years. Overall risk of suicide was nearly threefold higher among males than females, which was varied across age groups. Similarly, significantly higher risks were detected in the male groups as compared with the females in most suicide methods (more than twentyfold risk was the highest in the “Fire-arms and explosives” subgroup), except “Poison” and “Drowning” subgroups.

The negative binomial regression model for total annual data revealed a decreasing annual trend in the suicide rates during the study period, which remained significant after the Bonferroni p-value correction. The annual suicide rates data displayed segmented lines rather than a decreasing linear trend. Thus, the joinpoint regression analyses were applied to refine the annual trends. Overall, the joinpoint regression model fitted a segmented line indicating two main changes in the trend: a peak in 1982, followed by a significant decrease until 2006, and a basically constant period thereafter. From 1963 to 1982, there was a significantly increasing linear trend in the suicide data, and then a significant decline between 1982 and 2006, followed by a rather constant level from 2006 to 2011 (annual changes in percentages, slopes with p-values of each segment: 2.9%, 1.15 ($p<0.001$); -2.8%, -1.09 ($p<0.001$) and 0.2%, 0.06 ($p=0.84$) respectively).

The gender-specific analyses depicted turning points in 1981 and 2002 for females and 1983 and 2006 for males, and changes in segmented linear trends. The annual changes in percentages and slopes of the segments in the male cases for 1963-1983: 2.7%, 1.58 ($p<0.001$), for 1983-2006: -2.4%, -1.41 ($p<0.001$), and for 2006-2011: -0.2%, -0.10 ($p=1.00$), and in the female cases for 1963-1981 were: 3.2%, 0.75 ($p<0.001$), for 1981-2002: -4.1%, -0.86 ($p<0.001$) and for 2002-2011: -1.7%, -0.20 ($p=0.08$).

Significant joinpoint segmented line fits were found in each of the age groups except the 7-14-year old group. Detailed results of the joinpoint regression analyses are presented. Additionally, the regression estimations (slopes and significance level) of the joinpoint analyses in all age groups are demonstrated. Peaks were observed following significant increases in suicide trends in 1986, 1984 and 1980 in the age groups of 15-39, 40-59 and 60- years, respectively. The suicide rates declined in all the age groups 15 years and over, after the 1980s, this being more marked in those aged 60 years and over (annual change in percentage: -3.4%, slope -2.25; $p<0.001$) until 2005, but has remained constant since then.

4.2 Neurological studies

The used RM-ANOVA analysis models was able to describe the effect of BBG and electrical stimulation of the trigeminal ganglion in the first study, and also the effect of KYNA analogs in the second study in the modulation of trigeminal nociceptive processing considering the effect of time or space. Repeated measurements of c-Fos immunoreactive cell counts and area covered by CGRP-immunoreactive fibres in mild and robust stimulation groups were analyzed, and also in the orofacial formalin test spatially from the bregma of the rat. The number of c-Fos immunoreactive cells were analyzed to highlight the comparison between two KYNA analogs at different distance levels from the bregma in contralateral (left) and ipsilateral (right) sides of the rat.

Nociceptive scores were analyzed in time to monitor behavior in the study of BBG administration of trigeminal activation, and also in the comparative study of two KYNA analogs.

4.3 Neuroendocrine studies

Applied RM-ANOVA models highlighted the effect of hypokalaemia on hormone exocytosis. Analyses results showed significant difference for all K^+ treatment groups compared to the control at all time points. All pairwise comparisons were significant in all RM-ANOVA models, even with Bonferroni p-value correction for the 6 models run: in the first 4 models control versus any K^+ treatment group for all time points, in the last 2 models normal control AdH vs. PRLoma K0 for all time points.

The applied two marginal models fitted the data for both hormones, and model results interpreted the effect of low levels of Ca^{2+} on hormone release.

Five cases were extreme values in the ACTH data, from all n=120 subjects structured in a long format dataset. Using winsorization or deleting these cases resulted in similar differences in ACTH release between the Ca^{2+} treatment groups and the control at all time points by disease (normal and PRLoma AdH cell cultures).

4.4 Environmental study

The effect of ELF EMF was significant over time compared to the control group during weeks 3-5 (differences: week 3: 4.04 with 95% CI of (0.66 – 7.41); week 4: 8.29 with 95% CI of (5.61 – 10.97); week 5: 5.66 with 95% CI of (2.08 – 9.24) measured in nmol cAMP/ml red blood cell (RBC) suspension; $p < 0.01$). The values of the control group did not change (16.7 nmol cAMP/ml RBC suspension with the 95% CI of (15.3 – 18.2)) throughout the whole experiment.

The cAMP level of subjects in the ELF EMF treated group had a decrease ($p < 0.001$) of 2.6 nmol/ml RBC suspension per week (95% CI: (-2.8 – -2.4)) in the treatment period, while there was an increase (1.5 nmol cAMP/ml RBC suspension per week; 95% CI: (1.2 – 1.9); $p < 0.001$) in the regeneration period. At the intercept of the two fitted linear pieces (week 4), the mean cAMP level was 9.6 nmol/ml RBC suspension in the treated group, which was 7.1 nmol/ml RBC suspension (95% CI: (-8.6 – -5.5)) less than that of the control group (16.7 nmol/ml RBC suspension). As 60% of the variation of cAMP levels comes from individual characteristics of turkeys, 40% is from within subject effects.

The piecewise LMM revealed a decreased effectiveness of β-adrenoceptors, as the NE-activated cAMP level was getting lower and lower during the treatment with ELF EMF. After the end of the ELF EMF treatment, the β-adrenoceptor functions returned to the starting state in 5 weeks. As mentioned above, the recovery (increase) of β-adrenoceptor functions in the treated group was slower in the regeneration period (slope of cAMP: 1.5 nmol/ml RBC suspension) compared to the rate of decrease (slope of cAMP: -2.6 nmol/ml RBC suspension) in the treatment period. The treated group did not differ from the control at the end of the regeneration period (weeks 6-9, $p \geq 0.15$).

5 DISCUSSION

The trends in suicide rates in our *epidemiological study* were described by using segmented linear models overall, by gender and by age group in Hungary between 1963 and 2011. Results revealed an overall peak in 1982 and remained constant after 2006. There was a significant, approximately 3% annual increase until 1982 (an increment of ~20 suicides per 100,000 persons) during two decades. This was followed by a significant decrease during the next about two decades, when suicide rate decreased approximately to the same level where it started in 1963, and with relative constancy after 2006 overall. Similar trends were observed in both genders. The different numbers of turning points reported by age group in the segmented linear trends revealed significant, but shifted peaks in the 1980s.

Risk estimates of suicide rates were calculated by gender, age group and suicide method, and highlighted a more than twice as great risk in males than in females overall. The risk of

suicide in males was more than twofold in all age groups. A significant gender difference was found for most suicide methods, except “Poison” and “Drowning”. The highest risk, more than twentyfold in males relative to females, was observed in the “Fire-arms and explosives” subgroup. The most frequent method was “Hanging”, with a risk of more than fourfold in males.

Instead of reporting only the suicide rates, we applied regression models to estimate the trends in Hungarian suicide rates between 1963 and 2011, including joinpoint regression, which involves the best fit of segmented lines connected at the "joinpoints" (turning points) through use of a Poisson model of variation. As far as we know, this was the first utilization of joinpoint regression to investigate the pattern of trends in suicide rates in Hungary. In the joinpoint regression analysis, the slopes with annual changes in percentages of the segmented lines were used to determine the annual changes in suicide rates in Hungary, while other studies applying joinpoint regression for suicide data involved only the estimated annual percentage change. We consider that the use of slopes is more appropriate in the case of linear fit and generally gives a complete picture of trends with the annual percent change.

The risk of suicide in Hungary was calculated by negative binomial regression, which can handle overdispersed data common in rare discrete events, such as suicide frequencies relative to the population. Although suicide rates in Hungary have often been investigated, detailed statistics have scarcely been reported. The negative binomial regression method applied gives valuable risk estimates for the whole of the examined period, and joinpoint regression analyzes the pattern of the trends in detail.

The reference population was given only by gender and age group to calculate the suicide rates. We could investigate the risk of suicide by gender, age group and suicide method only from the data published in the Demographic Yearbooks of the Hungarian Central Statistical Office. However, the suicide methods were broken down in more detail than the violent and non-violent categories usually used in the literature.

In the *neurological studies* RM-ANOVA models characterized BBG administration and the effect of two KYNA analogs of trigeminal activation in the rat in the investigation of migraine through the animal models of trigeminal nociceptive processing. The number of c-Fos immunoreactive cells and separately the sums of the areas covered by CGRP-immunoreactive fibres were analyzed as repeated measurements spatially in different levels of the trigeminal nucleus in mild and robust electrical stimulation groups, and also in the orofacial formalin test to investigate the effect of BBG. KYNA analogs were compared based on the number of c-Fos immunoreactive cells as repeated measurements spatially at different distance levels from the bregma by paired aspects (contralateral and ipsilateral sides of the rat).

Effect of time was analyzed on nociceptive scores (number of seconds the rat spent rubbing the injected area in the orofacial formalin test) to compare BBG and saline treatments before saline and formalin injections and also in the comparison of two KYNA analogs.

In the case of the comparison of two KYNA analogs, related aspects could be analyzed by using 3-way RM-ANOVA, where not only the effect of space (adjacent measuring sites of the trigeminal nucleus) could be studied as repeated measurements, but also the effect of both sides (left: contralateral and right: ipsilateral) of the rat as another related factor in the analysis model. Both factors (measuring sections and sides) were used as within-subject factors in the model of comparison of pretreatment groups (two KYNA analogs and control), where the response variable was the number of c-Fos immunoreactive cells. By fitting this model to the data, we could take into account when several measurements were taken from the same individual.

On the other hand, using more factors in an analysis model increases model complexity because of the statistical interaction which can be detected in 2- or more ways in the coexistence of the used factor variables affecting the investigated response. In the case of statistically significant interaction, the effects of factors cannot be separated for the dependent variable, so these terms must be interpreted together. Besides statistical interactions the researcher also has to focus on biological interactions, which could be essential with no interaction in the analysis model being statistically significant.

A limitation of RM-ANOVA would be the inefficient treatment of missing data. In the neurological studies, there were no missing data, so we did not have to drop all repeated measurement data for any subject.

In the *neuroendocrine studies*, effects of hypokalaemia and hypocalcaemia on ACTH and PRL hormone release could be described by appropriate repeated measures analysis methods. RM-ANOVA resulted in significant difference in both ACTH and PRL hormone release of normal and PRLoma AdH cell cultures for all K^+ treatment groups versus the control across time. Marginal models fitted the exocytosis data for both hormones in case of normal and PRLoma AdH cell cultures in the comparison of low levels of Ca^{2+} treatment groups versus the control over time. For the ACTH release dataset the winsorization technique resulted in similar differences as the deletion of the extreme outlier cases in the comparison of hypocalcaemic groups and the control for all time points in normal and PRLoma AdH cell cultures.

In the case of the analysis of the hypocalcaemic effect on hormone exocytosis, applying the marginal model resulted in a better fit than the RM-ANOVA model, as unique covariance structure could be incorporated, rather than just the homogeneous structure for the observations on the same individual. Furthermore, the use of winsorization reduced the effect of outliers on

statistical analysis, at the same time, it did not give any other result than when we extracted the few extreme outlier values.

In our *environmental study*, marginal and mixed models confirmed the effect of *in vivo* 10 μT ELF EMF treatment on the NE-activated β-adrenoceptor function of physiological processes as cellular mechanisms. Additionally, fitting piecewise linear mixed model to the data, the treatment and regeneration periods could be characterized separately, which reflected a sharp linear reduction in NE-activated β-adrenoceptor function in the treatment period, and then a remarkable – likewise linear – growth in the regeneration period. The control group did not change throughout the whole experiment.

There were no missing values in our measured data, although mixed models can be used on longitudinal data with missing values. The difference in sample size (4 vs. 40) of the investigated groups, could cause biased estimates, though the mixed model is designed to handle unbalanced data sets. Different statistical methods were applied which basically revealed the same result concerning the significant effect of ELF EMF over time.

Piecewise linear mixed model could explain the regeneration phenomenon by the linear increasing trajectory of the ELF EMF treated group, which was returned to the control level.

6 SUMMARY AND CONCLUSIONS

Epidemiological study

1. Risk estimates of suicide rates were calculated using negative binomial regression models by gender, age group and suicide method in Hungary between 1963 and 2011. The risk of suicide in males was more than twofold of that in females overall and in all age groups. A significant gender difference was found for most suicide methods, except “Poison” and “Drowning”. The highest risk, more than twentyfold, in males relative to females was observed in the “Fire-arms and explosives” subgroup. The most frequent method was “Hanging”, with a risk of more than fourfold in males.
2. Segmented linear trends in annual Hungarian suicide rates were described using joinpoint regression models overall, by gender and by age group. Three linear segments were identified in suicide rates overall and by gender: a significant increase in the beginning which was followed by a significant decrease then a relative constancy period.
3. The reference population selection by age (> 6 years) in the calculation of suicide rates helps to avoid biased estimates.
4. Segmented trends and risk estimates were described using complex regression models for age-specific suicide frequencies in Hungary to present detailed analyses rather than purely descriptive statistics to reveal more specific and precise information. The practical application of negative binomial and joinpoint regression analyses was demonstrated on epidemiological data in preventive medicine.

Neurological studies

5. Repeated measures analysis models were applied on trigeminal activation data over time and spatially from the bregma in the brain. These statistical models were able to investigate the effect of time and space considering related data in models of pain in the rat.

Neuroendocrine studies

6. ACTH and PRL hormone release were investigated over time at different, low extracellular K^+ and Ca^{2+} levels on normal and PRLoma AdH cell populations using repeated measures statistical models, taking into account within-subject effects. Significant alteration in hormone exocytosis was detected over time applying marginal models in hypocalcaemic treatment groups compared to the untreated group even with using winsorization or excluding the extreme values.

Environmental study

7. The effect of ELF EMF on repeated measurements of intracellular mechanisms was investigated using marginal and piecewise mixed models on turkeys, so behavioral patterns defined by receptor functions could be characterized to compare treated and control groups over time, considering inter-individual differences and intra-subject correlations.
8. Different statistical methods could reveal the significant effect of ELF EMF over time. Piecewise linear mixed model described the segmented pattern (rate of change) of the treated group during the experiment. A sharp linear reduction was found in NE-activated β-adrenoceptor function in the treated birds in the treatment period, and then a remarkable linear growth in the regeneration period (no ELF EMF exposure) based on the fitted statistical analysis model.
9. Based on the marginal and piecewise mixed models we could highlight that the examined NE-dependent β-adrenoceptor function of the ELF EMF treated group converged to the starting condition in the regeneration period while data of the control system reflected no induced alteration.

10. Appropriate statistical analysis methods need to be used to help answer the scientific question, thus these are crucial in result interpretation in public health and may improve the evaluation of longitudinal biomedical and environmental studies from preventive aspects.

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