

ACUTE ISCHEMIC STROKE AND ITS COMPLICATIONS – IMMUNOLOGICAL STUDIES AND EPIDEMIOLOGICAL INVESTIGATIONS

Ph.D thesis

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INTRODUCTION

As in other fields of medical sciences clinicians experienced dramatic and breakthrough changes in neurology. This applies particularly to my closer field, stroke therapy. In addition to novel diagnostic modalities preventive efforts became more efficient and safe and more effective therapies are available for primary and secondary prevention. These improve significantly stroke victims' prognosis and outcome.

During my professional career of more than 3 decades I eagerly followed scientific literature and made efforts to implant international achievements into national practice. As a result I work in a Department presenting nationally outstanding clinical results.

During the past years I had the opportunity to participate in collection and publication of novel knowledge in an active manner. As a leading author I could participate in basic research and clinical studies that may provide a basis for further investigations.

My PhD theses summarize those 4 studies that I have recently done in the field of stroke research. In the field of basic research I investigated the pathophysiological background and the laboratory diagnosis of immune deficient status in post-stroke patients. In addition, we have performed an epidemiological study in Hungary that revealed the prevalence of stroke risk factors in populations of different socio-cultural background. In a human meteorological study we defined anomalous equivalent potential temperature (anomalous EPT) that may have an impact on stroke risk and outcome.

The link between neurological status and inflammatory complications is not always evident in acute stroke victims. Animal experiments clearly support the notion that acute stroke has an impact on peripheral immune status. This effect is biphasic. The early phase is characterized by a local then generalized inflammatory response accompanied by the extensive production of inflammatory agents and cerebral extravasation of peripheral lymphocytes. The early inflammatory phase is followed by systemic immune suppression developing within a few days after focal damage; this is termed as stroke-induced immune suppression (SIIS). The differences in T-cell subset prevalence have been described earlier; however, the dysfunction of T cell is not investigated fully and it is still unknown how the activation kinetics of T-cells alter in acute ischemic stroke (AIS) victims. The activation of T-cells has a central role in the maintenance of inflammatory reaction, the major cause of tissue damage in the post-stroke phase. The increase of cytoplasmic free calcium levels has a key role in lymphocyte activation. Potential-dependent Kv1.3 and calcium-operated IKCa1 potassium channels have an outstanding role in the regulation of lymphocyte activation. Following antigen presentation they contribute to the maintenance of the electrochemical gradient responsible for the

permanent influx of calcium ions; hence, they have an impact on cytokine production and other components of appropriate immune response.

Following acute stroke, influencing the immunological alterations can be successful if we have the markers that reliably indicates the process. Stroke is related to the classic inflammatory markers increase, including C-reactive protein (CRP) levels, erythrocyte sedimentation rate (ESR), peripheral total white blood cell count (WBC), peripheral neutrophil counts, and body temperature elevation. However, studies have demonstrated that elevated levels of these markers is independent of the presence of infection. Recently identified novel inflammatory markers are becoming more widely available in clinical practice. The soluble plasminogen activator receptor (Supar) as a biomarker of systemic inflammatory diseases is increasingly used. It is cleaved from the membrane bound protein, urokinase-type plasminogen activator receptor (uPAR), found on the surface of several cell types, including trophoblast, endothelial cells, smooth muscle cells, and some tumor cells especially immunologically active cells, monocytes, activated T cells and macrophages. CD177, which is also known as NB1 55-kDa receptor is a member of the uPAR family. It is expressed by certain subgroups of monocytes and neutrophils and is considered as a marker of extravasation. Migration of immune cells into tissues is an elementary step in tissue remodelling and the inflammatory response after injury. Activation of the immune system and the inflammatory response leads to an increase Supar plasma concentration. In neutrophils at rest the Fc gamma receptor I (FcγRI, CD64) is expressed only at a very low level, while during neutrophil activation its expression is strongly enhanced by certain pro-inflammatory cytokines, eg. IFN-γ and granulocyte colony stimulating factor (G-CSF), that are produced in large quantities during infection or endotoxin exposure. The CD64 is also found on the surface of monocytes and activation can also be observed when the receptor is upregulated. The upregulation of CD64 in a longitudinal pattern in neutrophils and monocytes is surprisingly similar, however, it is more pronounced in neutrophils. CD64 expression in neutrophils and monocytes monitored using flow cytometry is a suitable diagnostic marker for various infections and sepsis monitoring. CD64 expression in neutrophils has a high sensitivity (greater than 90%) and specificity (90-100%), and therefore is advantageous as an indicator of systemic infections and sepsis over CRP and hematological changes. The expression of CD64 is suitable for the identification of exacerbation of autoimmune diseases or infections caused by the inflammatory response. If we can determine the inflammatory

marker that early and reliably indicates a change in the immune system, it may support laboratory assessment of stroke victims admitted to emergency departments.

Literature data clearly indicate the association between low socioeconomic status and adverse stroke figures; poverty is strongly correlated with stroke incidence and mortality and stroke outcome. We analyzed the association between living conditions and stroke characteristics in Hungary. We compared stroke epidemiological parameters in two districts of Budapest.

All forecasts that might indicate an adverse effect of external factors may have importance in the management of stroke patients. The impact and significance of meteorological features on the outcome of acute stroke are largely unknown, and the interaction between the individual elements may also have importance. If meteorological indicators with adverse effect on the outcome of stroke can be identified, then closer monitoring of patients with very high risk is justified in such critical weather conditions. We evaluated if changes in the equivalent potential temperature (EPT) are associated with outcome in stroke. The value of EPT is high when it refers to a warm, humid air mass and low when it describes a cold, dry air mass. In meteorological practice, vertical structure of EPT is generally used for analyzing vertical stability conditions of the atmosphere in order to forecast convective systems leading to thunderstorms with heavy rainfalls, for instance supercells. We used EPT data for characterizing air mass inflowing from different regions into the Carpathian Basin. We developed a term called anomalous EPT period and tested whether the rate of fatal outcome is higher in such periods.

OBJECTIVES

Mortality following acute stroke is caused by inflammatory complications in many cases. Our goal was to identify a relationship that may explain the increased inflammatory risk of some patients, while others are relatively well protected against infection. Our goal was to investigate the functional differences of the T lymphocytes: T cells' activation kinetics change following acute ischemic stroke. The cytoplasmic free calcium concentration ($[Ca^{2+}]_{cyt}$) is the key to increase lymphocyte activation process. We hypothesized that the inhibition of lymphocyte potassium channels, in particular Kv1.3 potassium channel inhibitors may be useful in specific immunosuppression in AIS also. Thus, the aim was to judge the effects of stroke in the specific inhibition of T lymphocyte surface located potassium channels. Our goal

was to reply to the question whether therapeutic targets for each potassium channel inhibitors may have a selective immunomodulatory action during the treatment of acute stroke. We tried to find answers, if the IKCa1 inhibitors, for example the TRAM-34 may be clinically applied in that field.

A further study has set the goal to find a marker of inflammation, which is suitable to mirror the early subsequent inflammatory responses due to AIS and to clinical infection. We wanted to select a parameter, which indicates the inflammatory response. To achieve this, the temporal kinetics of C-reactive protein (CRP), total white blood cell count (WBC), neutrophils, the soluble plasminogen activator receptor (Supar), and the prevalence of CD4 + CD25^{high} regulatory T cells, CD64 + and CD177 + neutrophils and monocytes were investigated.

In the stroke epidemiological study, we aimed to investigate the role of socio-cultural factors in the development and course of the disease. We analyzed if the patient's social situation is reflected in the risk factor profile. We investigated whether the common diseases (ie. hypertension, diabetes, atrial fibrillation, etc.) known as a risk for stroke, or other factors (alcoholism, smoking) related to the life style increase the occurrence of AIS and the outcome of it. The age at the onset of stroke is characteristic, so it was a priority target of our investigation. The two major stroke types (ischemic and hemorrhagic) differ in the prognosis of the disease, we wanted to compare these two entities by the epidemiological characteristics. Therefore, our goal was to compare the results between districts. The assumption that men are breadwinners, women's role in social and other traditions also play a role, we wanted to analyze the results by sex. Assuming that the more severe patients have often fatal outcome, we aimed to analyze the fatal cases separately. Finally, in the case of the survivors we wanted to see, whether there is a role for the adherence to the required therapy after the stroke in the case of lower socio-cultural environment. Our target was to discover the risk population, which allows for the most cost-effective way of prevention among the available financial resources.

In human meteorological study the objective was to determine the parameters that reliably predict the occurrence of AIS, an unfavorable outcome. The meteorological parameters when choosing objective was also to be well-known, long-established and trusted among meteorologists.

METHOD

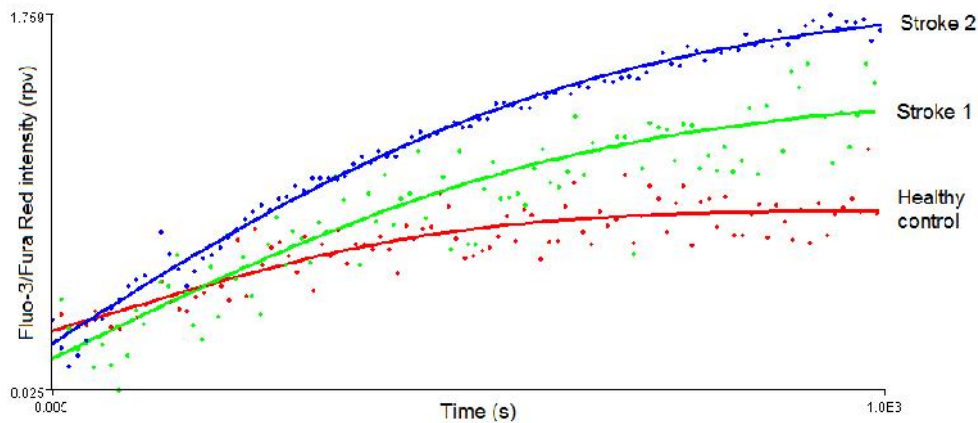
In the stroke immunological study we took peripheral blood sample from 12 acute ischemic stroke (AIS) victims within 6 hours (Stroke 1) and one week after (Stroke 2) the ictus. As control 14 age-matched non-AIS persons were enrolled with comparable cardiometabolic risk profile; they donated specimen for one occasion. We isolated mononuclear cells. We treated them with different cell surface antibodies labelled with fluorescent dyes and with intracellular calcium binding dyes. Cells were stimulated by phyto-hemagglutinine, then calcium signal was monitored for 10 minutes. We determined parameters including Max/maximal value, AUC/area under curve, tmax/time to reach max value in the presence or absence of potassium channel inhibitors.

To analyze the association between living conditions and stroke characteristics in Budapest, we compared stroke epidemiological parameters in two districts of the Hungarian Capital. Patients enrolled were AIS victims in 2007 and located in District 8 or 12 of Budapest. Patients' data were collected from their GPs. We reviewed the closing report and, also, asked GP to complete a questionnaire targeting patient's medical history, risk factor profile, hospital history, therapies applied etc. The 3-year follow-up data were collected from NHIF database.

In the human meteorological examination we analyzed the medical history of patients with ischemic strokes admitted to the Department of Neurology and Stroke Center of Szent János Hospital, Budapest, Hungary in January, February, and December of 2009. All of them had neuroimaging. The incidence of stroke was even in days with and without EPT anomaly.

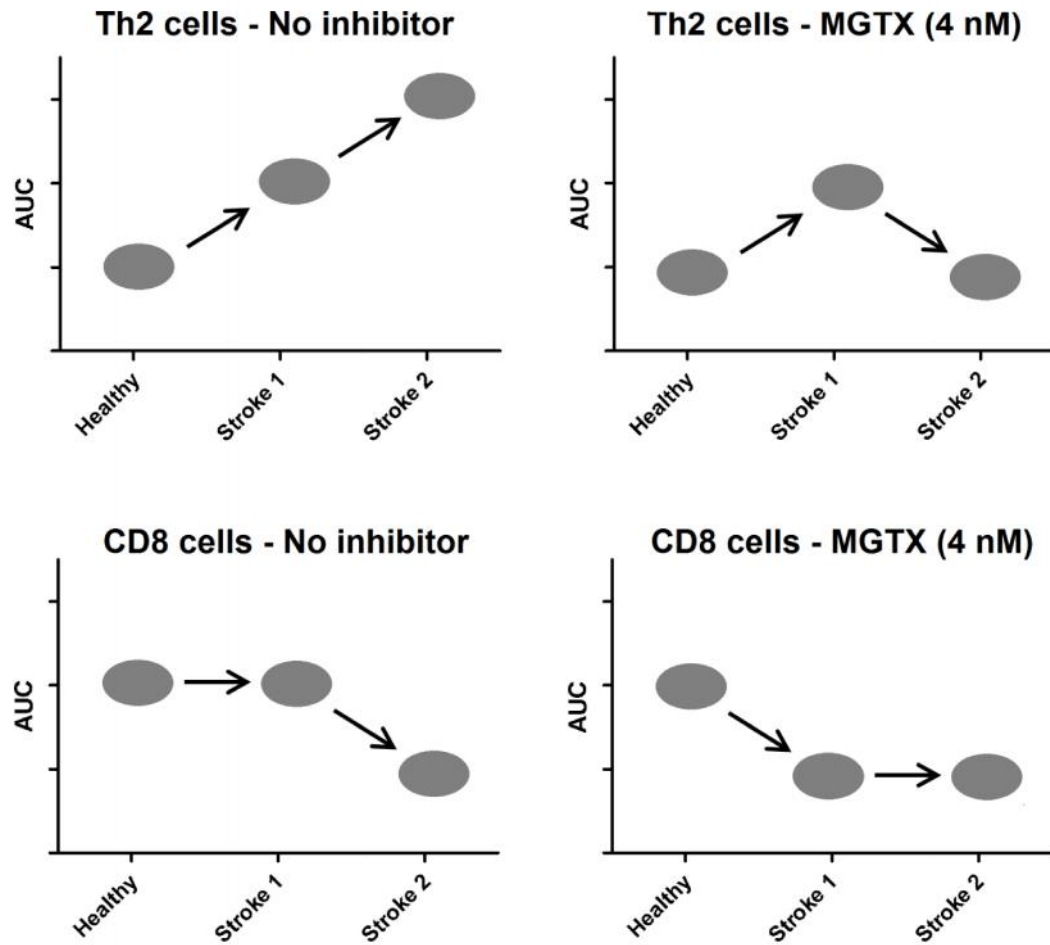
RESULTS

The representative figure of calcium kinetics in Th2 cells of individual groups:



Margatoxin (MGTX), the inhibitor of Kv1.3 potassium channel decreased AUC and Max values of Th2 cells in Stroke 2 and those of CD8 cells in Stroke 1 group. t_{max} values were decreased in control subjects' Th2 cells and in CD8 cells of Stroke 2. Triarilmethane (TRAM), the inhibitor of IKCa1 potassium channel decreased Th2 AUC values below to control in Stroke 1 and Stroke 2, while increased CD8 t_{max} values in Stroke 2 compared to Stroke 1. In AIS subjects the involved cell types expressed high level of Kv1.3 channels on cell surface.

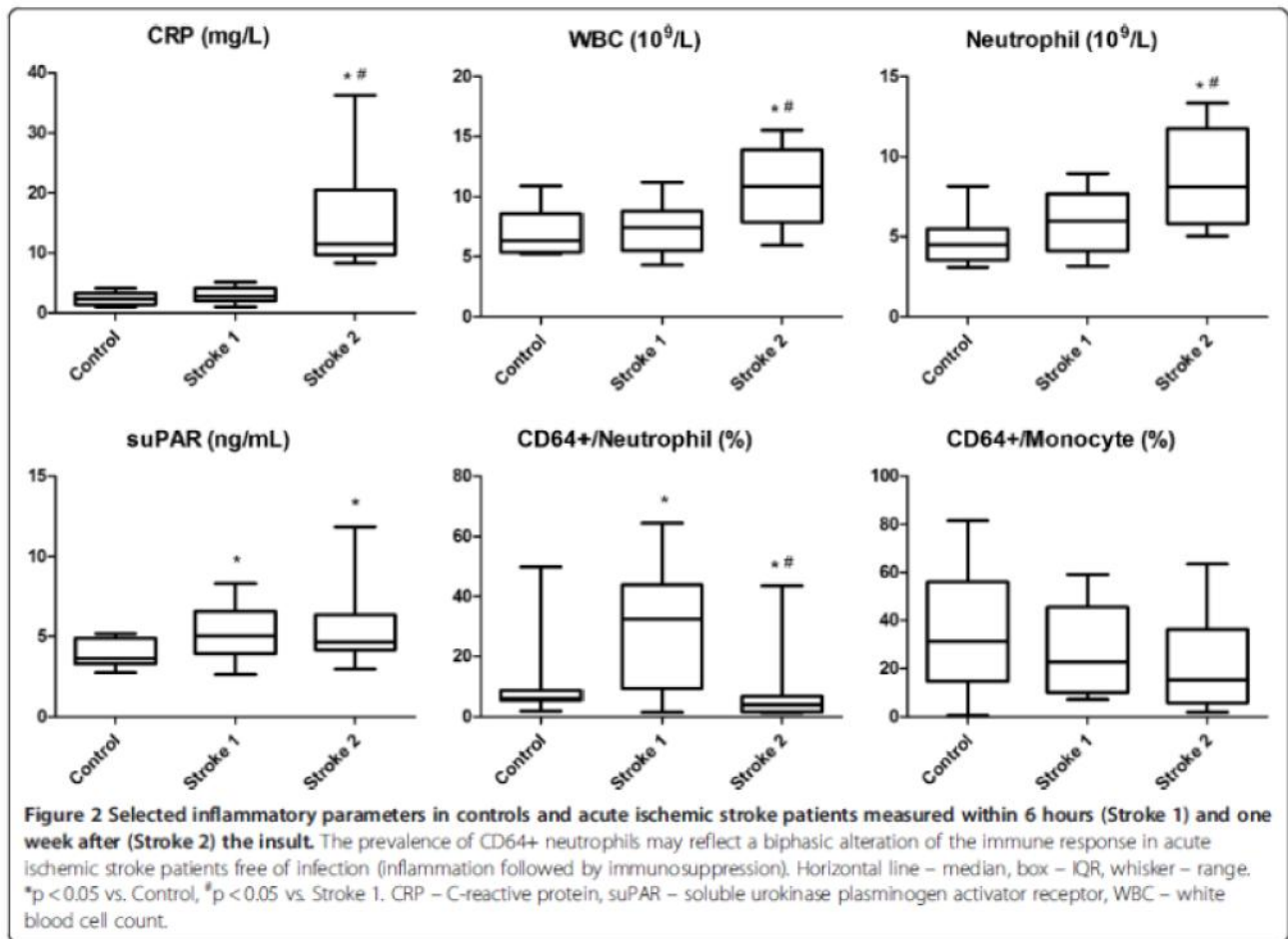
These results support the possible significance of altered Ca^{2+} influx kinetics in Th2 and CD8 in the development of postictal IIS. During the acute phase (i.e. a few hours after the ictus) the administration of MGTX, the selective inhibitor of Kv1.3 channel may have a possibly beneficial impact on immune functions and, theoretically, prevent the development of tissue damage that is mediated by Kv1.3 driven activation of cytotoxic CD8 cell. The administration of MGTX would not alter the function of other T-cell subsets. On the other hand, MGTX would decrease Th2 activation when is given 1 week after the ictus possibly leading to the improvement of immunodepression.



Relative change of calcium influx in peripheral Th2 and CD8 cells of healthy subjects and in AIS patients. Stroke 1 and Stroke 2 groups refer to AIS patients 6 hours and 1 week after the ictus, respectively.

In summary we demonstrated that the selective inhibition of Kv1.3 lymphocyte potassium channels may modulate beneficially T cell activation in AIS patients.

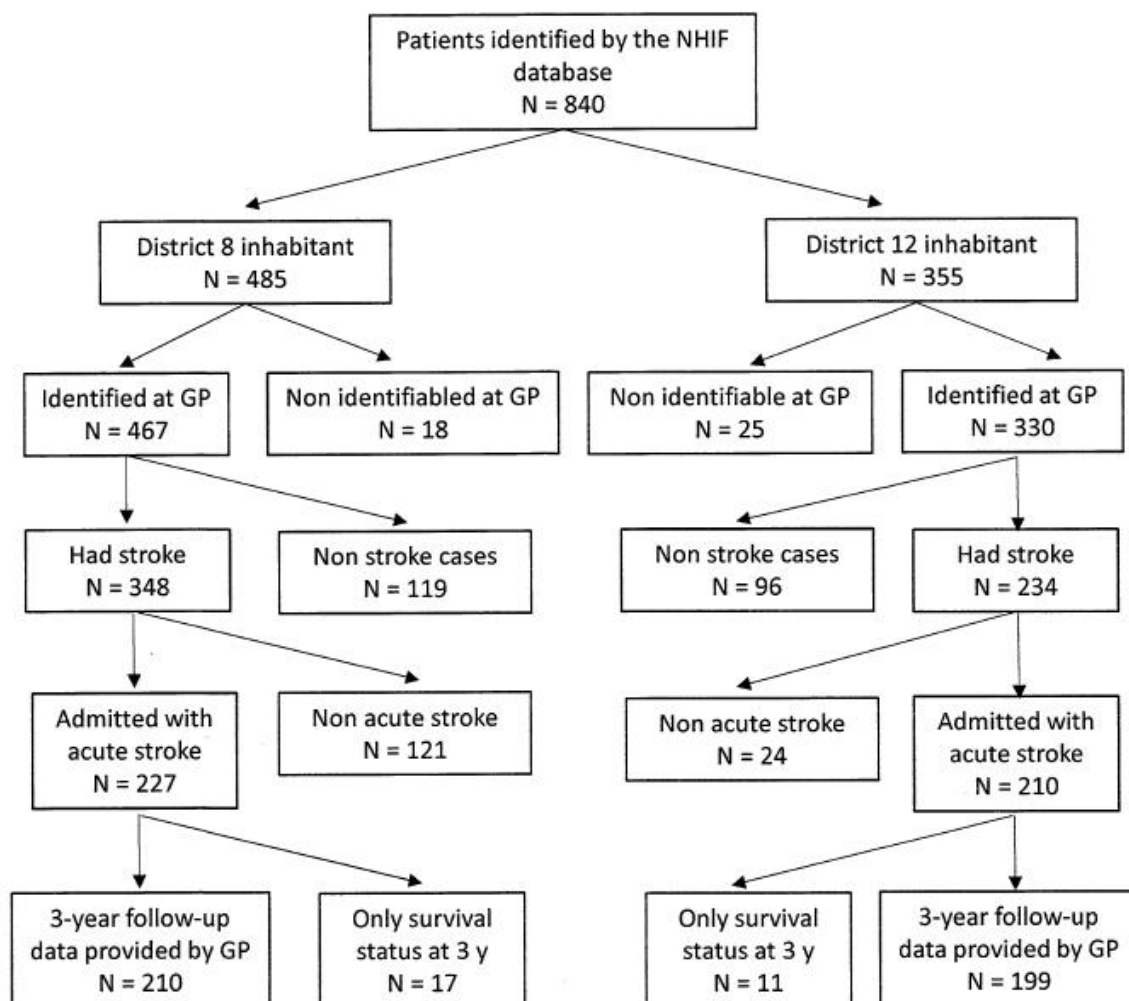
Our results are summarized by the figure below:



We demonstrated that the prevalence of CD64+ neutrophil cells reflect the immunological changes in AIS patients. As this marker decreases below normal 1 week after the stroke in subjects without infection, presumably its increased levels indicates early the risk of infection of AIS patients. The other parameters investigated are not suitable for this purpose.

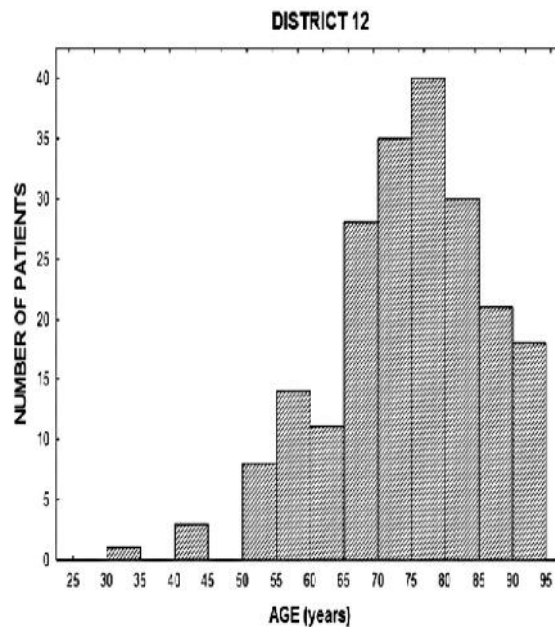
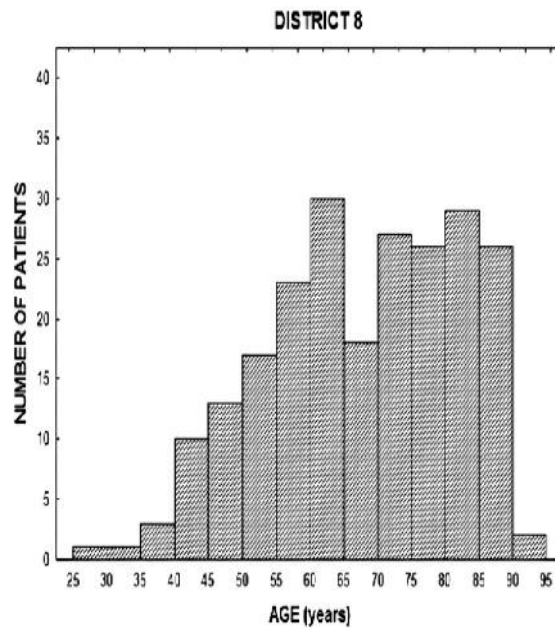
In the stroke epidemiology study we found, that the population density and average income were 5 times larger and 43% larger in District 8 compared to 12, respectively. (District 8 is one of the poorest area of Budapest, while District 12 is among the wealthiest one. The GPs are comparably available in both areas. The average age of stroke onset was 7 years lower in District 8 compared to 12. Stroke incidence peaked above 70 years in District 12, while in District 8 a secondary peak was observed between 55 and 65 years of age.

Patients are summarized in figure below:



Based on 3-years follow-up data case fatality index was 36 and 31 per cent in District 8 and 12, respectively ($p=0.24$). Stroke survivals' average age was somewhat lower in both gender; however, the age of men was lower than that of women in District 8.

The age distribution was comparable between men and women when the subgroup with fatal outcome was analyzed, irrespectively of the area; however, the average age of men with fatal outcome was lower than that of women with fatal outcome in District 8. The difference between District 8 and District 12 was considerably high, 12 years when the average age of men with fatal outcome was analyzed.



In District 8 stroke events occurred several years earlier, particularly in men with fatal outcome. In this area hemorrhagic strokes are also more frequent. Cardiovascular risk profile was comparable in District 8 and 12, while lifestyle risk factors profile differed significantly. The primary prevention (particularly blood pressure control) is less effective in the District 8, the poorer area.

All cases with ischemic strokes who were admitted in January, February, and December of 2009 to the Department of Neurology and Stroke Center of Szent János Hospital, Budapest, Hungary, and had neuroimaging were included in the analysis. During this period 184 patients

were admitted. The incidence of stroke was even in days with and without EPT anomaly. Of the 81 stroke deaths during the full year, 35 occurred during the 3 months of the study period. Average age at death was 82 ± 11 years; the number of women and men were 20 and 15, respectively. The risk of fatal outcome was higher in EPT days ($p = 0.017$): a 27 of the 35 fatalities occurred on these days. We postulate that EPT may have a considerable effect on stroke fatality.

Our results are summarized below:

Feature	Value
Number of days during anomalous periods (NA)	48.5
Total number of days observed (NT)	90
Number of deaths on anomalous days (DA)	27
Total number of deaths (DT)	35
Average number of deaths on anomalous days (DA/NA)	0.56
Average number of deaths on non-anomalous days $(DT - DA)/(NT - NA)$	0.19

CONCLUSION

Different lymphocyte subsets are activated in a different manner when measured at two different timepoints following acute ischemic stroke.

The selective inhibition of Kv1.3 potassium channels may normalize the abnormal lymphocyte activation following stroke

The measurement of CD64+ neutrophil granulocyte prevalence may support the differentiation of inflammation caused by the ischemic attack and that induced by later infection.

Living conditions have a major effect on stroke risk and characteristics. The adverse effect of poor socioeconomic situation is also detected when different districts within the same city are investigated.

Stroke events occur earlier in the poorer district; this effect is pronounced in men with fatal outcome.

The prevalence of lifestyle risk factor differs significantly. While the health care system is available in general, primary prevention, particularly blood pressure control is less effective in poorer populations.

Men living under poorer conditions form a population that should be selectively targeted by the national stroke prevention efforts.

Using publicly available meteorological data and comparing them to fatal stroke outcomes in a hospital stroke center we defined a novel parameter, the anomalous equivalent potential temperature (anomalous EPT).

Anomalous EPT may have an impact on risk and progression of ischemic stroke cases. Stroke-related mortality is three times higher during winter days with anomalous EPT, suggesting the clinical significance of this parameter.

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