Impaired nocturnal melatonin in acute phase of ischaemic stroke: cross-sectional matched case-control analysis.

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### IMPAIRED NOCTURNAL MELATONIN IN ACUTE PHASE OF ISCHAEMIC STROKE: CROSS-SECTIONAL MATCHED CASE-CONTROL ANALYSIS

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<th>Journal of Neuroendocrinology</th>
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<tr>
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<td>JNE-08-0145-OA.R2</td>
</tr>
<tr>
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<td>Original Article</td>
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</table>
| Complete List of Authors: | Atanassova, Penka; Medical University, Neurology
Terzieva, Dora; Medical University, Clinical Laboratory
Dimitrov, Borislav; BORDANI, Consultancy |
| Keywords: | Melatonin, Cortisol, Stroke, Modelling, Bulgaria |
IMPAIRED NOCTURNAL MELATONIN IN ACUTE PHASE OF ISCHAEMIC STROKE:
CROSS-SECTIONAL MATCHED CASE-CONTROL ANALYSIS

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Abstract

Quantitative data on melatonin in stroke patients are scarce. Gender- and age-matched cross-sectional case-control study in 33 patients with ischaemic stroke (IS) was performed and associations between nocturnal melatonin and other factors (e.g., cortisol) evaluated. Clinical and laboratory (e.g., melatonin and cortisol) measurements (3 and 8 a.m.) with statistical techniques [e.g., multifactorial regressions, receiver operating characteristics (ROC) curve and curvilinear estimations] were used. We identified mean value and 95% confidence interval (69.70 pg/ml, 95% CI 53.86-85.54) for control levels of nocturnal melatonin in healthy subjects. The patients with stroke had lower melatonin (48.1±35.9 pg/ml) and higher cortisol (297.3±157.8 nmol/l) at 3 a.m. (p<0.05) but not at 8 a.m. (p>0.05). The stroke was the strongest factor of disturbed nocturnal cortisol (p<0.001) while decreased melatonin depended on the stroke (p=0.010) and gender (p=0.018). In the same time, vice versa, only nocturnal measures were associated with increased probability of presence of stroke (accuracy>75%, p-model<0.001). Thus, a hypothesis that a decrease of melatonin with 1.0 pg/ml might be associated with >2% increase in the probability of presence of stroke [adjusted odds ratio = 1.020 (95% CI 1.002-1.037)] was also suggested. ROC curve (0.67, p=0.0119) and optimisation techniques indicated that a novel best cut-off<51.5 pg/ml for decreased nocturnal melatonin in the view of the presence of stroke (odds ratio = 3.12, p=0.0463) might exist. The classification performance of such cut-off might be confirmed by existing nocturnal melatonin and cortisol differences between the sub-groups; potential differences in diurnal melatonin were also suggested. In conclusion, a novel melatonin cut-off of 51.5 pg/ml may be associated with the presence of ischaemic stroke. As a single marker (84% sensitivity, 74% specificity), its hypothesised modelling performance was independent of age, gender and cortisol. These new results, including the suggested hypothesis, might be further tested in follow-up (cohort), longitudinal studies and be applied to further explore melatonin disturbances as targets in high-risk pre-stroke and post-stroke patients.

Key words: Melatonin, Cortisol, Modelling, Acute Ischaemic Stroke, Bulgaria
Introduction

Melatonin (N-acetyl-5-methoxytryptamine) was isolated in 1958 by Lerner and collaborators due to its ability to aggregate melanin granules in melanocytes, thus, decreasing the pigmentation in reptiles [1]. Produced by the pineal gland, melatonin is one of main pacemakers of circadian rhythms in vertebrates, including humans. It is a neuro-hormone – its synthesis occurs in a diurnal pattern with a night peak. The light-dark cycle is a prominent synchronising factor [2] of the circadian system; e.g., the decrease in melatonin in the light might be seen as an acute response that is associated with degradation of serotonin N-acetyltransferase (SNAT). The melatonin increases after the onset of darkness, peaks in the middle of the night (2-4 a.m.) and gradually decreases. Melatonin rhythm can be interrupted by light which, depending on the phase of its application, may provoke disturbances of its circadian cycle. Also, exposure to light, independently of phase, suppresses melatonin secretion [3-4]. After its synthesis, melatonin is released in blood and liquor of the third cerebral ventricle.

Earlier studies have shown that melatonin secretion profiles are disturbed in stroke patients. For instance, the maximal excretion of 6-sulphatoxymelatonin in urine occurs with a delay [5-7]. This effect is specific during the first 3-4 days after stroke where the mean melatonin excretion is lower but these changes recover at the tenth days and do not correlate with stroke severity, age or gender [6]. This study indicated decreased nocturnal values of melatonin in urine during the acute and chronic phases of stroke that were suggested to contribute to neuro-psychological changes in such patients. Of note, relationships were reported between the impaired melatonin/cortisol ratio, sleep disturbances and depressive symptoms [7].

We aimed at exploring that melatonin profile disturbances during the acute phase of ischaemic stroke, e.g., a decreased nocturnal melatonin at 3 a.m. (main, primary outcome) is independently associated with the presence of stroke. However, since within the framework of our cross-sectional design we could not be certain whether such melatonin disturbances (if any) were ex novo or existed prior to the ischaemic stroke, we also hypothesized that a possible association might exist (secondary outcome) between melatonin profile disturbances and increased probability of ischaemic stroke. Most of earlier studies reported various control levels for nocturnal melatonin in healthy subjects. If true, our secondary hypothesis may further indicate an increased probability of ischaemic stroke in patients with disturbed melatonin profile, e.g., at previously lower levels of nocturnal melatonin or, so called ‘hidden’ amplitude or rhythmic disturbances. To note, such stroke-related values were not quantified earlier in human subjects.

To formally test and quantify “stroke-melatonin” association and control for potential confounders (e.g., cortisol), we performed cross-sectional, matched case-control study on serum melatonin at two pre-specified time points (3 a.m., 8 a.m.) in 33 gender- and age-matched patients during the acute phase of ischaemic stroke. We evaluated the associations with impaired melatonin in 33 IS patients versus 33 healthy controls (33 matched pairs). The results of this modelling approach formed the basis of the present report.
Methods

Patients’ selection
We studied 33 consecutive ischaemic stroke patients referred during the period Jan–Dec 2006 to the Clinic of Cerebrovascular Diseases who satisfied the selection criteria and provided written informed consent according to the Declaration of Helsinki guidelines. Inclusion criteria were: age <60 years, occurrence of first ischaemic stroke, hospitalization up to 6 hours after the appearance of neurological symptoms and CT-scan or MRI-confirmed diagnosis of the ischaemic stroke. Exclusion criteria were: haemorrhagic stroke, arterial hypertension, traumatic brain injury, neurological infections, renal insufficiency, impaired liver function, malignant disease, systemic diseases of connective tissue, and surgical interventions up to 3 months prior to the index event (stroke), consciousness disturbances, abstinence reactions and drugs intake up to 3 months before hospitalization if influencing melatonin. The Ethical Committee approved the study protocol.

Study Design
Thirty-four patients with ischaemic stroke were identified as cases (n=34). For each case, one healthy subject without ischaemic stroke was identified as control (1:1). Identified controls satisfied same inclusion/exclusion criteria (n=34). Cases and controls were matched for gender and age (±5 years). Each case was matched with the first control as identified on the basis of above criteria (Figure 1). After identification and matching, 2 subjects (1 cases and 1 control) did not provide written informed consent and did not participate in the study. Out of 68 identified subjects, 33 cases and 33 controls entered and completed the study.

Clinical and laboratory methods
Each case and control subject who participated and completed the study underwent an extensive physical and laboratory evaluation. All relevant demographic, clinical and laboratory data were reported in case record form.

Clinical examination: History of the onset of the cerebrovascular accident and its development till the hospitalization, physical examination, mental status and neurological examination with assessment of neurological deficit (by modified Rankin scale [8].

Instrumental and neuro-imaging procedures: CT or MRI of the brain, 12-channel ECG, extra- and transcranial Doppler sonography for confirmation of ischaemic stroke diagnosis.

Laboratory tests: The biological material for all routine clinico-laboratory analyses was taken between 7:00 h and 9:00 h in the morning, unless stated otherwise, following all standard requirements for quality of laboratory results during pre-analytical stage. Blood tests (Coulter STKS, USA), coagulation tests (prothrombin time, activated partial thromboplastin time and fibrinogen by Sysmex CA 6000, Kobe, Japan), tests for serum creatinine and routine biochemical and enzyme parameters (Konelab 60i, Finland).

a) Melatonin assessment procedure: Blood samples were taken on day 3 from hospitalization, at 3:00 and 8:00 a.m. The procedure consisted of selecting a separate room for hospitalization with dedicated medical personnel. The same therapeutic protocol and regime were applied to all patients. During the daytime, the room was illuminated by normal daylight. During night, to ensure complete darkness, the
windows were covered by black paper. Artificial light source was switched-off at 22:00 h. For determination of melatonin levels, two sampling per patient were performed at specific time point – 3:00 a.m. and 8:00 a.m., in the morning. Subjects were instructed to keep their eyes closed during blood sampling at 3:00 a.m.

The blood sampling at 3:00 a.m. was performed at very weak light and with a maximally shortened procedure, without illuminating the face of subjects. The blood sampling at 8:00 a.m. was performed under normal daylight. The separated serum for determination of melatonin was preserved in conic plastic test-tubes with cover type Eppendorf at 2-8 degree Celsius for 24 hours or frozen at temperature ≤ -20 degrees Celsius for 3 months. Sera with haemolysis, lipaemia or icter were excluded from testing. For determination of melatonin, test kit for immunoenzyme assay in serum and plasma (IBL-Hamburg, Germany, cat. number RE54021) was applied by concurrent heterogeneous immunoenzyme ELISA-based method (analyzer “Sirio S” microplate reader, SEAC, Italy).

b) Cortisol assessment procedure: The blood for determination of serum cortisol was provided by closed system for biological sampling. The equipment Monovette Sarstedt (cat. numbers 05.1553.001) for clinico-chemical analyses was used. The serum was separated from forming elements by blood centrifugation (15 min, 3000 rotations/min) by centrifuge with horizontal rotor T230 Janetzi (Germany). The reagents for FPIA (fluorescent-polarization analysis) with test kit (Abbott Laboratories, USA, cat. number 9116) were used by analyzer Tdx/FLx (Abbott, reference values: 150-830 nmol/l at 7:00-9:00 a.m.).

Sample size estimation
The sample size was calculated on the basis of an expected difference in the primary outcome variable (e.g., nocturnal melatonin at 3.00 a.m.) between the cases with IS and controls (healthy subjects). We have taken as a basis the melatonin profile amplitude of 87 pg/ml and its standard deviation (SD) of 19.4 pg/ml in 8 normal, healthy subjects during a nocturnal surge of ≈12 hours (10.23 p.m.-10.23 a.m.) with 95% confidence interval (CI) 70.8-103.2 pg/ml (variation ≈55-95 pg/ml at about 3-4 a.m., [9]). Assuming the same standard deviation of 19.4 (pooled) for IS patients and a minimum average difference of ≈15.5% (>13.5 pg/ml) from controls, it was estimated that to give the study a power of 80% to detect such expected difference as statistically significant at p<0.05, 32 patients per group had to be included. By a preliminary estimate of IS prevalence for patients that would satisfy inclusion/exclusion criteria from all those referred to the Clinic of Cerebrovascular Diseases, it was predicted that 68 patients (34 cases and 34 controls) needed for the analyses should be identified throughout a screening period of about 12 months (estimated maximum drop-out ≈5%).

Statistical analyses
The characteristics of the cases and controls were assessed by methods of descriptive statistics and tests of normality, and the two groups were compared by two-tailed independent sample Student's t-test, χ² test or non-parametric tests, as appropriate. Prior to the analyses, the variables with skewed distribution were normalized by log-transformation. The associations among the main variables listed in Table 1 and the ischaemic stroke were evaluated by univariate analyses. Logistic and parametric regression analyses were applied by entry and backward stepwise methods with adjustment for covariate effects (logit link function
with likelihood ratio or conditional tests, as appropriate) to those variables that were significantly associated with the ischaemic stroke at univariate analyses or served as matching criteria. Logistic curve estimation function was used to fit the regression models. Receiver operating characteristics (ROC) curve analysis was applied to test the models’ internal validity as well as to define the best cut-off values that implied discrimination points between the sub-groups of participants with low or high probability for presence of ischaemic stroke. Data are mean (±S.D.) or number and frequency (percentage), unless otherwise stated. The statistical significance of all tests was assumed at p<0.05, unless stated otherwise.
Results

Patients’ characteristics

The main demographic, clinical and laboratory features of IS patients included in the present study are given in Table 1. As expected, since 33 patients with IS were matched with 33 healthy controls, no differences in gender and age distributions were observed (20 males vs. 13 females, 58.4±7.5 vs. 55.3±7.4 years, respectively). Only 2 patients in either cohort denied their consent to study participation and were not included. Their characteristics were similar to those of included patients (Figure 1).

According to the clinical data, the IS diagnosis in 33 patients was most frequent in the region of the carotid system (the middle cerebral artery - 72.7 % and the vertebrobasilar system – 27.3%), with subacute onset of the neurological deficit being also more frequent. The neurological examination had revealed typical abnormalities in the damaged region, e.g., hemipareses, motor/sensor aphasias, etc. Most frequently observed neurological impairments, mainly with paresis symptoms, had a Rankin score of 3 (48.4%). More than one-half of the IS patients had abnormal Doppler sonography findings, while the brain CT scan/MRI indicated abnormal findings in 60.6%.

Comparative analyses

The IS patients and healthy controls were similar not only for the matching variables, but also for melatonin and cortisol diurnal (at 8 a.m.) values and normal serum creatinine (Table 1). Patients with IS, however, had lower melatonin and higher cortisol nocturnal (at 3 a.m.) levels (p<0.05). Distribution of individual levels of melatonin at 3 a.m. is shown for all study participants (Figure 2). We identified a 95% confidence interval for a melatonin peak in our healthy subjects to be from 53.86 to 85.54 pg/ml that served as control levels for further analyses.

Having found a statistically significant difference in the nocturnal levels of melatonin (primary, main outcome), we then performed a multifactorial parametric regression (Table 2) which revealed that IS was the strongest factor of disturbed nocturnal cortisol (p<0.001). The decrease of nocturnal melatonin depended upon the presence of IS (p=0.010) and male gender (p=0.018) as independent factors. Because of the cross-sectional nature of our study, vice versa, we further tested our secondary hypothesis and found that at backward logistic regression only nocturnal melatonin and cortisol were independently associated with increased probability of IS [odds ratio (OR) of 0.30 and 6.22, respectively, model accuracy > 75%, \( p_{mod}=0.001 \), Table 3]. A decrease of melatonin with 1.0 pg/ml was associated with >2% increase in the probability for the presence of ischaemic stroke [adjusted OR = 1.020 (95%CI 1.002÷1.037)].

Further, the ROC curve analysis (ROC\(_{AUC}=0.67, \, 95\%CI\%= 0.54-0.78, \, p=0.0119\) and optimisation techniques (Figure 3) indicated a novel best cut-off value <51.5 pg/ml for a decreased nocturnal melatonin (at 3 a.m.) in the view of increased probability of IS. An additional analysis of IS patients versus controls, by “normal”/“abnormal” nocturnal melatonin profile in 4 sub-groups according to the new cut-off value of 51.5 pg/ml (i.e., below control subject levels) has confirmed its possible classification performance
(OR=3.12, p\text{two-tailed}=0.0463, Figure 4A) about probability of presence of IS. The nocturnal cortisol values were found to be different among the 4 groups (p<0.05, Figure 4B). According to this discrimination, a significant difference in melatonin values at 8 a.m. among some sub-groups was revealed (p<0.05) thus suggesting also a disturbed diurnal melatonin profile during the acute phase of ischaemic stroke, being similar to that in the nocturnal melatonin (not shown).
Discussion

In this analysis of a study cohort of young patients with ischaemic stroke we modelled the possible associations of the nocturnal melatonin disturbance with ischaemic stroke and we launched the hypothesis of its role of an eventually novel cerebro-vascular risk factor (CVRF). Notably, (i) we studied the differences in melatonin levels; (ii) modelled the dependence of melatonin disturbances upon the presence of stroke; and (iii) explored associations of presence/absence of stroke with the impaired nocturnal melatonin and established a new cut-off value of 51.5 pg/ml below which there might be a clear distinction in the continuous increase of the probability of the presence of stroke. However, follow-up, longitudinal studies are further needed to better clarify the eventual role of the impaired nocturnal melatonin (especially the deviations from its control subject’s 24-h pattern) in the IS pathogenesis. Thus, potentials and settings for successful prevention by add-on medications and/or melatonin supplementation, especially in high-risk patients, e.g., patients after transient ischaemic attack (TIA) or minor ischaemic stroke (MIS) [10], may be further addressed. Well-known and clinically defined conventional modifiable and non-modifiable risk factors for stroke are arterial hypertension, smoking, hyperlipidaemia, co-morbid heart diseases, diabetes, obesity, etc. Recently, however, new important factors emerged such as impaired nocturnal melatonin, impaired fibrinolysis, infectious agents and inflammation, sleep-related respiratory disorders, etc. [11-14]. Notably, as pathophysiological mechanisms, the impaired nocturnal melatonin may be related to suppressed natural melatonin production, impaired metabolism or decreased intake of melatonin-forming nutrients as well as to disturbed sleep-wake cycle (e.g., sleep-disordered breathing) and shifted working schedules.

The present study confirmed the patterns of a clinically established and instrumentally diagnosed ischaemic stroke in young patients in respect to its most frequent localizations and main focal neurological symptoms. Relatively early atherosclerotic patterns in our young IS patients and related pathological laboratory findings, if not observable but hidden, may be considered as deficiencies in the defence antioxidative systems associated with disturbed, decreased nocturnal melatonin. We found statistically significant differences in both melatonin and cortisol, but only in those measured at 3 a.m. (Table 1) and both maintained significant and independent role at the multivariate association with IS. It is possible that the impaired nocturnal melatonin in 23 of our IS patients (69.7%) be also explained by a higher extent of their lesions towards the intergeniculate leaflet (IGL) innervating both the suprachiasmatic nucleus and pineal gland as being involved mainly in the regulation of the nocturnal melatonin metabolism [15].

It is quite probable that decreased nocturnal melatonin may show interferences with increased cortisol and, in the same time, it may correlate with disturbed antioxidant status [16] leading to the progression of atherosclerotic changes and increased risk of cerebrovascular incidents, even in younger patients. The established differences of nocturnal melatonin in our young patients have confirmed earlier results [5-6, 17]. For instance, Beloosesky et al [5] have found that the mean excretion rate of urine sulfatoxymelatonin (6-SMT), as a major metabolite of melatonin, had lower nocturnal (2 and 6 o’clock)
values on 3rd-4th days post-stroke (mainly, in extensive cortical lesions). Similarly, Fiorina and collaborators [6] have measured urine melatonin and also found decreased nocturnal values on 3rd day post-ischaemic stroke that persisted at least till the 7th day in extensive cerebral injuries. Pang and collaborators [17] have also reported lower nocturnal rise of plasma melatonin in acute cerebral haemorrhages. However, none of these studies have not even attempted to specify any possible cut-off value in such nocturnal melatonin decreases that may have been eventually associated with the increased probability of the presence of stroke. Cortisol changes were not evident in the above reports, either, but in the present studies we were able to find also a significant parallel nocturnal increase in the cortisol levels. It is clear that disturbances of the melatonin rhythm (amplitude and/or phase) during the acute phase of stroke may be also associated with other parallel dysregulations of the neuroendocrine (hypothalamic-pituitary-adrenal) axis that can be seen early in hemispheric strokes.

Notably, it has been shown that the melatonin levels or their rhythm may recover later, in the reconvalescent (3rd) period in IS patients, but our current study reported that the associations of the disturbances of nocturnal melatonin during the acute phase of stroke were independent from cortisol. Cortisol has shown also increased nocturnal levels and even higher odds ratio (OR=6.22, see Table 3), however, both were independently associated to IS within the multivariate backward regression model. Moreover, the previous multifactorial backward parametric regression analysis (Table 2) indicated that only the age-adjusted presence/absence of stroke (p=0.010) and gender (sex, p=0.018) were able to model the melatonin, but not cortisol nocturnal levels (p=0.415).

It had been shown previously that impaired nocturnal melatonin levels might be related to worse IS prognosis and outcome [5-6,18]. Although we were not able to assess the prognosis after IS according to the decreased nocturnal melatonin, having established that decreased nocturnal melatonin (as measured during the acute phase of ischaemic stroke) was independently associated with the presence of stroke, we decided to further search for a potentially significant cut-off for this association. Thus, by logistic fitting and ROC analysis, we have established in younger patients and suggest a novel and well-sensitive cut-off value for nocturnal melatonin (51.5 pg/ml), irrespectively of age, gender and cortisol levels, to better discriminate and more precisely define and model correctly the patients with increased probability of presence of IS. We cannot assess the extent to which the decreased nocturnal melatonin that we measured during the acute phase has been due (as a consequence) to the index event (stroke) and/or it had been low (decreased) even beforehand, prior to the stroke occurrence (which, if not confirmed, cannot be excluded, either). In this regard, no studies were found in the literature and, although in experimental animals, a recent paper by Meng and collaborators [19] have shown that mainly the on-set time of melatonin nocturnal increase (MT-on: the time when melatonin rises to 20% of the daily maximum) increase (around 21-24 h) has changed from pre- to post-stroke. The authors described that middle cerebral artery occlusion caused a slight advance of MT-on that persisted through 4 days of monitoring, but no changes were observed for the peaked (amplitude) levels during the dark part of the circadian rhythm (from 22 pm to 6 am). To note, the decrease of nocturnal melatonin in our IS patients as compared to controls was by one-third (~21.6 pg/ml or -30.9%, Table 1). Assuming that our controls represent our IS patients before the occurrence of IS, this value for the
difference is too high to have been due only to the IS index event (post-stroke), without having been sufficiently lowered beforehand. Rather, the low/decreased nocturnal concentration of melatonin that we detected in our IS patients (i.e., during the acute phase of the ischaemic stroke) may have actually existed long or short before, to a large extent, as a pre-existing unknown pattern. The fact, that the pre- and post-stroke nocturnal levels (including also the peak values at 3 a.m.) in the above experimental animals [19] remain relatively stable as an amplitude, may be also seen as an immediate confirmatory finding in the view of our current postulate about the „dormant“ („hidden“) pre-existing low nocturnal melatonin in subjects, eventually being at increased IS risk. This strictly indicates that decreased nocturnal melatonin levels (before IS) might be seen as novel, important IS risk factor and surely need further confirmation in prospective cohort studies. Moreover, in such populations with high background risk of IS as the Bulgarian one, a valid and reliable cut-off level for decreased nocturnal melatonin (at 3 a.m.) may have even more relevant and informative clinical applications than usually applied laboratory control subject levels. Our finding is a very important contribution that more precisely indicates an eventual presence of potentially ‘hidden’ but increased risk prevalence within a usually-defined control subject levels of nocturnal melatonin thus allowing better clinical application of preventive and treatment strategies in younger, but “at risk” populations.
References

Legends to Figures

Figure 1  CONSORT flow-chart of study screening, enrolment and analysis.

Legend: IS, ischaemic stroke.

Figure 2  Distribution of melatonin levels, measured at 3 a.m.

Legend: X-axis: Study participants’ anonymous identification number - black triangles [patients with IS (cases)], black circles [subjects without IS (controls)]; Y-axis, Melatonin [pg/ml]; IS, ischaemic stroke

Figure 3  Models of ischaemic stroke (IS) and melatonin at 3 a.m.

A) Probability of IS expressed as a nonlinear association along the melatonin range at 3 a.m. (p_{model}<0.05). X-axis, Melatonin [pg/ml]; Y-axis, Probability of IS (where 0.0 = no IS; 1.0 = IS); Horizontal line, cut-off event probability of 0.5.

B) ROC curve of IS versus melatonin at 3 a.m.: for a given melatonin level, the ordinate values indicate the corresponding true-positive rate (fraction of IS patients with this melatonin) and the abscissa values indicate the corresponding part of the false-positive rate (fraction of patients without IS with this melatonin). The inflection point of the curve was chosen as the optimal diagnostic value. The larger area between the ROC curve and the diagonal line reflects the higher degree with which the melatonin shows a predictive benefit. X-axis, 1-Specificity; Y-axis, Sensitivity. Both estimates are expressed as a proportion of patients without or with IS (i.e., from 0.00 to 1.00).

Legend: IS, ischaemic stroke.

Figure 4  Analyses of ischaemic stroke (IS) by nocturnal values of melatonin and cortisol

A) Distribution of IS patients and controls according to the indicated cut-off level of melatonin (at 3 a.m.) of 51.5 pg/ml. X-axis, IS status; Y-axis, Number of subjects (where 0.0 = no IS; 1.0 = IS); Framed numbers on white area, Number of subjects in each sub-group (column); Asterisk(*) indicates the significant difference at p<0.05. Legend: Blue (light) column [normal melatonin ≥51.5 pg/ml]; Violet (dark) column [abnormal melatonin <51.5 pg/ml].

B) Box-plots of cortisol at 3 a.m. according to groups by combination of ischaemic status (IS patients, controls) and nocturnal melatonin cut-off values (normal, abnormal). X-axis, Subgroup combinations; Y-axis, Cortisol (at 3 a.m.) values [ng/ml]. The box-plot represents the median (thick horizontal line within the box), interquartile range (25th-75th percentile or H-spread, box) and min/max non-outlier (smallest/largest “whiskers”, short horizontal lines) values. Asterisks(*) indicate significant differences at p<0.05. Legend: IS, ischaemic stroke.
**Table 1.** Main characteristics of sex- and age-matched patients with ischaemic stroke (cases) and healthy subjects (controls)

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<td>69.7 ± 44.7</td>
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<td>Melatonin (at 8 a.m.) [pg/ml]</td>
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<td>27.2 ± 20.1</td>
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<tr>
<td>Cortisol (at 3 a.m.) [nmol/l]</td>
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<td>141.7 ± 120.6</td>
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<tr>
<td>Cortisol (at 8 a.m.) [nmol/l]</td>
<td>483.9 ± 158.6 #</td>
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<tr>
<td>Serum creatinine [µmol/l] &amp;</td>
<td>81.1 ± 27.1 $</td>
<td>79.2 ± 11.7</td>
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* Notes: Number or frequency (percentage) or mean ± SD, as appropriate; °p<0.05 vs healthy subjects as assessed by χ² test or independent sample Student’s t-test / $ non-parametric Wilcoxon’s test, as appropriate. # Comparison by log-transformed values. & Missing data for serum creatinine in 1 patient.
Table 2. Multifactorial backward parametric regression analysis for modelling of main endocrine parameters by presence or absence of ischaemic stroke in 66 sex- and age-matched cases and controls.

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<th>Dependent variable</th>
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<td></td>
<td></td>
<td>Sex (0.322)</td>
<td></td>
</tr>
<tr>
<td>Melatonin #</td>
<td>Stroke (0.010)</td>
<td>Sex (0.018)</td>
<td>Stroke+Sex (0.003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Age (0.270)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cortisol (0.415)</td>
<td></td>
</tr>
</tbody>
</table>

* Notes: Ischaemic stroke (yes/no) and sex (male/female) are categorical variables with dichotomous ordinal coding (1,2); $ Log-transformed values; $ Independent variables / covariates included in the initial models were: ischaemic stroke, sex, age, cortisol or melatonin (both at 3 o’clock), respectively; only variables at p<0.05 remained in the final regression models.
**Table 3.** Logistic backward conditional regression analysis for modelling of ischaemic stroke ($p_{\text{model}}<0.001$; accuracy 75.8%)*

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>Odds Ratio (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol #</td>
<td>6.22 (2.23,17.79)</td>
<td>0.001</td>
</tr>
<tr>
<td>Melatonin #</td>
<td>0.30 (0.12,0.76)</td>
<td>0.011</td>
</tr>
</tbody>
</table>

* Notes: Ischaemic stroke (yes/no) and sex (male/female) are categorical variables with dichotomous ordinal coding (1,2); # Log-transformed values; * Independent variables / covariates included in the initial model were: sex, age, cortisol and melatonin (both at 3 o’clock); only variables at $p<0.05$ remained in the final model.
Participants completing the study (n = 66)

All identified participants (n = 68)

Not completing the study (n = 2)

Subjects without IS (controls) (n = 34)

Patients with IS (cases) (n = 34)

Subjects without IS (controls) (n = 33)

Patients without IS (controls) (n = 33)

Patients with IS (cases) (n = 33)

Participants completing the study (n = 66)

Subjects without IS (controls) (n = 33)

Patients with IS (cases) (n = 33)

Figure 1
Figure 2

Study participants

Melatonin at 3 a.m. [pg/ml]
Figure 3

A) Logistic regression model of IS

Melatonin at 3 a.m. during the acute phase of IS [pg/ml]

B) ROC curve of IS

1 – Specificity (false positives)

Sensitivity (true positives)
A) Distribution of subjects by melatonin (3 a.m.) cut-off

B) Cortisol (3 a.m.) by IS and melatonin status

Figure 4