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RESEARCH ARTICLE



Values of vanillylmandelic acid and homovanillic acid in the urine as potential prognostic biomarkers in ischaemic stroke patients

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ABSTRACT

Background: Suitable biomarkers that have prognostic values are one of the key points of interest in ischaemic stroke. Increased sympathetic nervous system activity in ischaemic stroke causes multiple local and systemic effects that can be detrimental to the outcome. The mechanism of action is increased secretion and activity of catecholamines, whose end metabolic products are vanillylmandelic acid and homovanillic acid. Aim of our study was to determine whether these compounds can be used as potential prognostic biomarkers in ischaemic stroke, as a unique insight into the activity of the sympathetic nervous system.

Methods: Urine samples of 96 patients with ischaemic stroke and transitory ischaemic attacks were analysed. Values of vanillylmandelic and homovanillic acids in urine were tested using liquid chromatography on the first and third day post-stroke. Severity of stroke was determined using the NIHSS scale, while functional outcome was determined using the Modified Rankin Scale.

Results: Values of vanillylmandelic and homovanillic acids positively correlated with functional outcome of ischaemic stroke. Favorable outcomes correlated with decreased values, on contrary to increased values, which were associated with unfavourable outcomes.

Conclusion: Determining the values of these compounds in the urine is an easily available prognostic tool for the ischaemic stroke outcome, while also influencing potential therapeutic changes.

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Autonomic nervous system; ischaemic stroke; immunodepression syndrome; prognostic biomarkers; vanillylmandelic acid; homovanillic acid

Introduction

Ischaemic stroke (IS), a leading cause of death and disability in the world (Warlow 1998), is caused by disrupted blood flow to the brain. One of the key points of interest in research today is finding suitable biomarkers that have a bearing on the functional outcome of the disease (Song *et al.* 2012, Wakisaka *et al.* 2014). The complex interplay between the central nervous system (CNS), autonomic nervous system (ANS) and the immune system could be the source of such biomarkers, as this interplay has a significant impact on the neurological status and outcome of IS (Gelderblom *et al.* 2009). Brain ischaemia leads to a disruption in the homeostasis, causing neuronal cell death and the activation of the innate immune system. Acute inflammation can be considered as a double-edged sword, with its benefit being a debated topic. It is likely that the acute inflammation is needed during the early post-ischaemia period for the clearance of cellular debris, however it also seems that prolonged inflammation is neurotoxic and does not propagate cellular repair (Doyle and Buckwalter 2012). Two different effects of the sympathetic nervous system (SNS) activity occur in the post-stroke period: it drives both the local acute

inflammation and the Central nervous system injury-induced immune deficiency syndrome (CIDS) causing (or leading to) symptoms of secondary systemic immunodepression (Dirnagl *et al.* 2007). Ischaemic stroke causes an increase of catecholamines (CA), which activate the beta-adrenergic receptors (β -ARs) of microglia cells and thus stimulating a cytokine production as well as promoting a proinflammatory reaction (Johnson *et al.* 2013). Cytokines produced by innate immune cells like interleukin (IL)-1 β (Besedovsky *et al.* 1986) or tumour necrosis factor alpha (TNF- α) (Zielinski *et al.* 2013) are necessary for the communication between the immune and central nervous systems. TNF- α values are lowered after central inhibition of the SNS, which indicates that the communication between the systems goes both ways (Pöyhönen-Alho *et al.* 2008).

Sympathetic and parasympathetic nerve endings directly innervate primary and secondary lymph organs and are in close contact with the cells of the immune system. Leukocyte surface receptors for neurotransmitters (catecholamines, dopamine, acetylcholine and opiates), hormones (growth hormone, corticosteroids and prolactin) and neuropeptides (substance P and arginine vasopressin) have

immunoregulatory function, both *in-vivo* and *in-vitro*. The cytokines released from activated immune cells act on the hypothalamus–pituitary–adrenal (HPA) axis and affect the neuroendocrine regulation centres in the hypothalamus, enhancing the effects of HPA and the hypothalamic–sympatho–adrenomedullary axis (Chrousos 1995).

There is a systemic increase in epinephrine (EPI), norepinephrine (NE) and dopamine present in ischaemic stroke and other situations with major sympathetic activity such as major surgery. However, there are notable differences of dopamine levels in ischaemic stroke compared to other states due to direct stimulation of neurons and the paraventricular nucleus of the hypothalamus (Beley *et al.* 1991, Kao *et al.* 1994, Sarkar *et al.* 2017).

All of the released catecholamines have to be metabolised into inactive compounds, which represent an opportunity to identify them as possible biomarkers. The final metabolic products of EPI and NE is vanillylmandelic acid (VMA), after conversion via intermediates, metanephrine/normetanephrine and 3,4-Dihydroxyphenilglycol (DHPG) and 3-methoxy-4-hydroxy-phenylglycol (MHPG). On the other hand, homovanillic acid is the final metabolic product of dopamine, via intermediate 3,4-Dihydroxyphenylacetic acid (DOPAC). VMA values provide insight into the overall activity of the sympathetic nervous system, while HVA provides insight into the central dopaminergic activity and could be a differentiating factor of sympathetic activation in ischaemic stroke compared to other major sympathetic activation, such as major surgery (Scatton *et al.* 1983). VMA and HVA are usually present in the urine in small amounts, however they increase during and immediately after exposure of the body to stressors (Csaba 2014).

In physiological conditions, the increased synthesis of CA results in the synergy between Adrenocorticotrophic hormone (ACTH) excreted by the pituitary gland and cortisol excreted by the adrenal glands, which is present in chronic stress response, as a result of increased sympathetic reaction to the hormone (Bralley and Lord 2001). Cortisol values can impact the values of VMA and HVA indirectly, through the negative feedback loop in the HPA axis. Increased cortisol values can inhibit further ACTH release that occurs in stress. As ACTH increases production of both catecholamines and cortisol; the overall effect of increased cortisol values could be a reduction in catecholamine values (Stephens and Wand 2012). Similar results were found in a recent study, where elevated corticosteroid values had a negative feedback on the sympathetic axis (Mracsko *et al.* 2014).

Elevated levels of these metabolites are found in urine of patients suffering from certain diseases; for example neuroblastomas, pheochromocytomas and other neuroendocrine tumours that can produce large amounts of CA, resulting in a significant increase in levels of the hormones and their metabolites (Akimaru *et al.* 1994; Pacak 2011, Barco *et al.* 2014, van Berkel *et al.* 2014). Thus, finding these metabolites is an important diagnostic indicator of some other diseases, e.g. in children with undifferentiated tumours.

Creatinine, VMA and HVA concentration measurements are carried out on random samples of urine and results of these tests often list VMA, HVA and creatinine ratios. Creatinine is excreted mainly by glomerular filtration which

makes it an important endogenous indicator of kidney function. Creatinine in urine is also determined, especially in randomised urine testing. As creatinine is excreted in urine in constant values, it is used for normalising values of VMA and HVA excretion due to uncontrolled influences on their secretion. (Li *et al.* 2002).

A number of drugs (Shekim *et al.* 1982, Vaccarezza and Ruiz 1974) and foods increase values of urinary catecholamines and may affect VMA testing (Numata *et al.* 1997, Weldin *et al.* 2003), along with other factors such as acute stress and strenuous exercise (Filaire *et al.* 2002).

The aim of our study was to determine the values of VMA and HVA in random urine samples of ischaemic stroke patients, using them as possible indicators of the severity of stroke and to check whether changes in the ANS activity is related to the outcome of ischaemic injury.

Clinical significance

- Vanillylmandelic and homovanillic acids are sympathetic system activity biomarkers easily available in the urine.
- Values of VMA and HVA positively correlated with ischaemic stroke functional outcome.
- Higher TNF- α values in severe stroke indicate higher sympathetic system activity.
- VMA and HVA values provide unique insight into the course of the disease, which could lead to novel therapies and new windows to apply them.

Methods

Patients

Patient data and their urine samples were cared for in accordance with the published International Health Guidelines (Helsinki declaration, 2008). The study protocol was approved by the local ethics committee in accordance with World Medical Association outlines in the declaration of Helsinki. All patients included in the study signed informed consents (Figure 1).

In this study we examined patients suffering from ischaemic brain infarction. The patients were divided into three groups according to severity of stroke: patients with acute ischaemic brain infarction with values measured according to NIHSS (Kelly-Hayes *et al.* 1998) at 12 and lower, (NIHSS ≤ 12) patients suffering from acute ischaemic brain infarction with NIHSS values above 12 (NIHSS > 12) and patients suffering from transient ischaemic attack (TIA), who were treated at clinical hospital centre (CHC) Rijeka's department for neurology in the period between 2012 and 2015.

Ninety-six patients were involved in the study (46 men and 50 women, age group 65–85 years old, mean age 74.75 years and the mean \pm [SD]: 74.75 \pm 04.06), suffering from ischaemic stroke and TIA of middle cerebral artery (MCA) within the first 24 hours. Studies have shown no significant differences between the genders in VMA and HVA values. The reference value has been set as 4–10 $\mu\text{mol}/\text{mmol}$

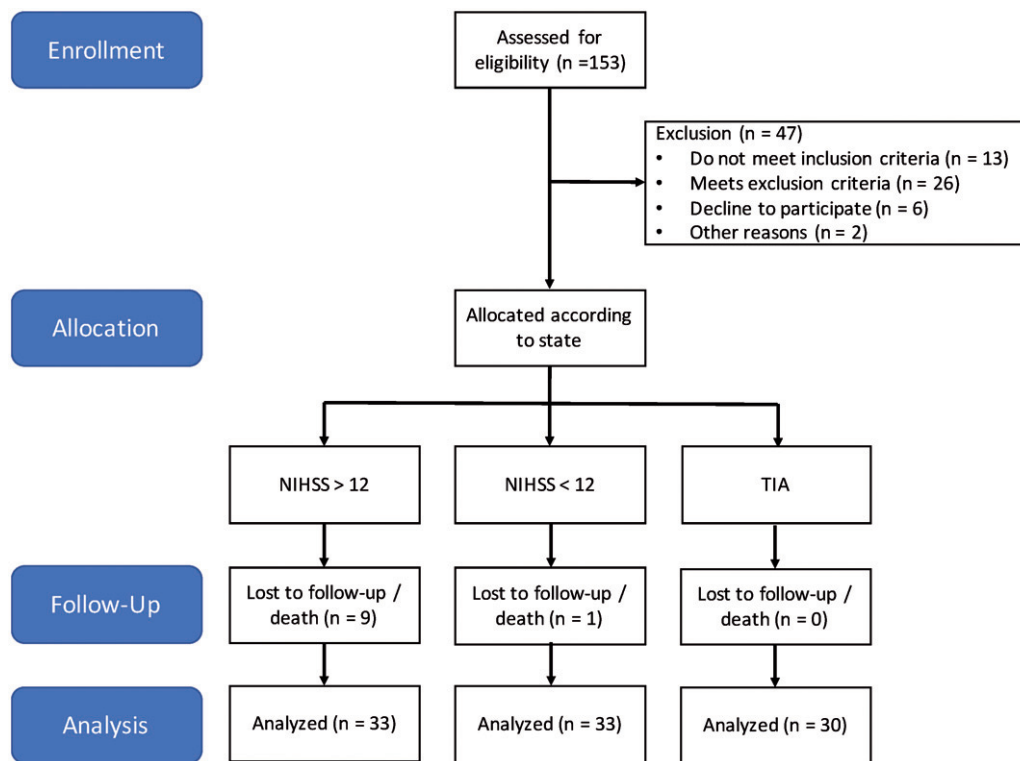


Figure 1. Consort flow diagram of the study.

creatinine, which has been suggested as a cut-off point in elderly patients by previous studies (Tohmola *et al.* 2015).

Diagnosis of ischaemic stroke was based on medical history, clinical picture, neurological examination and CT findings which confirmed the hypodensity in the MCA brain territory. Patients were not asked to fast before sample acquisition, but were informed to avoid nutrients that can affect the VMA and HVA values in the urine. Furthermore, the patients did not receive any serum IV replacements.

Exclusion criteria were: haemorrhagic stroke, clinical and laboratory signs of infection at admission, oncology patients, patients suffering from kidney and liver disease, patients suffering from alcoholism and treatment with corticosteroids, antidepressants, dopamine stimulators, clonidine and immunosuppressants. The study did not include patients who require admission to the intensive care unit and mechanical ventilation, patients who received corticosteroids in the treatment, as well as medications and nutrients which are confirmed to interfere with values of VMA and HVA in urine (Numata *et al.* 1997, Vaccarezza and Ruiz 1974).

Neurological function was monitored, as previously stated, in accordance with the NIHSS scale, on the first and third day in all subjects, while degree of disability and disease outcome were estimated in accordance with the mRS scale (Modified Rankin Scale) (Rankin 1957, Banks and Marotta 2007) 90 days after the IS.

Sample collection procedure

30 mL urine samples were taken to determine the concentration of creatinine (Cobas C501; Roche Diagnostic, Mannheim,

Germany), HVA and VMA, on the first and third day after stroke or TIA, between 8 and 9 a.m., in the Cerebrovascular Unit, Department of Neurology at the Clinical Hospital Centre Rijeka (CHC Rijeka), Croatia. Concentrations of VMA and HVA in urine were tested using high performance liquid chromatography (HPLC) with electrochemical detector (Chromsystem, Germany) at the Department of Clinical Laboratory Diagnostics at Clinical Hospital Centre Osijek (CHC Osijek), Croatia. A commercial kit detecting VMA, HVA and 5-HIAA (Chromsystem, Germany) in the urine was used. Urine sample preparation (solid-liquid extraction) was required prior to chromatography, which included the commercial column, flow rate of 1.0 ml/min, column temperature of 25 °C and the injection volume of 10 µL. The intra-assay and inter-assay coefficients of variations were <4 and 5%. The results were expressed as HVA and VMA /creatinine ratio (in text referred to as values) (Link *et al.* 1985).

TNF- α was measured from the serum of patients using the enzyme-linked immunosorbent assay as per the manufacturer's instructions (ELISA kit, Quantikine, R&D Systems, Europe, UK).

Statistical analysis

For statistical analysis, we used Statistica 12 software package (StatSoft Inc., Tulsa OK). Since the data are not distributed normally, we used non-parametric tests. Values in the three groups (NIHSS >12, NIHSS \leq 12 and TIA) on the first and third day were compared using the repeated measures analysis of variance (two-way RM-ANOVA). Post hoc test was conducted using Fisher's least significant difference (LSD) technique.

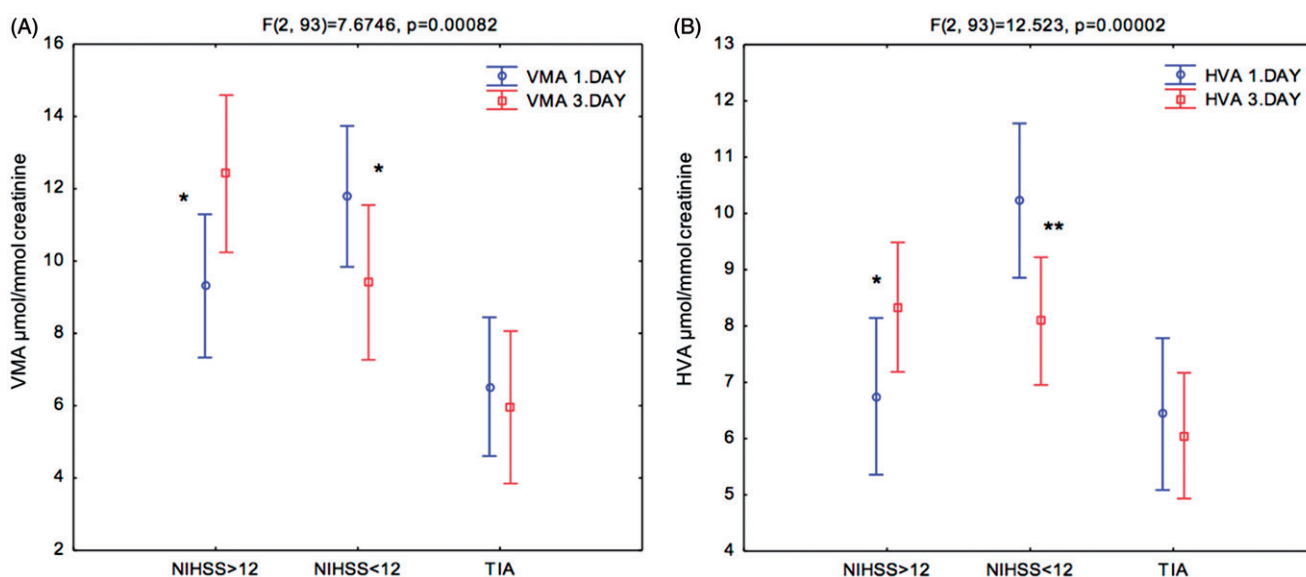


Figure 2. Vanillylmandelic acid (VMA) and homovanillic acid (HVA) values among the three groups on the first and third day post-stroke. VMA (A) and HVA (B) values in patients with severe IS (NIHSS >12) increased on the third day, while values in patients suffering from mild IS (NIHSS ≤12) decreased on the third day compared to the first day. The values were unchanged in patients with TIA. (* $p < 0.05$).

Connection with the degree of disability, which is a categorical variable (1–6), is shown using Spearman's rank correlation coefficient. Using the chi-square test, we compared the differences between various categories of disability in relation to increase or decrease of HVA and VMA ratios.

Statistical significance level was set to $p \leq 0.05$, i.e. with 95% confidence limit. TNF- α values were measured using the repeated measures analysis of variance (RM-ANOVA).

Results

Vanillylmandelic and homovanillic acid values in comparison with stroke severity

Values of VMA and HVA were determined by comparing the values obtained on the first and third day for all 96 patients. Results showed no significant differences in values of VMA ($p = 0.389$) and HVA ($p = 0.240$) in urine between the first and third day when comparing all patients together.

However, there is a significant difference ($p = 0.00082$) in VMA values among the three groups (milder form of IS [NIHSS ≤12], severe IS [NIHSS >12] and TIA) on the first and third day post-stroke period. Tests revealed a significant increase in VMA values on the third day in severe IS group ($p = 0.003$), as well as significantly reduced VMA values in mild IS group ($p = 0.019$) compared to the first day after stroke. As for patients suffering from TIA, no significant differences in VMA values were noted between the first and third day ($p = 0.564$) (Figure 2(A)). Comparison of HVA values from the first and third day between the three groups of patients (NIHSS >12, NIHSS ≤12 and TIA) revealed significant differences between the groups ($p < 0.001$). HVA values in patients suffering from severe IS significantly increased on the third day compared to the first ($p = 0.004$), while patients suffering from mild IS had a significant decrease in values in

the same time period ($p < 0.001$). Patients suffering from TIA showed no significant difference in HVA levels ($p = 0.458$) (Figure 2(B)).

Table 1. Correlation between vanillylmandelic acid (VMA) and homovanillic acid (HVA) values and disease severity according to the NIH stroke scale (NIHSS). There is a positive correlation between VMA/HVA values and NIHSS score on the third day, while the correlation on the first day post-stroke is not statistically significant.

Values	Spearman's rho	p value
NIHSS and VMA day 1 ($\mu\text{mol}/\text{mmol}$ creatinine)	-0.354	0.004
NIHSS and VMA day 3 ($\mu\text{mol}/\text{mmol}$ creatinine)	0.267	0.034
NIHSS and HVA day 1 ($\mu\text{mol}/\text{mmol}$ creatinine)	-0.0298	0.017
NIHSS and HVA day 3 ($\mu\text{mol}/\text{mmol}$ creatinine)	0.062	0.624

Table 2. Correlation between vanillylmandelic acid (VMA) and homovanillic acid (HVA) values and degree of disability estimated to modified Rankin scale (mRS). Spearman's rank correlation coefficients and their levels of statistical significance. The correlation includes both the patients who had severe and minor IS, after 90 days ($n = 66$).

Values	Spearman's rho	p value
MRS and VMA day 1 ($\mu\text{mol}/\text{mmol}$ creatinine)	-0.354	0.004
MRS and VMA day 3 ($\mu\text{mol}/\text{mmol}$ creatinine)	0.267	0.034
MRS and HVA day 1 ($\mu\text{mol}/\text{mmol}$ creatinine)	-0.0298	0.017
MRS and HVA day 3 ($\mu\text{mol}/\text{mmol}$ creatinine)	0.062	0.624

Furthermore, when correlating NIHSS scores and VMA ($p < 0.001$) and HVA ($p = 0.006$) values there is a statistically significant correlation on the third day post stroke, while there is no correlation between them on the first day (VMA, $p = 0.228$; HVA, $p = 0.805$) (Table 1).

Vanillylmandelic and homovanillic acid values as stroke outcome parameters

Nonparametric correlation was used for comparing VMA and HVA values from the first and third day with degree of disability expressed by the mRS. The Spearman's rank

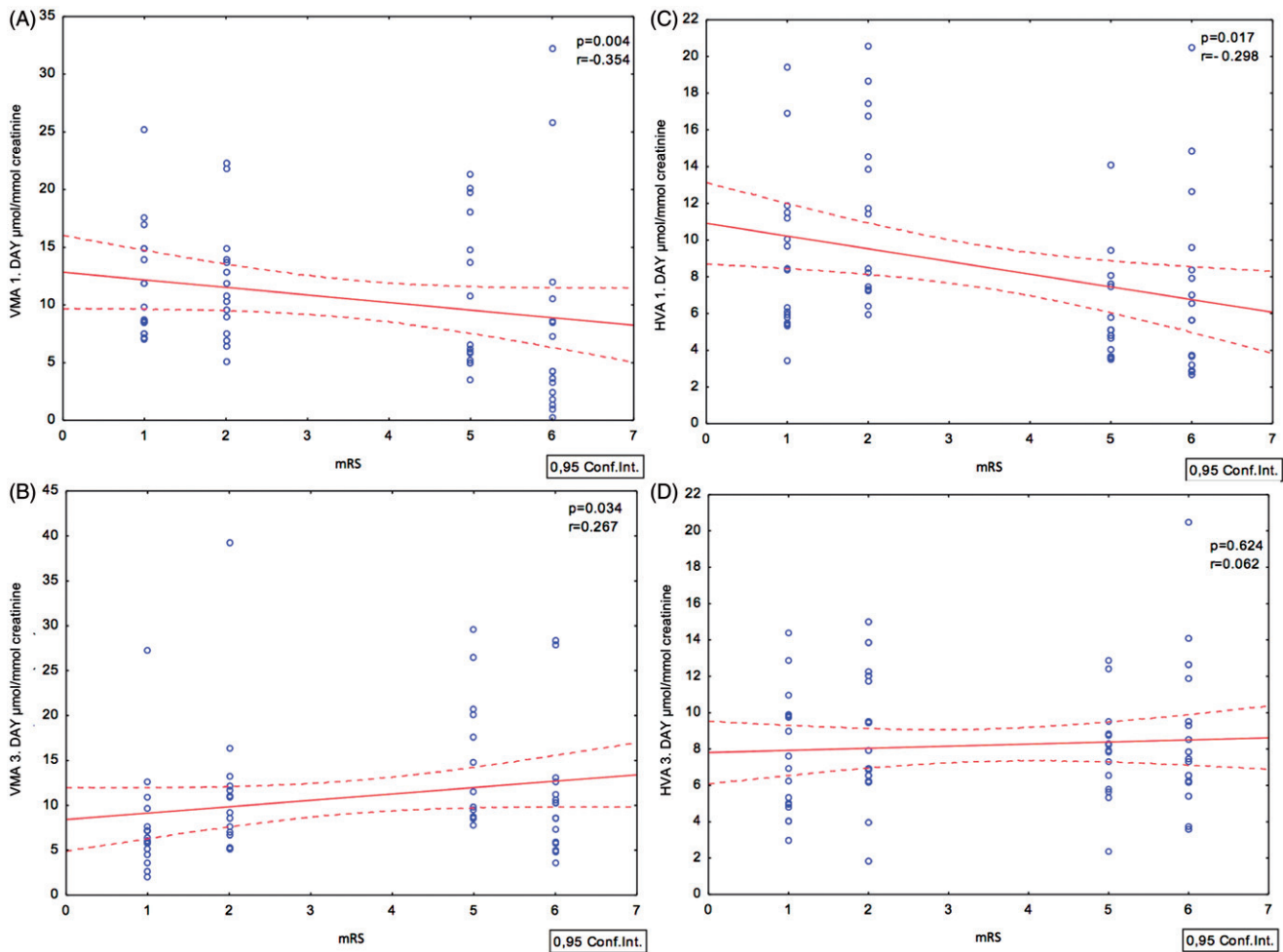


Figure 3. Correlation between vanillymandelic acid (VMA) and homovanillic acid (HVA) values and the degree of disability in mRS. VMA (A,B) and HVA (C,D) values from the patients on the first day post stroke reveal a negative correlation to outcome (modified Rankin scale – mRS). However, on the third day the correlation becomes positive, as it links the acid values to higher mRS score.

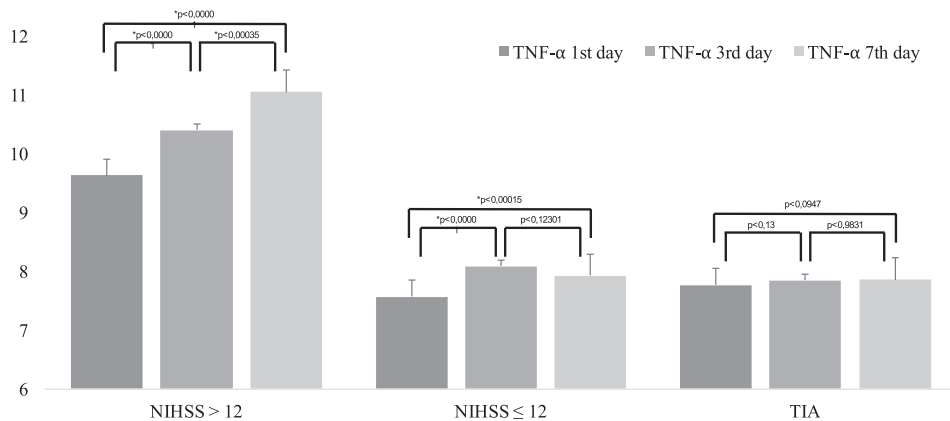


Figure 4. TNF- α values in patients with severe, mild and transitory ischaemic attack (TIA). TNF- α values in the severe ischaemic stroke group constantly increases through all three time periods (1st, 3rd and 7th day post-stroke). Interestingly, TNF- α values in the mild ischaemic group significantly increase on the 3rd day post-stroke, but decrease slightly on the 7th day post-stroke. Testing in patients with TIA didn't show any increase or decrease of TNF- α values, which remained constant through all time periods.

correlation coefficients and their levels of statistical significance is shown in Table 2. The correlation includes patients who had severe and minor IS, after 90 days ($n = 66$).

Comparing VMA and HVA values from the first day with the values of mRS for 90 days after IS, on a scatter plot shows that patients whose VMA and HVA values decreased

had higher mRS, due to the higher initial values in patients with less severe IS.

However, when comparing VMA and HVA levels from the third day, the correlation becomes positive as it links higher VMA and HVA values with more severe disability (Figure 3).

Using the chi-squared test, we compared results of patients whose HVA and VMA levels decreased or increased on the third day with degree of disability expressed in mRS. HVA ($\chi^2 = 38.5$, $p \ll 0.001$) and VMA ($\chi^2 = 35.7$, $p \ll 0.001$) levels decreased more in patients whose mRS score was lower (less severe disability), while levels increased in patients whose mRS was higher (severe disability).

TNF- α as an indicator of inflammation severity

Measuring TNF- α values was performed in three time periods: first, third and seventh day's post-stroke. In patients with severe IS (NIHSS >12) TNF- α values significantly increased on both third and seventh day post-stroke compared to the first. While the TNF- α values of patients with mild IS (NIHSS ≤ 12) significantly increased on the third day compared to the first, while on the seventh day post-stroke the values decreased compared to the third day. Testing in patients with TIA didn't show any increase or decrease of TNF- α values, which remained constant through all time periods (Figure 4).

Discussion

There is a growing body of knowledge regarding SNS activation in IS. Several papers already point out the increased catecholamine levels in patients after IS and even connect the increased levels to negative outcomes (Chamorro *et al.* 2007, Akil *et al.* 2015). Minimum information is known about the full effects of SNS activation in both the brain and the whole system. It is clear that SNS activity after stroke is increased, while the parasympathetic nervous system (PNS) activity is decreased, which leads to a proinflammatory state in the early post-stroke period, with peripheral immunodepression after the splenic reserves are depleted (Dirnagl *et al.* 2007).

Our results indicate that VMA and HVA values have the same tendency to change in both groups with IS and remain unchanged in patients with TIA. In the first group with more severe IS (NIHSS >12), we found that the values of VMA and HVA increase significantly from the first to the third day post-stroke. These changes point out to the activation of the SNS and increased release of catecholamines in the early post-stroke period. Interestingly, the group with less severe IS (NIHSS ≤ 12) had higher initial values of VMA and HVA in the urine, with them decreasing significantly on the third post-stroke day compared to the first. It appears that less severe strokes did not activate the SNS in the same strength as the more severe strokes, while the decrease in levels point out to the quick damage repair and subsequent SNS deactivation. The increased initial values in these patients can be explained by a large number of substances and foods that affect catecholamine levels along with individual variations (Numata *et al.* 1997, Shekim *et al.* 1982, Vaccarezza and Ruiz 1974, Weldin *et al.* 2003). Additionally, there is a positive correlation between the NIHSS scores and the metabolites values in the urine only on the third day, which further indicates the possible link between SNS activation and stroke severity.

Furthermore, we found that the increase or decrease of VMA and HVA values between the first and third day had a positive correlation on the outcome of patients. This data indicates that patients with unfavourable outcomes had an increase in acid values, while patients with better outcome had a decrease in acid values on the third day post-stroke in both groups. The worse outcome can be related to the various effects that increased SNS activity has an effect on the immune, cardiovascular and metabolic systems.

The innate immune system is responsible for the initial response to the ischaemia, with increased activation of microglial cells and recruitment of peripheral NK cells and monocytes due to the blood-brain barrier breakdown (Chamorro *et al.* 2012). This is also evident in the increase of proinflammatory cytokines like TNF- α , which we confirmed in our study as well. TNF- α is one of the first cytokines whose production and secretion has been linked with the occupation of alpha-adrenergic receptors (α -ARs) or β -ARs by catecholamines (Severn *et al.* 1992). Interestingly, TNF- α levels in our patients were dependent on the severity of IS, with the more severe group (NIHSS >12) levels constantly increasing from the first to third and seventh day, while the less severe group (NIHSS ≤ 12) had a significant increase from the first to third day, with a subsequent decrease in values on the seventh day. The patients with TIA did not have significant differences between measurements. These results indicate that the inflammation in the more severe group hasn't subsided, most likely due to the higher tissue damage and activation of the SNS.

Further systemic effects of SNS activation are focussed on the cardiovascular system, the spleen, liver, adipose tissue and bone marrow. The negative effects on the cardiovascular system, primarily through the activation of α -1 and β -1 adrenoceptors lead to reduced cardiac blood flow and constriction of the coronary arteries (Di Carli *et al.* 1997). Furthermore, increased SNS activity has been described as proarrhythmogenic, which in unison with previously mentioned effects, can cause myocardial infarction, sudden cardiac death and heart failure (Florea and Cohn 2014).

The effects on the cardiovascular system and the previously mentioned peripheral immunodepression can lead to negative outcomes if the SNS activity is significant. In our study, the patients with an increase in VMA and HVA values from the first and third day had negative outcomes compared to patients whose values decreased. A possible reason for that could be the higher SNS activation and the negative effects it has on cardiovascular and the immune system. On the other hand, parasympathetic nervous system is known to be anti-inflammatory, as acetylcholine attenuates proinflammatory cytokine release (Kenney and Ganta 2014). The cholinergic anti-inflammatory pathway has been described both in the CNS and in the peripheral blood through the known effector cells, the macrophages and the microglia. Acetylcholine activates the nAChR $\alpha 7$ receptors on macrophages and microglial cells, which attenuates the inflammatory response peripherally and centrally (Hao *et al.* 2011, Shytle *et al.* 2004). Furthermore, vagal nerve stimulation (VNS) has been shown to attenuate cerebral ischaemic injury in a transient model of focal cerebral ischaemia in rats (Ay

et al. 2009). VNS is used to treat epilepsy, a neurological disorder also followed by an increase in SNS activity (Connor et al. 2012). VNS and the activation of the cholinergic anti-inflammatory pathway could have therapeutic potential in patients with stroke, especially in patients who have more pronounced ANS disturbance, which we followed in this study using the HVA and VMA as markers of SNS activity. Measuring parasympathetic activity is possible in a similar way, by simultaneously measuring acetylcholine and its metabolite choline as markers in the urine (Kirsch et al. 2010). Combining the measurements of SNS and PNS could provide additional useful information regarding ANS interaction in patients with ischaemic stroke.

Conclusion

The prognostic value of measuring VMA and HVA values comes from the comparison of the first and third day of treatment, as the increase of values is linked to unfavourable outcomes, while a decrease is linked to favourable outcomes. Therefore, measuring levels of catecholamine metabolites can provide important or even unique insight into pathophysiology and course of the disease, while also helping to decide on possible therapeutic changes, especially if treatment that increases parasympathetic activity is found effective.

We conclude that determining VMA and HVA values in urine could be an effective and easily available prognostic tool for the outcome of patients suffering from IS, since taking diagnostic material is easy and sampling frequency is low.

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Disclosure statement

The authors report no conflict of interest.

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