Linear and non-linear indices of vagal nerve in relation to sex and inflammation in patients with Covid-19

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Received March 17, 2023; Accepted July 18, 2023

DOI: 10.3892/br.2023.1662

Abstract. Hyperinflammation is one of the most important pathophysiological risk factors for poor prognosis in patients with coronavirus disease-2019 (Covid-19). Low vagal neuro-immune modulation can lead into this kind of immune dysregulation. The association between vagal activity, sex and inflammatory markers were investigated in patients with Covid-19. A total of 19 patients with Covid-19 were included in the present study. Vagus nerve activity was indexed by heart rate variability (HRV) derived from electrocardiogram at hospital admission. Linear HRV parameters included the root mean square of successive RR interval differences (RMSSD) and high-frequency HRV (HF-HRV), while non-linear parameters included 2 UV%. Immune/inflammatory parameters included C-reactive protein (CRP), interleukin-6 (IL-6), neutrophil/lymphocyte ratio (NLR), systemic inflammatory index (SII), and procalcitonin (PCT). It has been revealed that both linear HRV indices HF-HRV and RMSSD, are significantly negatively correlated with CRP and IL-6, independent of age. The non-linear index of 2 UV% is significantly negatively correlated with NLR and SII, which reflect subtle changes in the response of immunocompetent cells. Patients that received high-flow nasal oxygen therapy had significantly higher IL-6 and CRP levels and lower levels of HF-HRV and RMSSD. These patients also had a significantly longer length of stay in hospital (LOS) than patients receiving low-flow oxygen therapy. Men had higher plasma PCT levels and longer LOS in hospital than women, and PCT statistically explained (mediated) the association between sex and LOS. The present study showed different correlations of linear and non-linear vagal indexes of HRV and inflammatory markers in patients with Covid-19. Significant sex differences in certain inflammatory markers were also observed, which may very well verify previous findings of poor prognosis in men with Covid-19. HRV reflects a continuous interaction between the sympathetic and parasympathetic autonomic nervous systems, which are affected by mental or physical stress, and certain disease states. The increased sympathetic and decreased parasympathetic vagal tone contribute to a higher risk of diseases associated with inflammation, cardiovascular disease, cancer, pulmonary diseases and other pathologies, including infectious diseases such as Covid-19. The present study showed that higher RMSSD (a marker of vagal activity) in Covid-19 patients is associated with lower levels of inflammatory biomarkers, a lower need for treatment and is negatively correlated with intensive care unit admission, leading to a shorter hospital stay. These findings support the idea that activation of vagus nerve may help certain Covid-19 patients by reducing the cytokine storm and excessive inflammation.

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Abbreviations: Ach, acetylcholine; CNS, central nervous system; CRP, C-reactive protein; HF-HRV, high-frequency heart rate variability; HFNO, high-flow nasal oxygen therapy; HPA axis, hypothalamic-pituitary-adrenal axis; HRV, heart rate variability; IL-6, interleukin-6; LF-HRV, low-frequency heart rate variability; LFO, low-flow oxygen therapy; NLR, neutrophil/lymphocyte ratio; NTS, nucleus tractus solitarius; RMSSD, root mean square of successive; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SDNN, standard deviation of the NN (R-R) intervals; SII, systemic inflammatory index; tVNS, transcutaneous vagus nerve stimulation; VN, vagus nerve; PCT, procalcitonin; LOS, length of stay

Key words: covid-19, heart rate variability, vagal activity, inflammation

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) outbreak began in the South East of China in

late December 2019. By 15 January 2023, a total of 6,730,187 individuals have succumbed and over 679 million individuals have been infected (1). One of the most important pathophysiological consequences and risk factors for poor prognosis in Covid-19 is hyperinflammation (2).

In an effort to control this infection, there is a strong activation of the Th1 immune system and macrophages, leading to the production of a large amount of pro-inflammatory cytokines and chemokines. Developed exaggerated immune response and hyper-inflammation may lead to vital organs failure and even death. In aged and polymorbid patients, suppression of anti-virial immunity on the one hand and hyperinflammation on the other, may predominate (2). Immune system activity and inflammation is regulated by both immune mechanisms and the nervous system-both the autonomic (ANS) and the central nervous system (CNS). The sympathetic and parasympathetic nervous systems are usually balanced for maintaining homeostasis. However, this balance is disrupted in various pathologies, and impaired parasympathetic vagal nerve activity may precede the development of inflammatory disorders (3).

A possible and very plausible cause of such immune system dysregulation is low neuro-immune modulation by the vagus nerve (VN). In acute stages of disease, activation of the sympathetic ANS enhances the release of proinflammatory cytokines and other inflammatory mediators, and to balance this response, the anti-inflammatory activity of the vagus may enhance the anti-inflammatory immune response (4).

The VN is the major branch of the parasympathetic nervous system. It is the longest nerve of the organism, providing the connection between the central nervous system and the body by innervating important visceral organs such as the heart, lungs and gastrointestinal (GI) tract (5). At least three major anti-inflammatory pathways are then orchestrated by the VN (6-10). The first is via the hypothalamic-pituitary-adrenal (HPA) axis. Inflammatory signals (mainly IL-1β) in the periphery activate vagal afferents by binding to glomus cells of most VN paraganglia that express IL-1 receptors. The afferent VN fibres inform the brain about peripheral inflammation. The increased electric activity of the neurons transmits this information to the nucleus tractus solitarius (NTS) and the area postrema (AP) in the CNS. The neurons activated in the NTS send projections to hypothalamus, from where the corticotropin-releasing hormone is released. This leads to the release of adrenocorticotropic hormone by the pituitary gland, which initiates a humoral anti-inflammatory pathway (9) that stimulates the secretion of glucocorticoids by the adrenal glands, hormones with strong anti-inflammatory effects (7,8). In this way, cortisol suppresses the excessive activity of the Th1 immune system and systemic inflammation.

The second anti-inflammatory pathway of the VN occurs via vagal efferent fibres ('vago-vagal reflex') by means of the cholinergic anti-inflammatory pathway, vago-splenic, i.e. a vago-sympathetic pathway (8,9,11). Afferent vagal nerves inform the CNS of the presence of peripheral inflammation, and in turn activate efferent vagal nerves. Since no vagal nerves have been found in the spleen, cooperation with the splenic sympathetic nerves is required. The efferent VN then activates the splenic nerve to release its neurotransmitters including norepinephrine in the spleen. Subsequently, norepinephrine activates choline acetyltransferase-expressing T cells, possibly via adrenergic receptors, and promotes the production and release of T cell-derived acetylcholine (ACh), which interacts with the α7-subunit-containing nicotinic receptor (α7nAChR) on macrophages and other immune cells (11). Activation of the transcription factors STAT3 reduces the production of proinflammatory cytokines tumor necrosis factor α, IL-1 beta, interleukin-6 (IL-6), interleukin-18 IL-18 and thus protects the body from damage (12-13).

Finally, a non-neuronal cholinergic pathway is involved in this effect (5,10). Classically the parasympathetic (i.e. the VN) and the sympathetic nervous systems have an opposite effect. However, in vago-splenic pathway, this effect is synergistic through a connection between the VN and the splenic nerve, a sympathetic nerve from the celiac ganglion (13-15). ACh is also released at the distal end of the efferent VN from vagal postsynaptic neurons and binds to alfa7nAChR on macrophages and other immune cells.

Vagus nerve activity is indexed by heart rate variability (HRV), the fluctuations in the intervals between normal heartbeats (16,17). HRV is usually measured by indices in the time and frequency domains; for example, root mean square of the successive differences (RMSSD), high-frequency HRV (HF-HRV), low-frequency HRV, and various non-linear indices, such as entropy or 2UV%. HRV is associated with important outcomes in Covid-19. Decreased HRV has been found to precede and correlate with increased C-reactive protein (CRP) in patients with Covid-19 admitted to the intensive care unit (ICU) (18). In another study of 14 patients, both higher HF-HRV and lower standard deviation of the NN (R-R) intervals (SDNN) predicted an increased risk of death (19). However, the sample of the later study was very small. In a larger study conducted on a broader sample of patients (n=271), higher HRV SDNN was found to predict a doubling of survival risk in patients with Covid-19, particularly in patients aged 70 years and older, independent of confounding factors (20). However, it remains unclear whether HRV correlates with finer indices of inflammation and whether there are differences between the linear and non-linear HRV parameters.

Another important topic to which some research has been devoted is sex differences in Covid-19. A review of 57 studies found that symptomatic Covid-19 is more prevalent in men than in women, possibly because men smoke and consume alcohol more (21). In addition, a recent study found a 2.4-fold higher risk of death in men than in women with Covid-19 (22). Another study examined sex differences in background and inflammatory biomarkers in 132 patients with Covid-19 and pneumonia. While no sex differences were found in age, total white blood cell count, CRP, albumin and d-dimer, men had higher levels of polymorphonuclear leucocytes and haemoglobin and longer length of hospital stay (LOS), and lower levels of lymphocytes and platelets than women, among others (23). However, the aforementioned study did not examine more specific types of pro-inflammatory and anti-inflammatory cytokine markers.

The aim of the present study was to investigate the relationship between the linear and non-linear indices of HRV and sex, with selected inflammatory markers and with treatment decisions for patients with Covid-19. It was hypothesized that

HRV would be inversely related to inflammatory markers and that men's HRV and inflammatory markers would be worse than those of women with Covid-19.

Materials and methods

Sample characteristics. Patients from the ICU and the Infectious diseases ward of the University Hospital in Bratislava (Bratislava, Slovakia), were consecutively enrolled in the study in January and February 2021. During this period, the alpha variant of SARS-Cov-2 was prevalent in Slovakia. Inclusion criteria was a COVID-19 diagnosis within 10 days prior to study enrolment, with hypoxemia treated with low-flow oxygen (LFO) therapy or high-flow nasal oxygen (HFNO) therapy. Exclusion criteria included coronary artery disease, left ventricular hypertrophy, moderate or severe valvular heart disease, history of or existing cardiac arrhythmias, and implanted pacemaker. Individuals taking medications that interfere with ANS function (beta blockers, inhaled beta-mimetics, atropine, glycosides, selective serotonin reuptake inhibitors, angiotensin-converting enzyme inhibitors, etc.) were also excluded from the study. A confirmed case of COVID-19 was defined as a positive result of the real-time reverse transcription PCR assay of nasopharyngeal swabs for SARS-CoV-2.

The study group consisted of 20 Covid-19 patients (10 women and 10 men), with one patient's data excluded from the study due to the high number of artefacts in the ECG recording. The study was conducted in concordance with the World Medical Association Code of Ethics (Declaration of Helsinki) for human experimentation and was approved (approval. No KRO22020) by the local Ethical Committee of the University Hospital in Bratislava, (Bratislava, Slovakia). Written informed consent was obtained from each participant prior to participation in the study. A blood sample was taken for biochemical analysis of CRP, IL-6 and blood count. The ECG was recorded with the Faros Bittium at a sampling rate of 1,000 Hz.

CRP levels were measured by immunoturbidimetry (Cobas Integra 400; Roche Diagnostics), and serum IL-6 level was determined by immunoassay (Elecsys, Roche Diagnostics). Blood counts and differential blood counts were analysed with an automated digital morphologic analyser and cell counter (Cobas m 511, Roche Diagnostics).

HRV. HRV was measured in the supine position at 23±2˚C room temperature. The ECG lasted between 7-10 min (Bittium Faros) and was checked for the presence of ectopic beats and artefacts. Artefact-free 5 min ECG segments with a stable signal were analysed for each patient by the linear analysis method using Kubios premium software Version 3.5.0 (Kubios Oy). Non-linear analysis including pattern classification was performed with a symbolic dynamics' algorithm according to Porta *et al* (2001) (24). From the linear parameters, HRV was represented by the high-frequency (HF) domain of spectral analysis in the band of 0.15-0.40 Hz (16) after applying the smoothness priors detrending (25). From time-domain analysis of RMSSD was selected. Both HF-HRV and RMSSD reflected vagally mediated HRV. Of the non-linear parameters, 2 UV% which indicates the percentage of sequences of three cardiac periods with two significant unlike variations, was selected to characterize cardiac vagal modulation (26).

Table I. Main continuous study variables.

Variable, units	Mean ± SD
Age, years	56.26±9.91
Body-mass index, kg/m^2	32.86±7.91
Inerleukin-6, pg/ml	131.63±128.98
C-reactive protein, mg/l	108.73±80.02
Eosinophils, 10^9/l	0.02±0.09
Neutrophil/lymphocyte ratio	11.94±13.83
Systemic inflammatory index, 10^9/l	2,671.43±3,501.10
High frequency heart rate variability, ms^2	9,370.05±9,869.23
Route mean square of successive differences, ms	103.62±65.82
Two-UV, %	30.67±10.01

Statistical analysis. Sample characteristics were described using the mean and standard deviations (SD) for continuous variables and percentages for categorical variables. The main analysis consisted of a series of partial Pearson correlations, where the correlations between each HRV index and inflammatory markers were examined, after statistically adjusting for the effect of age, as HRV decreases with age (27). Sex differences were examined using independent t-tests for all inflammatory and immune markers. $^*P<0.05$ was considered to indicate a statistically significant difference.

Results

Sample characteristics. The characteristics of the patients are listed in Table I. The study group of 19 patients comprised 52.6% women and 47.4% men. Their mean age (± SD) was 56.26 (±9.91) years. A total of 75% of patients were treated with HFNO and 78.9% were admitted to the ICU. In addition, the mean body mass index of the patients exceeded the threshold for obesity (32.86±7.91 kg/m^2) and the mean CRP level substantially exceeded the reference range (108.73±80.02 mg/l).

Correlations between HRV and inflammatory markers. The correlations between the linear frequency-domain HRV parameter of HF-HRV, the linear time-domain HRV parameter of RMSSD, and the non-linear HRV parameter of two-UV with inflammatory/immune markers are demonstrated in Table II. The two-UV are significantly negatively correlated with neutrophil/lymphocyte ratio (NLR) and with systemic inflammation, adjusting for the effects of age. By contrast, HF-HRV is significantly negatively correlated with CRP and IL-6 levels and showed a positive correlation with the number of eosinophils (an early marker of infection and inflammation), adjusting for the effects of age. Finally, the time domain HRV parameter of RMSSD also significantly negatively correlated with CRP and IL-6, adjusting for age.

Table II. Correlations between HRV parameters and inflammatory markers, statistically controlling for effects of age.

	Inflammatory markers				
HRV index	CRP	NLR	SII	Eo	IL-6
Two-UV	-0.11	-0.50	-0.49	0.11	0.06
HF-HRV	-0.71	-0.24	-0.29	0.52	-0.49
RMSSD	-0.56	-0.23	-0.28	0.32	-0.55

HRV, heart rate variability; CRP, C-reactive protein; NLR, neutrophil/lymphocyte ratio; SII, systemic inflammatory index; Eo, eosinophils; IL-6, interleukin-6; HF-HRV, high frequency heart rate variability; RMSSD, route mean square of successive differences.

When testing treatment capacity, comparison of HRV and inflammatory marker values (Table III) revealed that patients receiving HFNO had significantly higher levels of IL-6 [t(15.62)=2.92, P=0.01], CRP [t(17)=2.37, P=0.03], lower levels of HF-HRV [t(17)=4.73, P<0.001] and lower levels of RMSSD [t(17)=4.26, P=0.001]. In addition, patients admitted to the ICU had significantly higher levels of IL-6 [t(16)=16.78, P<0.05], lower levels of HF-HRV [t(17)=3.81, P=0.001], and RMSSD [t(17)=3.60, P<0.005]. Finally, the association of treatment type with LOS showed that those receiving HFNO stayed in hospital significantly longer (21.0 days) than those receiving LFO treatment [9.2 days; t (16.98)=2.74, P<0.05].

Sex and inflammatory markers. Sex differences in HRV and inflammatory markers were examined. Using log-transformed data for most biological data, it was found that men tended to have lower SDNN levels than women [t(17)=1.76, P<0.10], and significantly higher PCT levels than women [t(10.04)=2.81, P<0.05], and significantly longer LOS than women [t(8.75)=2.36, P<0.05]. No other significant differences were found between men and women. These results are shown in Table IV.

Since PCT, reflecting inflammation, was associated with sex, and since PCT was also correlated with LOS (r=0.63, P<0.005), it was examined whether PCT statistically mediated (explained) the association between sex and LOS. Indeed, after statistically controlling for PCT, sex was no longer associated with LOS (r=-0.24, P>0.05). By contrast, after statistically controlling for the effects of sex, PCT was still significantly correlated with LOS (r=0.47, P<0.05). Thus, PCT statistically mediated or explained the association between sex and LOS.

Discussion

In the present study the relationships between various vagus nerve indices of HRV and immune and inflammatory markers in patients with Covid-19 were investigated. Sex differences in HRV and inflammatory and clinical variables were also examined. The findings of the present study revealed that both linear HRV indices of the frequency-domain (HF-HRV) and time-domain (RMSSD) are significantly and negatively correlated with inflammatory markers CRP and IL-6. By contrast, the non-linear HRV index two-UV are significantly and negatively correlated with NLR and systemic inflammatory index (SII), independent of the effects of age. Regarding sex differences, it was found that men had elevated PCT levels and longer LOS in hospital than women, and that the PCT marker statistically explained (mediated) the association between sex and LOS.

SARS-CoV-2 infection activates innate and adaptive immunity responses that can lead to a massive and uncontrolled inflammatory response with the development of a 'cytokine storm', eosinopenia and lymphopenia (28). Since macrophages can contribute to the excessive inflammation by producing IL-6 and other pro-inflammatory cytokines, 'macrophage activation syndrome' may explain the high serum CRP levels (29). IL-6, often together with CRP, have been reported as predictors of the development of severe Covid-19 not only in Slovakia (30), but also elsewhere (31-33). However, these humoral indicators of disease severity are often preceded by a decrease in lymphocyte and eosinophil counts, reflecting the recruitment of antiviral immune cells to fight infection. This is associated with frequent neutrophilia (34) in which leads to elevated immune-cell-based inflammatory markers, such as NLR and SII. The relationship between these indices (for example, NLR) and disease severity is not as unequivocal as that of IL-6 and CRP. Increased NLR levels has been observed in patients with Covid-19 in Johannesburg, South Africa but this parameter was not significantly related to prolonged LOS (35). The NLR has been shown to be useful in identifying young patients with severe Covid-19 (≤40 years) (36). The NLR, but not the SII, had a significant predictive value for Covid-19 mortality in a study of 108 hospitalized patients from Timisoara, Romania (37). By contrast, both NLR and SII were able to predict severe disease in Ratlam district of Madhya Pradesh State, India, where both indices had a significantly higher value in ICU-admitted patients compared with non-ICU admitted patients (38).

In the present study, the diagnostic value of IL-6 and CRP levels in predicting the severity of the disease in terms of the need for more sophisticated treatment and ICU admission was demonstrated. In addition, HFNO treatment was associated with longer LOS. As aforementioned, these two inflammatory indices correlated negatively with time and frequency domain HRV parameters in the present study, suggesting the clinical significance of these associations. These findings regarding the relationship between HRV and inflammation reflect and extend recent findings showing that a reduction in HRV in Covid-19 patients admitted to the ICU is preceded in time and correlates with an increase in CRP (18). The findings of the present study are also consistent with the known inhibitory role of the vagus nerve in inflammation via activation of the HPA axis, cortisol secretion (39), and via splenic T-cells that inhibit the production of inflammatory cytokine by macrophages (14).

The cell-based immune inflammation markers NLR and SII were both significantly and negatively correlated with the non-linear VN-associated parameter of symbolic dynamics, namely 2 UV%. Both NLR and SII were not related to the need for HFNO therapy, ICU admission or LOS. In general, it is considered that the linear HRV parameters do not to fully

Table III. Mean values of HRV and inflammatory indexes as function of medical treatments.

	Treatment		Intensive care unit	
Index, unit	Low	Heavy	No	Yes
Inerleukin-6, pg/ml	47.40	161.71	54.50	152.20
C-reactive protein, mg/l	43.80	131.91	46.50	125.32
Neutrophil/lymphocyte ratio	6.84	13.77	4.98	13.80
Systemic inflammatory index, 10^9/l	1,264.27	3,173.99	871.00	3,151.54
Eosinophils, 10^9/l	0.08	0.01	0.10	0.01
High frequency heart rate variability, ms^2	21,848.84	5,401.91	22,349.35	6,364.91
Route mean square of successive differences, ms	180.67	76.11	185.23	81.86
Two-UV, %	32.82	29.90	33.25	29.98

Table IV. Main study variables in men and women.

Variable, unit	Men Mean ± SD	Women Mean ± SD
Age, years	54.0±9.7	58.3±10.2
Body mass index, kg/m^2	34.6±8.4	31.3±7.5
Standard deviation of N-N intervals, ms	77.2±51.1	131.1±65.1
Root mean square of successive differences, ms	71.2±53.1	132.8±64.5
High frequency heart rate variability, ms^2	4,434.5±3,538.0	14,496.1±11,419.0
Two-UV, %	27.6±12.6	33.4±6.4
Interleukin-6, pg/ml	174.2±148.0	93.3±101.8
C-reactive protein, mg/l	128.6±81.6	90.8±78.3
Sepsis-related organ failure assessment	2.9±2.0	2.0±0.0
Procalcitonin, ng/ml	0.5±0.7	0.0±0.0
Neutrophil/lymphocyte ratio	12.3±17.0	11.6±11.2
Systemic inflammatory index, 10^9/l	2,585.8±4,219.7	2,748.5±2,945.1
Length of hospital stay, days	24.9±16.5	11.6±3.8

characterize the complex dynamics of the ANS activity, which is non-linear in humans. The non-linear indices may therefore better reflect the complexity, irregularity, and dynamic properties of HRV signals and are often more sensitive than the linear ones (40-42). This may explain why 2 UV% correlated significantly with two cellular-based inflammatory markers NLR and SII that reflect more subtle changes in immunocompetent cells that normally precede the excessive production of IL-6 and CRP. Future studies with larger samples need to replicate the findings of the current study and investigate the prognostic value of these different inflammatory markers in Covid-19.

Due to the small sample size and the fact that all immune parameters in the present study were measured when the patients were already hospitalised, these interpretations should be taken with caution. The cytokine storm was already present and determined the clinical outcome. The need for HFNO treatment and admission to the ICU correlated positively with serum levels of IL-6 and CRP, suggesting that these immune parameters reflect stronger and less subtle indices of inflammation. It has already been established that time-domain HRV indices SDNN and RMSSD predict survival and ICU admission of patients with Covid-19, respectively, although they were measured using only 10 sec ECG recordings (17). In the present study, using 5 min ECG recordings, we confirmed that a higher RMSSD value is closely associated with a lower need for treatment and negatively correlates with ICU admission, which in turn leads to a shorter LOS. Thus, the present data support and extend the findings of a recent study describing that higher vagal activity is associated with a shorter hospital LOS in the under-40 age group (43).

The sex differences found in the present study correspond in part to the results of previous studies. As in the study by Akkurt *et al* (23), it was also found that men have a longer LOS than women. These findings also extend to more precise inflammatory markers such as PCT. In addition, it was found that PCT mediated the association between sex and LOS, indicating the crucial role that the inflammatory response plays in sex differences in LOS. Future studies should also investigate whether sex differences in inflammation are also responsible for the higher risk of death in men compared with women with Covid-19 (22).

The present study had several limitations. First, as aforementioned, the sample size was small, possibly masking additional associations. Second, it was a cross-sectional study rather than a longitudinal or experimental study, so causal inferences were not possible. Third, a 24-h ECG would give more weight to the data and conclusions. Finally, a large number of statistical tests relative to the sample size, possibly leading to a Type-1 error was performed. Future studies should replicate these results with a larger sample and a longitudinal design.

If replicated, the results of the present study, in agreement with others, suggest that activation of the vagus nerve using transcutaneous vagus nerve stimulation (tVNS) may reduce the cytokine storm in patients with Covid-19 and improve their prognosis. In a recent clinical trial, tVNS was found to help patients with Covid-19 (44). In addition, another recent study found that vagal activation through slow-paced breathing also reduced inflammation in Covid-19 (45). Taken together, these studies point to the importance of the vagus nerve in neuro-immunomodulation in Covid-19.

Acknowledgements

Not applicable.

Funding

The present study was supported by The Ministry of Education, Science, Research and Sport of the Slovak Republic (grant. nos. VEGA 1/0090/22 and 2/0020/17) and the Slovak Research and Development Agency (grant no. APVV-22-0231).

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LH and YG conceptualized the study. PS developed methodology and conducted investigation. MZ performed software analysis. MZ and YG analyzed and validated data. LH and YG wrote the original draft. IM and MB made contributions to the conception and design of the study, reviewed and edited the manuscript. MB supervised the study. LH and MB acquired funding. LH and PS confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study was carried out in concordance with The Code of Ethics of the World Medical Association (the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards) for experiments involving humans and was approved (approval no. KRO22020) by the local Ethical Committee of University Hospital Bratislava (Bratislava, Slovakia). Written informed consent was obtained from each participant before enrolment.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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