


Cerebrovascular pattern improved by ozone autohemotherapy: an entropy-based study on multiple sclerosis patients

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Abstract Ozone major autohemotherapy is effective in reducing the symptoms of multiple sclerosis (MS) patients, but its effects on brain are still not clear. In this work, we have monitored the changes in the cerebrovascular pattern of MS patients and normal subjects during major ozone autohemotherapy by using near-infrared spectroscopy (NIRS) as functional and vascular technique. NIRS signals are analyzed using a combination of time, time–frequency analysis and nonlinear analysis of intrinsic mode function signals obtained from empirical mode decomposition technique. Our results show that there is an improvement in the cerebrovascular pattern of all subjects indicated by increasing the entropy of the NIRS signals. Hence, we can conclude that the

ozone therapy increases the brain metabolism and helps to recover from the lower activity levels which is predominant in MS patients.

Keywords Ozone autohemotherapy · Near-infrared spectroscopy · MANOVA · Multiple sclerosis · Time–frequency · Entropy · Empirical mode decomposition · Cerebrovascular pattern

1 Introduction

Ozone therapy has witnessed an increasing set of applications in recent years. Ozone can be administered by major ozone autohemotherapy, which consists in drawing a given amount of venous blood (approximately 240 g) and then in reinfusing it after it has been added to a mix of oxygen and ozone [46]. Ozone is effective as anti-bacterial, anti-fungal, and anti-viral agent [46] to treat vascular pathologies [7] and generally to improve tissue oxygenation and microcirculation [11, 41]. Ozone plays a fundamental role in inflammation: it induces a mild oxidative stress that, in turn, promotes the generation of anti-oxidative species [40]. In fact, animal studies have shown that ozone therapy is effective in protecting organs from reperfusion damages [10, 14]. Hence, ozone shows marked vascular and metabolic therapeutic properties.

Multiple sclerosis (MS) is a degenerative neurological pathology characterized by both vascular and metabolic impairments. If the vascular implications of chronic venous insufficiency in MS are still debated [12, 34], the increased level of oxidative stress found in MS patients has been clearly demonstrated [26]. Therefore, ozone therapy has a rationale of application as an adjuvant treatment for MS patients.

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The quantification of vascular and metabolic cerebral effects of ozone therapy on patients is not a trivial task. In fact, cerebral autoregulation maintains a constant the level of oxygen which is supplied to the brain, independently with possible changes in the systemic oxygen saturation. Therefore, complex methods and techniques are needed to investigate the cerebrovascular and metabolic effects of ozone therapy. In our previous study, we analyzed the long-term effects of ozone autohemotherapy in MS patients and controls by using transcranial Doppler sonography and near-infrared spectroscopy (NIRS) [25, 33]. Near-infrared spectroscopy (NIRS) is a non-invasive technique that can potentially measure in real time the vascular and metabolic characteristics of the brain tissues. By using infrared light, NIRS can monitor the changes in the concentrations of the oxygenated (O_2Hb) and reduced (HHb) hemoglobin in brain tissues [16], thus providing vascular and flow related information. To the best of our knowledge, only two studies used the NIRS technique to monitor the long-term cerebrovascular changes occurring during ozone hemotherapy [25, 33].

Some NIRS devices help to measure the concentration changes of the cytochrome-c-oxidase, whose concentration is related to the level of mitochondrial activity of the neurons [5], thus to brain metabolism. We documented that ozone autohemotherapy increased the level of oxygen in the brain up to 1.5 h after the reinfusion of the ozonized blood, without any adverse vascular effect [33]. Moreover, we showed that the MS subjects had an increase in the cytochrome-c-oxidase activity and concentration about 40 min after the end of the treatment. This result demonstrated the positive metabolic effect of ozone in reducing the level of oxidative stress and promoting brain metabolism.

Despite its potentialities, the reliability of the NIRS measurements can be decreased by confounding factors: (1) the contribution of skin and superficial vasculature to the absorption of infrared light [9], (2) concentration of the molecule to track and (3) noisier and less reliable signal. In fact, some recent NIRS devices introduced a spatially resolved technique to lower the effect of the confounding factors [9] and removed the possibility of measuring the concentration of the cytochrome-c-oxidase.

The NIRS signals, however, carry considerable information in the frequency domain as well. Obrig et al. [35] showed that cerebral NIRS signals allow the evaluation of the cerebral vasomotor reactivity and autoregulation. In our previous studies, we combined time and frequency analysis of NIRS signals in order to extract physiological information while trying to avoid the possible noise sources [24, 28, 32]. Furthermore, cerebral NIRS signals acquired when the subject is not in resting conditions might be nonstationary in nature; hence, ad-hoc time–frequency analysis

should be used [24, 30]. Given the complex interaction between infrared light and tissues, NIRS signals are nonlinear in nature. Therefore, the time and time–frequency analysis might be insufficient to capture the subtle variations from the signals, whereas nonlinear techniques can provide more accurate information [3, 17, 31, 32].

In this paper, we performed a linear and nonlinear analysis of cerebral NIRS signals acquired from MS patients and controls during major ozone autohemotherapy. The principal aim of this work is to assess the effect of the ozone autohemotherapy on the subjects' cerebral pattern even without monitoring the cytochrome-c-oxidase concentration. The secondary aim is to propose a general framework for the processing of NIRS signals that can lessen the effects of confounding factors, by relying on a robust and local structure based such as the empirical mode decomposition (EMD).

2 Materials and methods

This section describes the essential steps of the ozone autohemotherapy protocol and the corresponding registration windows. The overall therapy lasted about 3 h per patient, but the signals were acquired only at specific time steps. This section describes the time, time–frequency, and nonlinear analysis methods used.

2.1 Ozone autohemotherapy

The major ozone autohemotherapy protocol consists of the following steps:

1. drawing of 240 grams of whole blood from the subjects (in our case from the antecubital vein) by using SanO3 bags (Haemopharm, Milan, Italy);
2. mixing of the blood with 240 ml of O_2/O_3 , composed by O_2 at 50 %, with an O_3 concentration equal to 40 $\mu\text{g/ml}$ (Medical 95 CPS, Multiossigen, Gorle (BG), Italy);
3. slow blood re-infusion in the same vein.

During the ozone therapy, we acquired NIRS cerebral signals from the left and right forefront of the subjects. We used a NIRO200 device (Hamamatsu Photonics K.K., Japan), equipped by two probes, which consisted of a photo-detector and four infrared LED sources (wavelengths equal to 775, 810, 830 and 910 nm). Each probe was placed on the subject's forehead. To avoid the sinuses, we placed the probes 2 cm away from midline and 1 cm above the supraorbital ridge [28]. The sampling frequency of the system was set to 2 Hz. The pre-processing consisted in a IIR digital 8th order Butterworth band-pass filter, with

lower frequency equal to 20 mHz and an upper bound set to 250 mHz.

The subjects were asked to stay supine, keeping eyes closed and breathing normally. The NIRS device measured in real time the concentration changes of the O₂Hb and HHb for both the hemispheres. Since we did not find any statistically significant difference in the concentration changes of the two hemispheres (paired *Student's t test*; $p > 0.8$), we averaged the signals of the two hemispheres. Hence, for each subject, we recorded the O₂Hb and HHb concentrations. We analyzed the acquired signals in 7 different time intervals, lasting 256 s each:

1. baseline recording;
2. blood drawing;
3. middle of reinfusion;
4. end (last 256 s) of reinfusion;
5. 40 min after reinfusion;
6. 1 h after reinfusion;
7. 1.5 h after the reinfusion.

We chose these analysis windows in order to observe specific event-related and long-term changes. For each window and on each patient, we performed time domain analysis of the signals, by computing the average of the signal values in each of the observation windows.

2.2 Time–frequency analysis

The NIRS signals are characterized by a marked nonstationary nature and hence time–frequency analysis is needed [24, 30, 33]. We have used the Choi–Williams (CW) distribution, which belongs to the Cohen’s class of bilinear time–frequency transforms [6, 30]. The mathematical formulation of the CW is the following:

$$D_x(t, f) = \iiint_{-\infty}^{+\infty} x\left(t' - \frac{\tau}{2}\right) x^*\left(t' + \frac{\tau}{2}\right) \times g(\tau, \theta) e^{j2\pi\theta(t'-t)} e^{-j2\pi f\tau} dt' d\theta d\tau \quad (1)$$

where $D_x(t, f)$ is the time–frequency representation; t and f represent time and frequency, respectively; θ and τ the frequency and time lag, respectively; $g(\tau, \theta)$ the kernel of the CW distribution, which can be expressed as:

$$g(\tau, \theta) = e^{-\frac{\theta^2 \tau^2}{\sigma}} \quad (2)$$

The parameter σ in Eq. (2) is called kernel selectivity, and it can be tuned in order to avoid interference terms. In previous studies, it was shown that a value in the range 0.1–1 was suitable for EMG analysis [29] and NIRS signals [24]. In our study, we chose the value of 0.5 in order to

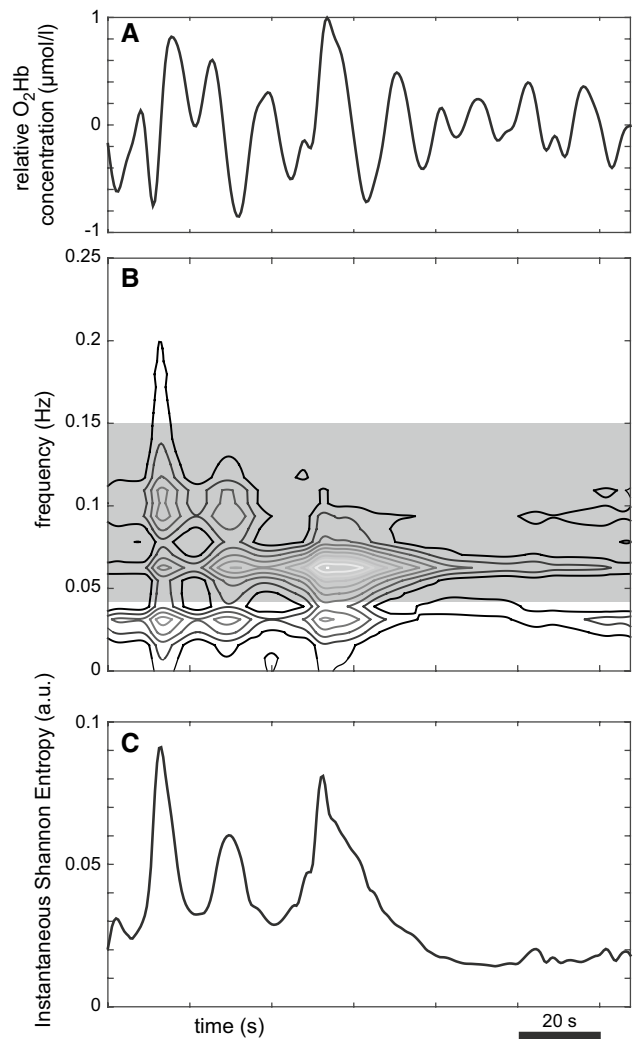


Fig. 1 a Time course of the concentration of O₂Hb during baseline. b CW transform of the signal. The gray zone represents the LF band. The gray rectangle depicts the LF band. c Corresponding instantaneous SE derived from the CW transform

balance between spectral resolution and time localization of the signal components.

We computed the CW distributions for O₂Hb and HHb signals in every analysis window. Figure 1a reports the O₂Hb concentration changes during the baseline recording of healthy subject, and Fig. 1b shows its corresponding CW representation with function of time and frequency. From the time–frequency representation, we computed the relative power of the low-frequency (LF) band, which means the ratio between the power in the LF band and the total signal power. The relative LF power was computed in each time window. The LF band spans from about 40 to 150 mHz, and it reflects spontaneous oscillations in the cerebral cortex [35]. Such oscillations have a debated physiological meaning; however, they are called as “vasomotor

waves” because they reflect the physiological vasomotor reactivity. They are correlated with the firing frequency of the baroreceptors, which are the triggers of the regulation of the vascular system [35]. The LF band is depicted by the gray box in Fig. 1b.

2.3 Instantaneous Shannon entropy

The Shannon entropy (SE) is a measure derived from the field of information theory [23]. SE is based on the probability distribution; hence, to make it applicable in signal processing, the power spectral densities must be normalized. However, our signals are nonstationary, so we defined the SE based on the time–frequency representation $D_x(t, f)$ of the signals. First of all, we normalized the power of the $D_x(t, f)$ as follows:

$$d_x(t, f) = \frac{D_x(t, f)}{\iint_{-\infty}^{+\infty} D_x(t, f) dt df} \tag{3}$$

in order to make the overall power equal to 1. Then we defined the instantaneous SE as:

$$SE(t) = - \int_{-\infty}^{+\infty} d_x(t, f) \cdot \ln(d_x(t, f)) df \tag{4}$$

Figure 1c reports the SE for the signal in Fig. 1a. The time course of the SE was averaged to compute a single value for each signal.

2.4 Empirical mode decomposition (EMD)

The empirical mode decomposition is a form of time–frequency representation of the signal that does not require linearity. Similar to the Fourier transform, the EMD decomposes the signals into a series of intrinsic mode functions (IMF), which should all have zero mean and must be orthogonal [20]. EMD is adaptive and considers the local characteristics of time and scale of the signal.

The core of the EMD is the *sifting* procedure, which consists in an iterative algorithm that, at each step, removes from the signal the mean of the envelope of the maxima and of the minima of the signal itself. By summarizing, the *sifting* procedure can be described as follows. Let’s consider a signal $x(t)$. Let $M(t)$ be the spline interpolation of the maxima of the signal, and $m(t)$ be the spline interpolation of the minima of the signal. The mean of the two maxima and minima envelopes is computed as $\bar{M}(t) = [M(t) + m(t)]/2$, and it is subtracted from the signal to generate $h(t) = x(t) - \bar{M}(t)$. The signal $h(t)$ now becomes the new signal, and the procedure is repeated until the $\bar{M}(t)$ is nearly null. At this point, the resulting $h(t)$ is

named as the first IMF. The extracted IMF is subtracted by the original signal $x(t)$, and the procedure is repeated to find the other IMFs.

In our work, we considered the first three IMFs of O₂Hb and HHb signals. We have computed two nonlinear metrics: the Sample Entropy (SampEn) and the Hurst Exponent (HE) from the extracted IMFs.

2.5 Sample Entropy (SampEn)

The rationale to compute the SampEn is that this entropy metric is based on the temporal time series instead of relying on the power spectral density [39]. The time series is used to derive vector sequences of length m (called embedding dimension) [36, 37], so that each vector sequence has the form:

$$u(n) = \{h_i(n), h_i(n + 1), \dots, h_i(n + m - 1)\} \tag{5}$$

where $h(1), h(2), \dots, h(N)$ are the N samples of the time series. The distance between each vector segment, and the others is computed by using the Chebyshev distance [43]. Given a distance threshold equal to r , each vector sequence is considered and the number of other segments with a distance lower than r is computed. This number, divided by the total number of vectors, indicates the probability of locating another vector not beyond the distance r from the considered vector $u(n_i)$. Let’s call this conditional probability as $C_{n_i}^m$. The overall conditional probability is given by the sum over the total number of vector sequences:

$$\phi^m(r) = (N - m + 1) \sum_{i=1}^{N-m+1} C_{n_i}^m(r) \tag{6}$$

The SampEn is then defined considering also the overall probability found for an embedding dimension equal to $m + 1$; hence, SampEn is defined as:

$$\text{SampEn}(m, r, N) = - \ln \left[\frac{\phi^m(r)}{\phi^{m+1}(r)} \right] \tag{7}$$

The basic concept of this metric is that in time series with a high degree of entropy, the vectors (epochs) of length m that match point-wise within a tolerance r also match at the next point. In our study, we considered a value of m equal to 2 [44] and a distance threshold r equal to 0.2 times the standard deviation of the data. The embedding dimension was chosen shorter than the oscillation period of the NIRS frequencies, whereas the r parameter was chosen large enough not to be excessively selective in discarding similarities among the time series. Similar values were used in previous works dealing with biomedical signals [42].

2.6 Hurst exponent (HE)

The Hurst exponent (HE) is proposed to measure the presence/absence of long-range in a signal and dependence degree [21]. We adopted this metric because it has been shown that when the physiological system modifies its behavior, the HE shows corresponding changes. In neurological studies, as examples, the HE was used to discriminate between interictal and epileptic EEG [18] and to characterize the EEG in different sleep stages [1].

The Hurst exponent is a value $0 < H < 1$, and the main interest of its use is the detection of long-range dependency in the data, which is given with a HE significantly greater than 0.5. A HE around 0.5 indicates random data (white noise), and $HE < 0.5$ indicates short-range dependency. Normal signals are usually characterized by HE values that decrease as the system is normalizing its activity [22].

Given a time series $h(n)$ of time length equal to T , the mean of the time series is computed and then subtracted by the time series itself. This produces a zero *mean* time series $h'(n)$. The cumulative deviation of $h'(n)$ is then computed by summing up all the elements. Let's define R as the range of the cumulative deviation of $h'(n)$ (i.e., the difference between the maximum and the minimum value) and S as the standard deviation of $h'(n)$. The HE is then defined as:

$$HE = \frac{\log(R/S)}{\log(T)} \quad (8)$$

2.7 Patients demographics

The experiment was conducted after having obtained the approval from the Review Institutional Board of the “Un Passo Insieme ONLUS Foundation” (Valdellatorre, Torino, Italy), of the “KAOS ONLUS Foundation” (Caselle Torinese, Torino, Italy), and of the Scientific Society of Oxygen Ozone Therapy (SIOOT, Gorle (BG), Italy). The approved informed consent was read and explained to the subjects before the tests, and they were required to sign it.

We recruited 10 subjects suffering from remitting–relapsing (RR) multiple sclerosis, with a confirmed diagnosis of pathology whose onset was at least 5 years before. Two MS-RR subjects successfully underwent therapy, but interrupted the recording and did not reach the end of the monitoring. These two subjects were excluded from the analysis. Thus, our sample group consists of 8 MS-RR and 10 controls. The average age of the 8 patients who completed the therapy was 40.2 ± 3.9 years, and 4 patients were females. The years from the onset of the pathology were 7.3 ± 3.3 years, and the EDSS average score was in the range 1.5–3.5. All the subjects followed a therapy consisting in beta interferon injections, and corticosteroids administered in correspondence of attacks. In control

group, we enrolled 10 volunteers (age: 60.1 ± 4.2 years, 6 females), without any neurological, metabolic, or cardiovascular disease. Heavy smoking and hypertension subjects were excluded.

2.8 Statistical analysis

Grouped data are expressed by mean \pm SD. The Kolmogorov–Smirnov test is used to test normality of the data, followed by a Student's t test to check differences in the mean values. For all the tests, the first error species probability is set to 5 % (i.e., confidence level of 95 %).

Multivariate analysis of variance (MANOVA) is used to compare the entropy data among the two groups and their change from the beginning to the end of the monitoring. MANOVA represents the subjects in function of the so-called canonical variables, which are linear combination of the original features. The overall number of the original features is 98 for each subject, 14 for each analysis window. The 14 features are the SE of O_2Hb and HHb signals (2 features), and SampEn and HE of the three IMFs from O_2Hb and HHb signals (12 features). Feature reduction is performed by removing the variables that explained a statistically low amount of overall data variability by using the Wilk's lambda [15]. The number of features decreased from 98 to 35 and the MANOVA is performed on these remaining variables. The original features that contributed the most to the canonical variables were considered as the most important to assess the changes in the cerebrovascular pattern induced by the ozone therapy and captured by NIRS.

3 Results

In this work, we studied the temporal changes, spectral changes, and the complexity changes of NIRS signals acquired from the brain of MS patients and control subjects during major ozone autohemotherapy. The purpose of this work is to document the vascular and metabolic changes induced by ozone therapy in normal and pathological cerebral tissues.

3.1 Time changes in brain oxygenation

Figure 2 depicts the average concentrations of O_2Hb (Fig. 2a) and HHb (Fig. 2b) signals. The concentrations are normalized and scaled with respect to the first window (baseline), which is thus zero valued. In accordance with previous studies, we found that ozone autohemotherapy increased the level of brain oxygenation both in controls and MS subjects [25, 33] (Fig. 2a). There are, however, some differences in the two groups. The MS subjects

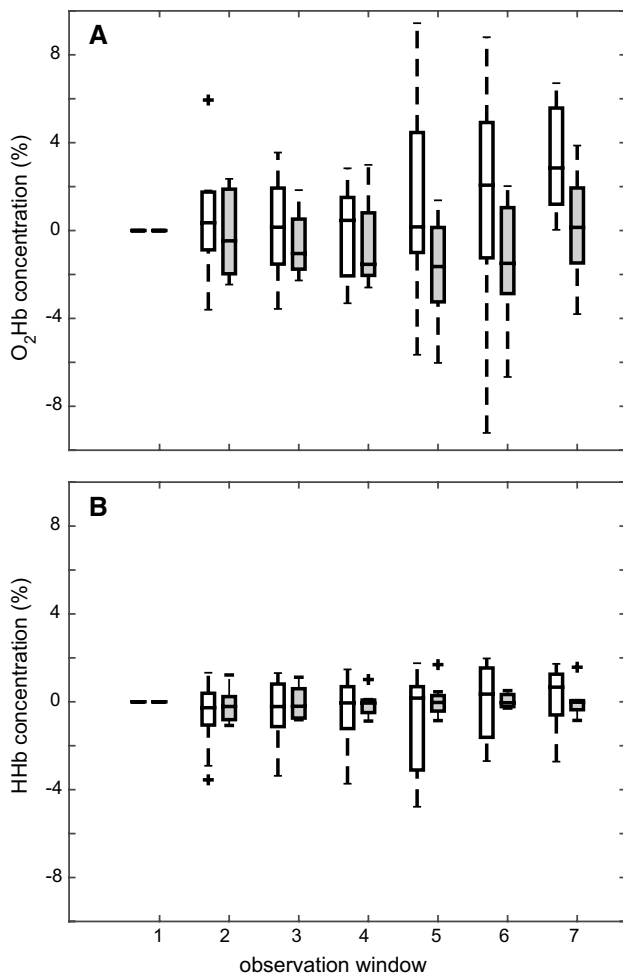


Fig. 2 Boxplot showing the time evolution of the average concentration of O₂Hb and HHb in the seven analysis windows. All the values are normalized and scaled w.r.t. the first window. Controls are depicted in *white*, MS patients in *gray*

showed a decreasing concentration of O₂Hb, more evident in the fifth window (40 min after the end of blood reinfusion). Then, the oxygen concentration raised and reached positive levels (compared to window 1) at the end of the

monitoring, i.e., 1.5 h after the end of blood reinfusion. Controls, conversely, showed increased O₂Hb concentrations in windows 5 to 7. This difference in the two groups might be explained by an increase in the oxygen consumption caused by the therapy on MS subjects. In fact, previous studies [25, 33] showed that ozone therapy increased the level of mitochondrial activity, which lead to a higher oxygen consumption by neurons. Hence, a hypothesis is that the increase in the metabolic brain activity, occurring in MS subjects, lowered the overall O₂Hb concentration, despite the reinfusion of highly saturated blood. Figure 2b demonstrates that the concentration change of HHb, which is not relevant during the therapy and that its variations are statistically equal for MS patients and control subjects. In none of the analysis windows, the concentrations of O₂Hb and HHb are statistically different in MS patients and controls (always $p > 0.2$). The numerical values of concentrations of O₂Hb and HHb are reported in Table 1. By using a Student's *t* test, we observed that none of the concentrations in the different windows were statistically different from zero (i.e., from the baseline; $p > 0.1$).

3.2 Frequency changes in NIRS signals

Obrig et al. [35] shown that the spontaneous oscillations of NIRS signals can be used to assess the subject's cerebrovascular reactivity. Our time–frequency analysis showed how the power associated in the LF band, thus in the vasomotor tone, changed during the therapy. Figure 3 represents the changes in the LF power (normalized to the total power of the signal) of control and MS subjects during the therapy. It can be noticed that during blood drawing (window 2), the power in the LF band increased significantly compared to baseline ($p = 0.01$) for the O₂Hb signal of control subjects, and for the HHb signal of MS patients ($p = 0.02$). This effect is due to the vagal response to the needle insertion for blood drawing. In the rest of the therapy, we did not observe significant LF changes in the O₂Hb and HHb signals. Table 2 summarizes the LF relative powers in the seven analysis windows.

Table 1 Relative concentrations of O₂Hb and HHb in the analysis windows

	Controls		MS-RR group	
	O ₂ Hb (%)	HHb (%)	O ₂ Hb (%)	HHb (%)
Window 1	–	–	–	–
Window 2	0.362 ± 2.657	–0.633 ± 0.545	–0.151 ± 1.963	–0.180 ± 0.755
Window 3	0.029 ± 2.394	–0.386 ± 1.436	–0.625 ± 1.463	–0.050 ± 0.750
Window 4	–0.247 ± 2.126	–0.593 ± 1.716	–0.644 ± 2.002	–0.100 ± 0.574
Window 5	1.453 ± 4.549	–0.791 ± 2.223	–1.768 ± 2.474	0.062 ± 0.771
Window 6	1.334 ± 5.004	0.037 ± 1.766	–1.410 ± 2.882	0.045 ± 0.308
Window 7	1.997 ± 4.743	0.201 ± 1.449	0.160 ± 2.489	0.009 ± 0.715

The first window is taken as reference

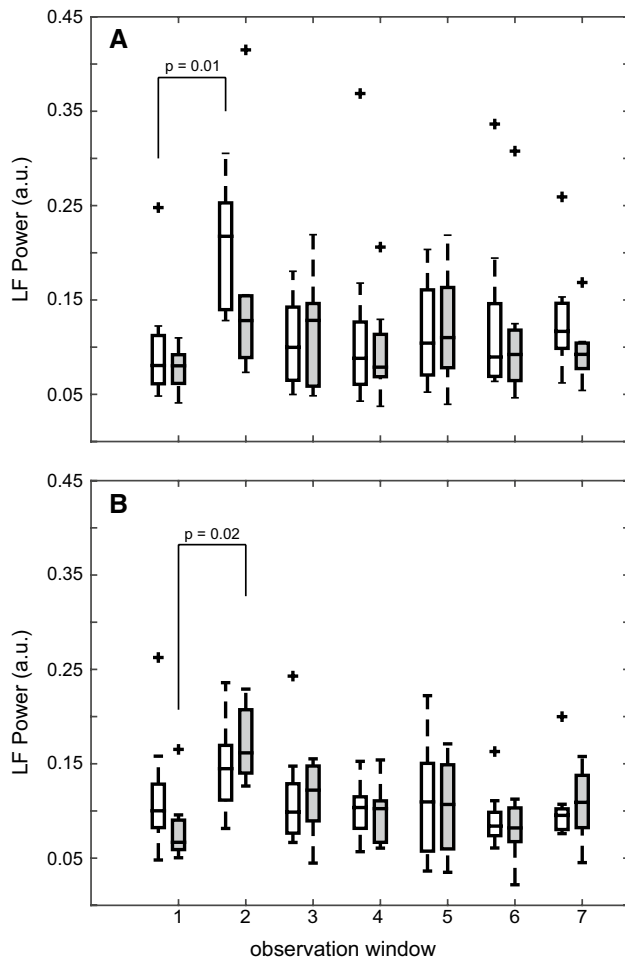


Fig. 3 Changes in the power associated to the LF band compared to the overall signal power. Controls are depicted in *gray*, MS patients in *white*. The *vertical bars* superimposed to the histogram represent the standard error. **a** Power in the LF band for the O₂Hb. **b** Power in the LF band for the HHb

3.3 Signals' entropy and HE changes

The total of 98 descriptors of the signals in the analysis windows are reduced to 35 after the feature selection procedure. These 35 features are considered as descriptive of the

complexity of the cerebrovascular pattern of the subjects. The MANOVA is then applied to the set of 35 features per subject in order to evidence differences in the overall cerebrovascular patterns of MS patients versus controls. Figure 4 reports the representation of the subjects in the canonical plane of the first and second variables as computed by MANOVA for three different analysis windows: Fig. 4a is relative to the baseline (first window), Fig. 4b is relative to the end of the blood reinjection (fourth window), and Fig. 4c to the end of the monitoring (seventh window, approximately 1.5 h after the end of the reinfusion). In baseline conditions (Fig. 4a), the MANOVA reveals that it is possible to reject the null hypothesis that the subjects belonged to the same group ($p = 1.9 \times 10^{-4}$). In fact, the MANOVA dimension, which intuitively corresponds to the number of variables sufficient to separate groups, is equal to 1. It can be noticed that the MS patients have a positive value of first canonical variable, whereas the controls have a negative one. At the end of the reinjection of the ozonized blood (Fig. 4b), the dimension of the MANOVA already lowered to zero ($p > 0.5$), thus indicating that it is not possible to reject the hypothesis that the subjects belong to the same group. At the end of the monitoring (Fig. 4c), the MANOVA dimension is still zero and the subjects belonging to the two different groups are even closer ($p > 0.5$).

Table 3 reports the statistical analysis results of three most discriminant features resulting from the Wilk's Lambda analysis. In all the three considered windows, the most significant features are the SampEn of first IMF obtained from HHb signal, the SampEn of the third IMF from O₂Hb signal, and HE of the first IMF from HHb signal. It can be observed that the entropy of the IMF is increasing as effect of ozone therapy, whereas the HE is decreasing. This result is observed on controls and on MS patients as well, although only for controls the increase in the SampEn of IMF3 from O₂Hb signal and HE of the IMF1 from HHb signal is statistically higher at the end of the monitoring compared to the baseline conditions ($p < 0.001$). The increase in the SampEn and decrease of the HE are both indicators of increasing complexity of the physiological system under analysis.

Table 2 Relative power in the LF band for the O₂Hb and HHb signals in the analysis windows

	Controls		MS-RR group	
	O ₂ Hb (%)	HHb (%)	O ₂ Hb (%)	HHb (%)
Window 1	0.098 ± 0.059	0.115 ± 0.060	0.077 ± 0.023	0.081 ± 0.037
Window 2	0.231 ± 0.219	0.163 ± 0.128	0.142 ± 0.115	0.159 ± 0.115
Window 3	0.104 ± 0.046	0.115 ± 0.052	0.117 ± 0.059	0.115 ± 0.038
Window 4	0.119 ± 0.096	0.102 ± 0.033	0.096 ± 0.052	0.097 ± 0.031
Window 5	0.116 ± 0.052	0.117 ± 0.064	0.120 ± 0.061	0.105 ± 0.052
Window 6	0.126 ± 0.085	0.091 ± 0.030	0.113 ± 0.083	0.080 ± 0.029
Window 7	0.128 ± 0.053	0.102 ± 0.036	0.096 ± 0.034	0.145 ± 0.131

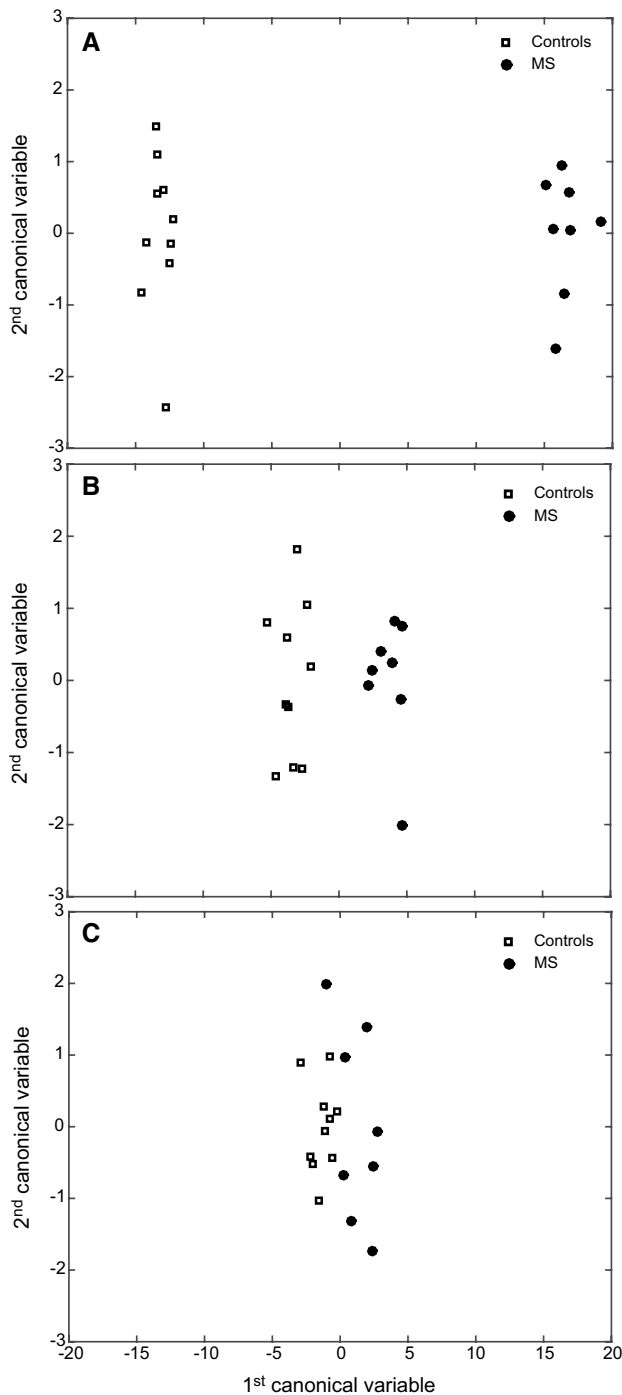


Fig. 4 Representation of the MANOVA for the subjects in three windows during therapy: **a** baseline (first window); **b** end of the blood reinfusion (fourth window); **c** end of monitoring (seventh window). Controls are represented by *white squares*, MS patients by *black circles*. In **(a)** the subjects belong to two different groups ($p = 1.9 \times 10^{-4}$), whereas in **(b)** and **(c)** the hypothesis that the subjects belong to the same group cannot be rejected ($p > 0.5$)

Table 3 Numerical values of the three most discriminant features in the first, fourth, and seventh analysis windows

	Window 1	Window 4	Window 7
Controls			
SampEn IMF1 (HHb)	0.370 ± 0.109	0.401 ± 0.132	0.431 ± 0.059
SampEn IMF3 (O ₂ Hb)	0.201 ± 0.057	0.244 ± 0.098	$0.266 \pm 0.091^*$
HE IMF1 (HHb)	0.529 ± 0.200	0.406 ± 0.137	$0.315 \pm 0.132^*$
MS-RR group			
SampEn IMF1 (HHb)	0.387 ± 0.081	0.410 ± 0.109	0.429 ± 0.113
SampEn IMF3 (O ₂ Hb)	0.281 ± 0.104	0.287 ± 0.073	0.318 ± 0.076
HE IMF1 (HHb)	0.518 ± 0.156	0.471 ± 0.148	0.408 ± 0.184

The first three rows are relative to controls, the bottom three rows to the MS patients. Increasing values of sample entropy and decreasing values of the Hurst exponent are observable

* Indicates a statistically significant difference compared to the first window

Figure 5 reports the results of three IMFs extracted from the HHb signal of a MS subject. The left panel (Fig. 5a) depicts the signal and its three IMFs during the baseline (first window). The right panel (Fig. 5b) depicts the signal and its IMFs at the end of the therapy (seventh window). It can be noted that three IMFs actually show a markedly higher complexity at the end of the monitoring (Fig. 5b). More specifically, the IMF1 during baseline (Fig. 5a) results in a quasi-periodic signal, clearly dominated by a single frequency. In this condition, it is expected that the entropy is low, due to the repeatability of the signal. Conversely, at the end of the ozone therapy (Fig. 5b), the same IMF1 is characterized by random bursts of different amplitude and frequency, which make the signal less repeatable.

4 Discussion

4.1 Ozone therapeutic effects

There are many literatures discussing the positive effects of the oxygen and ozone therapy on different pathophysiological process [10, 14], tissues [31], and organs [7, 10, 46]. However, there is the need of robust techniques for the in vivo and real-time monitoring of the ozone effects during a therapy. When ozone is used for therapy on specific tissues or systems, most of the times the validation

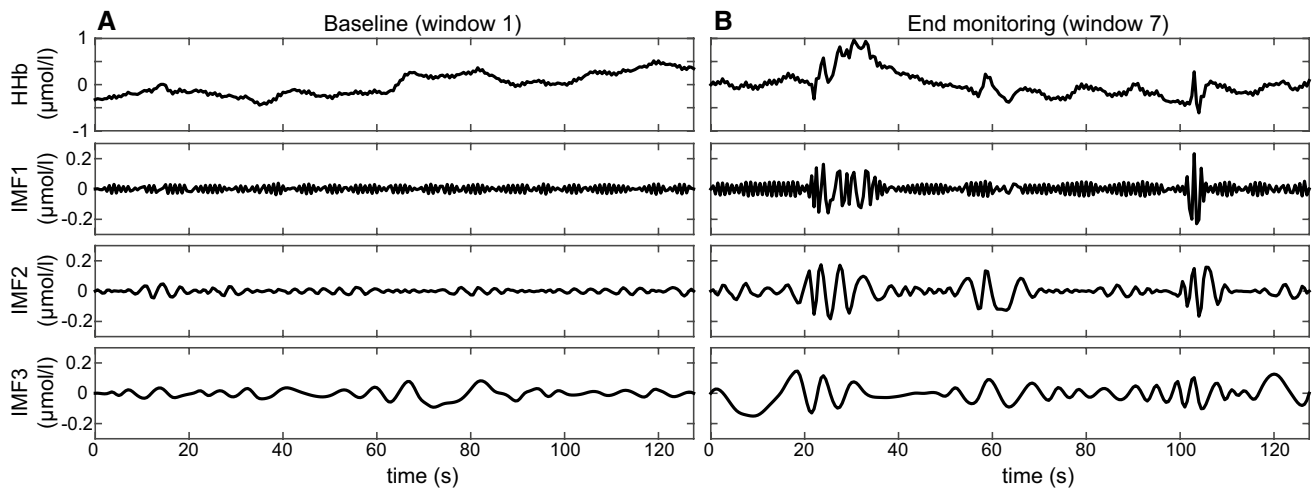


Fig. 5 Results of the empirical mode decomposition applied to the HHb signal acquired in baseline conditions (a) and at the end of the monitoring (b) from a MS subject. The upper panels report the HHb

time course; then the three IMFs are depicted. It can be observed that in panel (b) the IMFs are characterized by random bursts and changes, which make the signals less predictable

of the therapeutic effect is done by assessing either the overall improvement of the tissue, or by quantifying some functional markers. As an example, He and Ma [19] documented an improved distal fallopian tube recanalization and consequent higher number of conceptions in 400 infertile women with tube obstruction treated with ozone. When antibacterial properties are exploited, the validation is usually done by measuring the concentration changes of infective agents or the healing process of tissues. Shah et al. [41] showed how ozone autohemotherapy is effective in healing the leg of a patient with the post-surgical complications due to *staphylococcus aureus* infection causing a necrotic wound. At a molecular level, Re et al. [38] first demonstrated that ozone autohemotherapy helped the synthesis of proteins which collectively favored cell survival.

4.2 Characterization of the cerebral effects of ozone therapy

When the effects of ozone therapy have to be monitored at a cerebral level, there are limitations on the number of biomarkers that can be non-invasively quantified and on the minimally invasive techniques that can be used. Cerebral autoregulation masks most of the vascular effects of therapies, since it tends to let the cerebrovascular pattern in a steady state even in the presence of systemic changes in the concentration of blood gases or in presence of alterations of the velocity–pressure field. Brain–blood barrier blocks the exchange of many chemical compounds between brain tissue and blood; thus, many markers of inflammation, oxidation, and metabolic functions cannot be directly measured by blood samples. In this study, we adopted the NIRS as a real-time and non-invasive monitoring system to assess

the changes induced by the major ozone autohemotherapy in the cerebrovascular pattern. We previously showed that during this kind of therapy, MS patients underwent an increase in the activity level of the cytochrome-c-oxidase of the brain tissues [25, 33]. The study is based on the spectral analysis of the NIRS signals by using a time–frequency approach. However, from a signal processing point of view, time–frequency transforms can only capture changes in the power distribution of the signals. But they cannot capture subtle changes in the signal. Also, as mentioned in the introduction, in most of the commercially available NIRS devices, the measurement of the cytochrome-c-oxidase is not available. Hence, to satisfy our first aim, we conducted an extensive time and frequency analysis, and nonlinear analysis of the NIRS signals.

In accordance with previous studies, the ozone therapy increased the overall level of tissue oxygenation [11, 14, 33]. The oxygen increase is more evident in controls than in MS patients (Fig. 2a). The latter group of subjects reached an actual increase in the level of oxygen concentration only at the end of the monitoring, about 1.5 h after the end of the reinfusion of the ozonized blood. This result might be explained by the metabolic boost induced by ozone. It is proved that MS patients are characterized by lower level of mitochondrial activity, possibly due to an oxidative damage to the DNA [8, 26]. Since ozone improved the level of mitochondrial activity [33], it may be that the increment in the metabolic function of neurons caused an increase in the level of oxygen consumption. Therefore, just after the end of blood reinjection, the oxygen demand could have increased, thus leading to vascular response to increase the concentration at a later stage (about 1 h after the end of the reinfusion).

This consideration is supported also by the analysis of the relative power in the LF band of the NIRS signals (Fig. 3). It can be noticed that a slight (statistically not significant, $p > 0.05$), increase in the LF relative power in the last three observation windows (compared to the first window), more evident in the last window of the HHb signals analysis (Fig. 3b). Being the LF band a fingerprint of the vasomotor reactivity, this observation supports the hypothesis of increased metabolic demand by the brain cells, which triggers the cerebral autoregulation.

4.3 Nonlinear NIRS signal analysis

We have coupled a nonlinear analysis of the NIRS signals structure to the time and time–frequency analysis. We showed that complexity of the cerebrovascular pattern increased during the therapy (Table 3). Among all the descriptors used, the most significant are the SampEn of the first IMF of the HHb signals and the third IMF of the O₂Hb signals, along with the HE of the first IMF of the HHb signals. These three features explained about 85 % of the total variance of the data in the three analysis windows of Fig. 4. The first IMF obtained from EMD characterizes higher frequencies (Fig. 5), whereas the third IMF lower frequencies. Interestingly, the most important feature for the MANOVA is the SampEn of the first IMF of the HHb, which reveals a more complex structure indicating the rapid changes of the signal. Conversely, for the O₂Hb signal, we found that the structure change of the signal was found in the third IMF. The third IMF is characterized by lower frequencies and higher amplitude compared to the first one. This result is in accordance with the time changes reported by Fig. 2: the HHb signal did not show major changes during the therapy, while the O₂Hb concentration changed considerably in both two groups. Therefore, the complexity and entropy-based analysis can capture subtle and more accurate differences in the overall cerebrovascular pattern, which are difficult to observe by time and/or time–frequency analysis alone.

We computed the SampEn and HE also on the raw NIRS signals. However, we did not find any significant difference of these descriptors when comparing controls to MS subjects, and when comparing the signals acquired on the same subjects in different windows. This may be due to the fact that the NIRS signals are a combination of low amplitude and low-frequency oscillations and of higher amplitude and higher frequency ones [35]. The computation of the structural characteristics of all the oscillations together, as in the raw NIRS signals, provides an “averaged” result, which is unable to capture the changes occurring during the therapy and the differences between controls and patients.

It is well known that the complexity of biosignals is linked to the state and properties of the underlying

physiological system. In several studies, it is shown how the entropy of the EEG signal can be used to classify the sleep stages [2], and identify [4] and predict [27] epileptic seizures. It is also shown that diabetes mellitus decreased the complexity of the EMG signals [45] of the subjects and that physical activity helped to restore more complex muscular pattern in diabetic subjects [31]. Complexity of biosignals is usually connected to the unpredictability of the control mechanisms. In this case, since we are monitoring brain vascular and metabolic conditions, an increased entropy or decreased HE could be the effect of an increased level of control by means of cerebral autoregulation. Since the level of cerebral autoregulation is linked to the functional state of neurons, it can be hypothesized that, after a major ozone autohemotherapy, there is a boost in the metabolic and functional response of MS patients which brings the overall cerebrovascular pattern close to that of controls (Fig. 4). The entropy of the NIRS signals increases in control subjects. Hence, we can conclude that the ozone autohemotherapy is beneficial to improve the overall cerebrovascular pattern of subjects.

Our study shows that changes in the cerebrovascular pattern cannot be properly observed by relying only on time and/or time–frequency analysis. Major changes in oxygen or carbon dioxide concentrations in the brain tissues are clearly linked to blood ozonization and reinfusion. But they cannot explain which are the positive effects of the therapy at a functional and molecular level. Therefore, more complex frameworks for data analysis are required. In this work, we have shown that EMD is an efficient tool for the analysis of the cerebral NIRS signals. Thus, the principal innovative contribution of this work is the demonstration that linear and nonlinear NIRS signal analysis are needed for the characterization of the cerebrovascular pattern changes occurring after ozone autohemotherapy. This could have a direct impact in the numerical characterization of the cerebrovascular changes of subjects undergoing ozone therapy and could lead to personalized and more effective therapies.

4.4 Study limitations and further perspectives

A limitation of this study is the number of enrolled subjects, which is scarce. However, being the protocol focused on the assessment of long-term effects of ozone therapy, it is difficult to find MS patients that can hold the NIRS monitoring for about 3 h. NIRS requires continuous monitoring. It is not possible to interrupt the monitoring, detach the probes, and then apply them once again. This will cause a change in the optical coupling that introducing random variability in the signals. In our previous experience, we noted that about 50 % of the MS subjects could not complete

the monitoring due to pain, physiological needs, or excessive stress. Another limitation is that the NIRS equipment we used did not measure the concentration changes of the cytochrome-c-oxidase. To the best of our knowledge, the only reliable biomarker of brain metabolism which can be non-invasively monitored is by NIRS signals. Nevertheless, we believe that our results documented an improvement in the cerebral pattern of the subjects, which also correlated with the subjective sensation of patients and clinical observations. Finally, the two groups are not exactly age-matched. We compared the cerebrovascular pattern of MS patients to a group of controls that was older. Actually, this was a specific choice aimed at balancing the level of oxidative stress (with consequent reduction of the cytochrome-c-oxidase activity), which is typical of healthy brain cells during aging [13]. It would not have been proper to demonstrate a difference in the cerebral pattern of MS patients compared to healthy subjects of same age, since, in that case, the original level of cerebral oxidative stress would be very different. Elderly people show a progressive increase in the cerebral oxidative stress, which is physiological. Therefore, we believe that the O₃ therapy is actually acting on this overt oxidative stress induced by MS, and by rebalancing it for a relatively long period (over 3 h monitoring), we can document an increase in the O₂Hb consumption (indicating a metabolic boost).

Hence, further studies comparing age-matched groups could be useful to better analyze the cerebrovascular recovery caused by ozone therapy on MS sufferers.

5 Conclusions

In this study, we have analyzed the changes in the cerebrovascular pattern of MS patients and normal subjects after a major ozone autohemotherapy. We have observed an increase in the complexity of the cerebrovascular pattern caused due to an increase in the metabolism. The changes in brain metabolism and oxygenation are monitored by a NIRS system. We have shown that time analysis of the NIRS signals is not able to explain the complex effects of ozone therapy. Hence, we have proposed nonlinear and structural features to assess the functional cerebrovascular changes using NIRS signals.

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