Biochimica et Biophysica Acta 1842 (2014) 1137–1143

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Serum lactate as a novel potential biomarker in multiple sclerosis

Angela M. Amorini [a], Viviana Nociti [b,c], Axel Petzold [d], Claudio Gasperini [e], Esmeralda Quartuccio [e], Giacomo Lazzarino [a], Valentina Di Pietro [f], Antonio Belli [f,g], Stefano Signoretti [e], Roberto Vagnozzi [h], Giuseppe Lazzarino [i,*], Barbara Tavazzi [a]

[a] Institute of Biochemistry and Clinical Biochemistry Largo F. Vito 1, 00168 Rome, Italy
[b] Institute of Neurology, Catholic University of Rome, Largo F. Vito 1, 00168 Rome, Italy
[c] Fondazione Don C. Gnocchi, Onlus, Piazzale Morandi 6, 20121 Milano, Italy
[d] Department of Neurology, VU Medical Centre, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands
[e] Department of Neurosciences, S Camillo Forlanini Hospital, Circonvallazione Gianicolense 87 00152 Rome, Italy
[f] Neurotrauma and Neurodegeneretion Section, School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham, Edgbaston B15 2TT, Birmingham, UK
[g] National Institute for Health Research Surgical Reconstruction and Microbiology Research Centre, Queen Elizabeth Hospital, Edgbaston B15 2TH, Birmingham, UK
[h] Department of Biomedicine and Prevention, Section of Neurosurgery, University of Rome "Tor Vergata", Via Montpellier 1, 00133 Rome, Italy
[i] Department of Biology, Geology and Environmental Sciences, Division of Biochemistry and Molecular Biology, University of Catania, Viale A. Doria 6, 95125 Catania, Italy

ARTICLE INFO

Article history:
Received 21 November 2013
Received in revised form 13 March 2014
Accepted 4 April 2014
Available online 13 April 2014

Keywords:
Clinical disability
Energy penalty
Mitochondrial dysfunction
Multiple sclerosis
Serum lactate

ABSTRACT

Multiple sclerosis (MS) is a primary inflammatory demyelinating disease associated with a probably secondary progressive neurodegenerative component. Impaired mitochondrial functioning has been hypothesized to drive neurodegeneration and to cause increased anaerobic metabolism in MS. The aim of our multicentre study was to determine whether MS patients had values of circulating lactate different from those of controls. Patients ($n = 613$) were recruited, assessed for disability and clinically classified (relapsing–remitting, secondary progressive, primary progressive) at the Catholic University of Rome, Italy ($n = 281$), at the MS Centre Amsterdam, The Netherlands ($n = 158$) and at the S. Camillo Forlanini Hospital, Rome, Italy ($n = 174$). Serum lactate levels were quantified spectrophotometrically with the analyst being blinded to all clinical information. In patients with MS serum lactate was three times higher (3.04 ± 1.26 mmol/l) than that of healthy controls (1.09 ± 0.25 mmol/l, $p < 0.0001$) and increased across clinical groups, with higher levels in cases with a progressive than with a relapsing–remitting disease course. In addition, there was a linear correlation between serum lactate levels and the expanded disability scale (EDSS) ($R^2 = 0.419$; $p < 0.001$). These data support the hypothesis that mitochondrial dysfunction is an important feature in MS and of particular relevance to the neurodegenerative phase of the disease. Measurement of serum lactate in MS might be a relative inexpensive test for longitudinal monitoring of "virtual hypoxia" in MS and also a secondary outcome for treatment trials aimed to improve mitochondrial function in patients with MS.

1. Introduction

Multiple sclerosis (MS) is a primary inflammatory demyelinating disease of the central nervous system, associated with a probably secondary progressive neurodegenerative component, causing accumulation of disability to the patients. MS is believed to be of autoimmune pathology [1] although the reasons for the autoimmune attack towards myelin are not yet totally clear [2]. As it occurs in other chronic neurodegenerative disorders (Alzheimer's disease, Parkinson's disease), MS is characterized by various molecular alterations, including change in ionic homeostasis [3,4] and overproduction of reactive oxygen (ROS) and nitrogen species (RNS), with consequent oxidative/nitrosative stress and induction of apoptosis [5–7]. All these events seriously compromise several fundamental neuronal functions and certainly play a role in the disease progression and clinical deterioration of the patients.

Abbreviations: CPK-BB, creatinphosphokinase brain specific isoform; CrP, creatine phosphate; EDSS, expanded disability status scale; ETC, electron transport chain; ^{1}H MRS, proton-magnetic resonance spectroscopy; MS, multiple sclerosis; PP, primary progressive; RNS, reactive nitrogen species; ROS, reactive oxygen species; RR, relapsing remitting; SP, secondary progressive

* Corresponding author. Tel.: +39 0957384095; fax: +39 095337036.
E-mail addresses: angela.amorini@rm.unicatt.it (A.M. Amorini), viv.nociti@libero.it, viviana.nociti@tiscali.it (V. Nociti), a.petzold@ion.ucl.ac.uk, a.petzold@vumc.nl (A. Petzold), c.gasperini@libero.it (C. Gasperini), e.quartuccio@libero.it (E. Quartuccio), g.lazzarino@libero.it (G. Lazzarino), v.dipietro@bham.ac.uk (V. Di Pietro), a.belli@bham.ac.uk (A. Belli), stefano.signoretti@tiscali.it (S. Signoretti), vagnozzi@uniroma2.it (R. Vagnozzi), lazzarig@unict.it (G. Lazzarino), btavazzi@rm.unicatt.it (B. Tavazzi).

http://dx.doi.org/10.1016/j.bbadis.2014.04.005
0925-4439/

In addition, these molecular changes seem to have, as a common feature, the malfunctioning of mitochondria which has led to the "mitochondrial hypothesis" of axonal degeneration in MS [8–10], as well as the concept of "virtual hypoxia" in which chronically demyelinated axons of MS patients are forced to operate [11].

This hypothesis is supported by many experimental and clinical data showing that MS is characterized by a remarkable energy penalty due to the imbalance between energy production and consumption [10–12]. This is determined by a decreased mitochondrial ability to supply adequate ATP concentrations for the various energy-dependent functions crucial for neuronal survival.

In particular, imbalance in creatine phosphate (CrP) homeostasis [13] and decreased activity of the brain specific isoform of the enzyme creatinphosphokinase (CPK-BB) detected in MS [14] should be responsible for the reduced export of the mitochondrially generated ATP to the cytoplasm, with consequent decreased availability of cytoplasmic ATP. Contribution to a net decrease in ATP cellular content is also due to an impairment in the activity of the mitochondrial electron transport chain (ETC). In fact, it has been demonstrated that MS patients have decreased expression of several subunits of complexes I, III, IV and V of the ETC in different brain regions [15], with consequent diminution in the electron flow through the chain and inevitable decrease in ATP formation by the electron-dependent oxidative phosphorylation. The overall decline in cytoplasmic ATP negatively influences all the ATP-dependent reactions, including the activity of ATP-ases involved in ionic homeostasis [16], causing imbalance in intracellular calcium and membrane depolarization [16,17].

In line with the hypothesis of mitochondrial malfunctioning, we have previously demonstrated that MS patients have increased levels of compounds deriving from ATP catabolism (hypoxanthine, xanthine, uric acid, uridine, creatinine) in their cerebrospinal fluid (CSF) and blood [18–20]. These data strongly supported the concept that MS patients have impaired energy metabolism, with significant alterations in the production and consumption of ATP [13,14] ultimately causing an overproduction of its catabolites in the cerebral tissue [21]. Thanks to their ability to freely cross the neuronal membrane, these low molecular weight compounds are initially released into the CSF of MS patients, subsequently reaching the blood stream, and finally leading to a significant rise in their concentrations in the biological fluids with respect to the values found in healthy controls [18–20]. With this current knowledge of an energy penalty in MS, it is reasonable to hypothesize that, in order to counteract the diminished ATP supply caused by altered mitochondrial functions, the cerebral tissue would tend to increase the glycolytic pathway to rates exceeding the already compromised capacity of mitochondria to metabolize pyruvate. As a consequence of an increased glycolytic rate and pyruvate accumulation, an increase in lactate production would be expected. Recently, by using proton-magnetic resonance spectroscopy (^{1}H MRS), it has been shown that MS patients had increased lactate in their CSF, suggesting increased extra-mitochondrial glucose metabolism due to mitochondrial dysfunction [22]. A correlation between lactate concentration in the CSF and the number of inflammatory plaques reinforced the indication of increased glycolysis in MS due to mitochondrial malfunctioning [23,24]. Notwithstanding, data reporting decrease in CSF lactate in the early stages of MS have also been published [25], casting doubts over the role of this compound and, therefore, over the importance of its measurements. Furthermore, to compound this uncertainty, further ^{1}H MRS studies indicated either elevated [26] or no change [27] of brain lactate in MS patients. To date, no data are available concerning the concentration of circulating lactate in MS patients. Interestingly, a retrospective evaluation of the chromatographic runs of serum samples of MS patients enrolled in our previous studies [18,20] showed an impure peak having the retention time of true lactate which, in the majority of samples, was much higher than that found in controls (data not shown).

In this study, we measured the concentration of serum lactate in a large cross-sectional cohort of MS patients recruited in three different centers, comparing these values with those recorded in a control group of healthy subjects matched for age and sex. We reasoned that, firstly, an energy deficit in MS would lead to higher serum lactate levels than controls and that, secondly, any rise of serum lactate in MS would be clinically relevant and related to clinical disability and disease course.

2. Materials and methods

2.1. Selection and clinical evaluation of the MS patients

Patients (n = 613) fulfilling the 2005 revision of the diagnostic panel criteria for MS [28] were recruited at the Institute of Neurology of the "Policlinico Gemelli" of the Catholic University of Rome (center 1; n = 281), at the Department of Neurosciences, S. Camillo Forlanini Hospital, Rome, Italy (center 2; n = 174) and at the Department of Neurology, VU Medical Centre, Amsterdam, The Netherlands (center 3; n = 158). They were clinically assessed using the Extended Disability Status Scale score (EDSS) [29]. At the same time of the clinical assessment, patients were asked to undergo to a venipuncture for the blood samples to be used for this research study. Only patients that were clinically stable at the time of the blood sampling were included in the study. A clinical relapse within one month before withdrawal was used as an exclusion criterion.

Patients were classified into relapsing remitting (RR), secondary progressive (SP) or primary progressive (PP), according to Lublin and Reingold [30]. The control group consisted of 625 healthy subjects, matched for age and gender, and recruited among the students of the Catholic University of Rome and the University of Catania, as well as among the personnel of these two Universities who underwent their annual health check-up. Subjects suffering from any acute or chronic systemic disease, which might have influenced the serum lactate levels, were not included in the control group. The study was approved by the local Ethic Committees and written informed consent was obtained from all patients according to the Declaration of Helsinki.

2.2. Preparation of samples

In both controls and MS patients, peripheral venous blood samples were collected after at least 15 min of complete rest, using the standard tourniquet procedure, from the antecubital vein into a single VACUETTE® polypropylene tube containing serum separator and clot activator (Greiner-Bio One GmbH, Kremsmunster, Austria). After 30 min at room temperature, blood withdrawals were centrifuged at 1890 ×*g* for 10 min and the resulting serum samples were saved at −80 °C until the analysis. To measure lactate concentration, an aliquot of all serum samples was used with no further processing. This protocol for blood withdrawal and serum preparation was strictly observed in the three centers involved and was equally used in both controls and MS patients.

2.3. Spectrophotometric assay of serum lactate

The spectrophotometric determination of lactate was carried out using an Agilent 89090A spectrophotometer (Agilent Technologies, Santa Clara Ca, USA) and following the method described by Artiss et al. [31]. Briefly, the reaction mixture contained 100 mM Tris–HCl, 1.5 mM N-ethyl-N-2-hydroxy-3-sulfopropyl-3-methylalanine, 1.7 mM 4-aminoantipyrine, and 5 IU horseradish peroxidase. Fifty microliters of serum were added to the mixture, let to stand for 5 min and read at 545 nm wavelength. The reaction was started with the addition of 5 IU of lactate oxidase to the cuvette (finale volume = 1 ml) and it was considered ended when no change in absorbance was recorded for at least 3 min. To calculate lactate in serum samples, the difference in absorbance at 545 nm wavelength (Δ_{abs}) of each sample was interpolated with a calibration curve obtained by plotting Δ_{abs} measured in standard solutions of lactate with increasing known concentrations.

To compare analytical results of serum lactate obtained with the aforementioned method, 150 samples from the control group and 150 samples from the groups of MS patients were randomly selected and assayed applying the same lactate oxidase-based method and using conventional apparatus routinely used in the clinical biochemistry setting (Cobas c 702, Roche Diagnostics).

The analysis of lactate in the 1238 samples collected in the three centers, as well as the comparison between the two analytical methods, was performed at the Institute of Biochemistry and Clinical Biochemistry of the Catholic University of Rome, Italy. When assaying samples of MS patients, the analyst was blinded to all clinical information.

2.4. Statistical analysis

Normal data distribution was tested using the Kolmogorov–Smirnov test. For normally distributed data, group differences were assessed by the Student's *t*-test for unpaired observations. Due to the unbalanced study design the Kruskal–Wallis one-way ANOVA by ranks followed by the Friedman test was used for comparison of MS subgroups (RR, SP, PP) and controls. Correlation and regression analyses of serum lactate levels with the EDSS were followed by ANOVA of the regression coefficients. Only two-tailed p-values of less than 0.05 were considered as statistically significant.

3. Results

The demographic and clinical data of the group of control healthy subjects and of MS patients enrolled in this study are summarized in Table 1. When subgrouped on the basis of the EDSS scores, 64 patients were EDSS 0, 72 EDSS 1, 26 EDSS 1.5, 76 EDSS 2, 25 EDSS 2.5, 75 EDSS 3, 37 EDSS 3.5, 58 EDSS 4, 16 EDSS 4.5, 26 EDSS 5, 10 EDSS 5.5, 72 EDSS 6, 20 EDSS 6.5, 24 EDSS 7, 6 EDSS 7.5 and 6 EDSS 8. The majority of patients with MS had a RR disease course (n = 430; 70.1%), with smaller numbers of them being affected by the SP (n = 153; 25.0%) and the PP (n = 30; 4.9%) forms. The group of RR-MS patients had significantly lower mean values of EDSS and disease duration compared to the corresponding values recorded in the groups of both SP-MS and PP-MS patients ($p < 0.01$).

3.1. Serum lactate in controls and MS patients

Data referring to the concentration of circulating lactate detected in the serum of controls and MS patients are illustrated in Fig. 1. In our group of 625 resting healthy controls we found a mean lactate value of 1.09 ± 0.25 mmol/l serum (Panel A), with 615 of them having lactate ranging between 0.5 and 1.5 mmol/l serum and 10 outliers (1.6%) with a lactate range of 1.76–2 mmol/l serum. Irrespective of the clinical subtype and the EDSS score (Panel A), we detected a mean lactate value of 3.04 ± 1.26 mmol/l serum in resting MS patients, i.e. about 2.8 times higher than that of controls ($p < 0.0001$).

As shown in the scatterplot of Panel B, 101 (16.5%) of the 613 MS patients had serum lactate falling within the range of values determined in controls (0.5–2 mmol/l serum). The remaining 502 (84.5%) MS patients had values higher than the maximal concentration of circulating lactate (2.0 mmol/l) recorded in one control healthy subject only. In MS patients, serum lactate ranged from 0.60 mmol/l to 7.56 mmol/l. Analysis

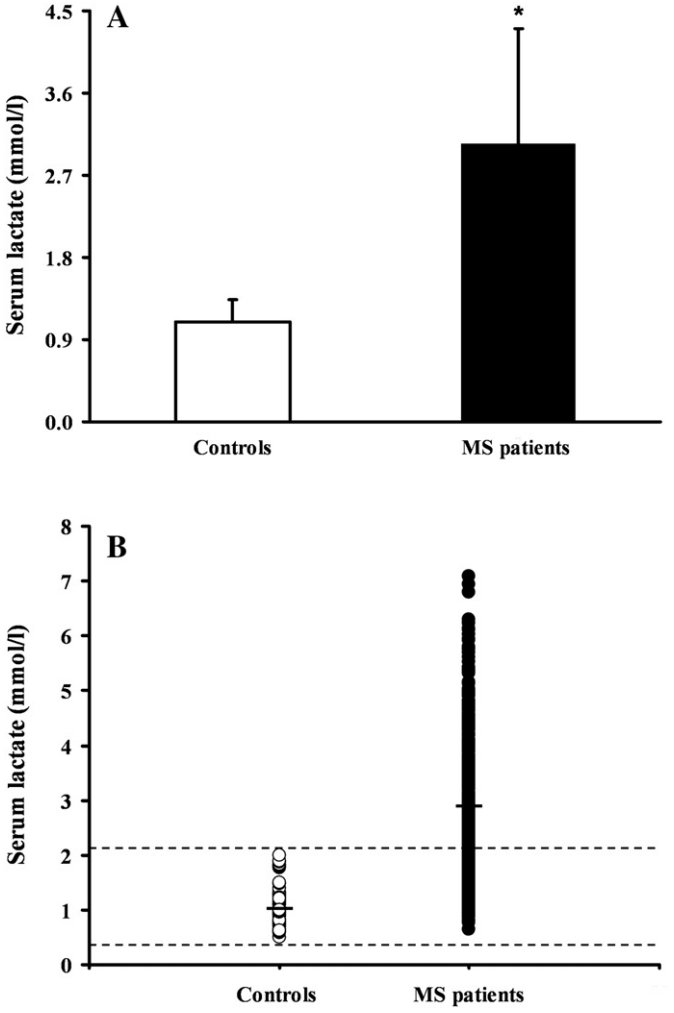

Fig. 1. Concentration of serum lactate determined in peripheral venous blood samples of 625 control healthy subjects and 613 MS patients. In Panel A, mean values with standard deviations represented by vertical bars are reported. In Panel B, the individual values of each subject of both groups, with medians represented by horizontal marks (1.10 and 2.87 in controls and MS patients, respectively), are reported. Dashed lines represent the range of the serum lactate values of controls. *Significantly different from the group of sex and age matched healthy controls, $p < 0.0001$.

of the clinical and biochemical results considering MS patients as three separate cohorts (one for each recruiting center) is reported in Table 2. We found that the patients recruited at center 2 had significantly lower EDSS scores compared to both centers 1 and 3 ($p < 0.05$), with center 3 having the highest mean EDSS score. Additionally, center 3 recruited the highest % number of PP patients with respect to both centers 1 and 2. The very relevant finding is that MS patients of the three centers had lactate values of 3.38 ± 1.83 mmol/l serum (center 1), 2.74 ± 1.39 mmol/l serum (center 2) and 2.94 ± 1.26 mmol/l serum (center 3), i.e. each of the three subsets of MS patients had significantly higher lactate values than those found in healthy controls (1.09 ± 0.25 mmol/l serum; $p < 0.0001$). Because of the differences in the EDSS score, lactate

Table 1
Clinical neurological data of MS patients and controls.

	CTRL (n = 625)	MS (all) (n = 613)	RR (n = 430)	SP (n = 153)	PP (n = 30)
Age (years)	44.8 ± 11.7;46.0 (15–73)	45.4 ± 12.8; 46.5 (15–80)	43.1 ± 12.5; 42.0 (15–70)	49.7 ± 11.7; 50.2 (28–72)	51.3 ± 13.4; 53.0 (26–80)
Gender	F 420: M 205	F 411: M 202	F 276: M 154	F 110: M 43	F 21: M 9
Disease duration (years)	N/A	12.9 ± 9.5; 12.0 (0–44)	10.8 ± 9.7; 9.0 (0–38)	18.8 ± 10.0; 18.2 (1–44)	14.8 ± 9.8; 15.1 (0.6–35)
EDSS	N/A	3.3 ± 2.1; 3.0 (0–8)	2.1 ± 1.6; 2.0 (0–8)	5.8 ± 1.5; 6.0 (0–8)	5.2 ± 1.5; 6.0 (3.0–8)

Values are expressed as mean ± S.D. and median (range). CTRL = controls; MS (all) = total number of multiple sclerosis patients; PP = primary progressive; SP = secondary progressive; RR = relapsing remitting; N/A = not available; EDSS = Extended Disability Status Scale score.

Table 2
Clinical status and serum lactate in MS patients recruited per center.

	MS (all)	RR	SP	PP	EDSS	Serum lactate
Center 1	n = 281	202 (71.9%)	72 (25.6%)	7 (2.5%)	3.82 ± 2.23	3.31 ± 1.83
Center 2	n = 174	132 (75.9%)	37 (21.2%)	5 (2.9%)	[a]2.18 ± 1.97	[b]2.81 ± 1.39
Center 3	n = 158	96 (60.8%)	44 (27.8%)	18 (11.4%)	4.21 ± 1.79	2.94 ± 1.26

MS (all) = number of all multiple sclerosis patients in each center; PP = number of primary progressive patients (% in each center), SP = number of secondary progressive patients (% in each center); RR = number of relapsing remitting patients (% in each center); EDSS = Extended Disability Status Scale score. In the EDSS and serum lactate columns, values represent mean ± S.D. and lactate is expressed in mmol/l serum.
[a] Significantly different from centers 1 and 3 ($p < 0.05$).
[b] Significantly different from center 1 ($p < 0.05$).

values recorded in patients recruited at center 2 were significantly lower than those measured in patients recruited in center 1, thus reinforcing the notion of a correlation between serum lactate and clinical status in MS.

To confirm these analytical results and to corroborate their relevance for the clinical biochemical monitoring of MS patients, we reported in Table 3 values of serum lactate determined in 150, randomly selected, samples of controls and 150, randomly selected, samples of MS patients, in which lactate was assayed both with the method described above and with the conventional method routinely applied in the clinical biochemistry setting. Data indicate that no significant differences exist in the lactate values obtained with these two methods, thereby demonstrating the analytical validity of the present results.

To asses whether circulating lactate was correlated to clinical subtypes, MS patients were divided into RR, SP and PP and values of serum lactate in these groups were then calculated. As shown in Fig. 2, all three subgroups of MS patients had higher values of lactate (2.72 ± 1.12, 3.86 ± 1.29 and 3.32 ± 1.16 mmol/l serum, respectively; $p < 0.0001$) than those detected in controls (Panel A). Significant differences were recorded in the comparisons between RR and SP ($p < 0.0001$), RR versus PP ($p < 0.01$), and SP versus PP ($p < 0.05$). Significantly, the subgroup analysis with all progressive patients pooled together (SP and PP) demonstrated higher serum lactate levels (3.77 ± 1.28) compared to either RR-MS patients or control subjects ($p < 0.0001$ for both comparisons), thus suggesting a correlation between MS subtype and serum values of this glycolytic end product (Fig. 2, Panel B).

In order to investigate the correlation with the disease progression, values of serum lactate were plotted as a function of the EDSS scores (Fig. 3). Since the number of subjects in the different EDSS subgroups did not exceed the 76 units, in order to avoid an excess weight of the control group in the calculation of the regression coefficients, we reduced the number of controls accordingly, for the further statistical comparisons (analysis of regression and analysis of variance on the regression coefficients). Therefore, we randomly selected 76 values from the 625 lactate values of controls. This reduced in size control group (mean = 0.95 ± 0.26, median = 0.88) had the same distribution (calculated using the Kolmogorov–Smirnov test) of the original larger control group and was used in the linear regression analysis. Lactate values in the control group were tabulated as the 0 values (no disease), while those in the MS patients were tabulated as the original EDSS score + 1. In Fig. 3, the equation describing the best fitting regression line ($y = 0.3657\ x + 1.4289$; $R = 0.6473$; $R^2 = 0.419$; $p < 0.0001$) indicates a strong positive correlation linking serum lactate with disease progression (increase in EDSS scores), thus suggesting that the worsening of the clinical conditions of MS patients is associated with evident deterioration in their cell energy metabolism.

It is however worth underlining that 16.5% of MS patients had values of circulating lactate falling within the range of variability of control

Table 3
Comparison of the analytical results obtained with a standard laboratory spectrophotometer and an apparatus in use in the clinical biochemistry setting and referring to serum lactate recorded in 150 randomly selected samples of controls and MS patients.

	Controls (n = 150)	MS patients (n = 150)
Serum lactate (mmol/l) laboratory spectrophotometer	1.06 ± 0.22[a]	3.29 ± 1.18[a,b]
Serum lactate (mmol/l) clinical biochemistry apparatus	1.11 ± 0.25	3.38 ± 1.26[b]

Values are expressed as mean ± S.D.
[a] Not different from the values of the same group assayed by the clinical biochemistry apparatus (longitudinal identity).
[b] Significantly different from controls ($p < 0.0001$) (latitudinal difference).

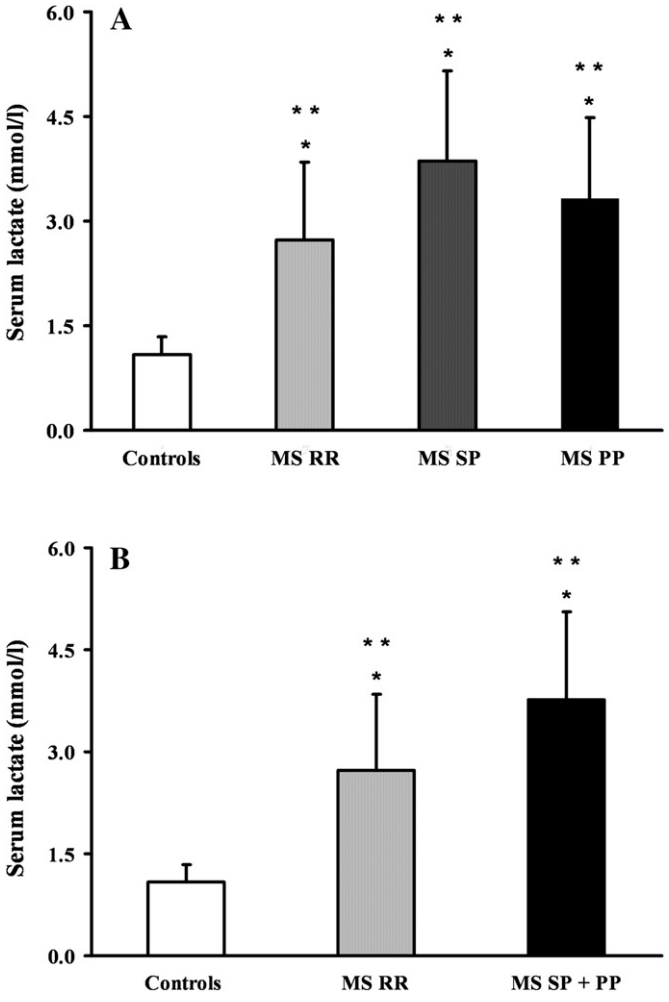

Fig. 2. Concentration of serum lactate in controls and MS patients divided according to their different clinical subtype. In Panel A, patients were divided into three groups (RR = relapsing remitting; SP = secondary progressive; PP primary progressive) of very different sizes (RR = 430; SP = 153; PP = 30). In Panel B, progressive patients were grouped (SP + PP = 189) to allow a better statistical comparison. Values are reported as means, with standard deviations represented by vertical bars. *Significantly different from the group of sex and age matched healthy controls, $p < 0.001$. **Significantly different from the group of RR patients, $p < 0.0001$.

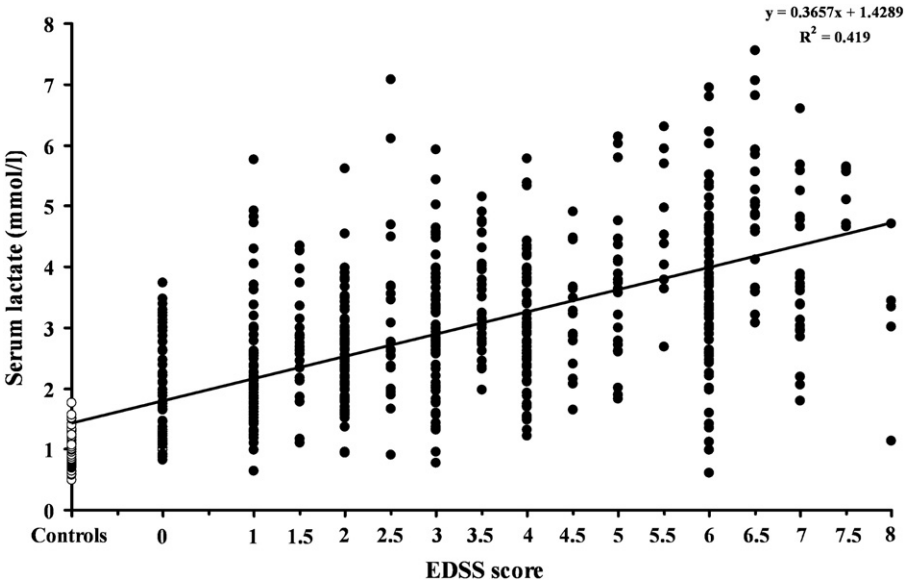

Fig. 3. Dispersion graphics in which 76 values of serum lactate of controls (randomly selected in the lactate values of the 625 healthy subjects) and each of the 613 patients were plotted as a function of the EDSS scores. To the group of the 76 controls, the real 0 on the y axis was assigned. The value of the correlation coefficient ($R^2 = 0.419$; $p < 0.0001$) indicated that the two parameters were linearly correlated.

healthy subjects (Fig. 1). These 101 "low lactate" MS patients were not found in MS patients scoring 3.5, 5.5, 6.5 and 7.5 on EDSS. Where present, they represented the 54.7% (EDSS 0), 29.1% (EDSS 1), 15.4% (EDSS 1.5), 21.0% (EDSS 2), 20.0 (EDSS 2.5), 16.1% (EDSS 3), 17.2% (EDSS 4), 10.0% (EDSS 4.5), 6.0% (EDSS 5), 11.1% (EDSS 6), 9.7% (EDSS 7) and 16.7% (EDSS 8) of the respective EDSS class, with an evident clustering in the EDSS 0, characterized by the less severe clinical symptoms. It is important to note that if the 10 outliers of the control group (having lactate ranging between 1.76 and 2 mmol/l serum and representing the 1.6% of the 625 control values) are excluded, the number of MS patients falling within the range of controls drops from 101 to 70 (11.5%),

The box plot in Fig. 4 shows the best fitting linear regression calculated on the medians of the different EDSS scores. According to the equation $y = 0.3935\,x + 0.83$ ($R = 0.9859$; $R^2 = 0.972$; $p < 0.0001$) it is possible to appreciate how medians of serum lactate in MS patients linearly increased as a function of the increase in EDSS score. It should be observed that, since in a box plot the abscissa is equally divided on the basis of the number of x values, patients with the intermediate EDSS scores (1.5, 2.5, 3.5, 4.5, 5.5, 6.5 and 7.5) were grouped with those of the upper EDSS class (1.5–2, 2.5–3, 3.5–4, 4.5–5, 5.5–6, 6.5–7 and 7.5–8), in order to have equally spaced x values. In addition, even in this case, the control group used in Fig. 3 ($n = 76$) was considered as the 0 group (no disease) and the EDSS scores + 1 were tabulated to perform the regression analysis.

4. Discussion

Data reported in the present study show, for the first time to the best of our knowledge, that MS patients have remarkably elevated concentration of serum lactate respect to control healthy subjects. Furthermore, we also found that increased levels of circulating lactate in MS patients are tightly correlated with the EDSS scores and with the clinical

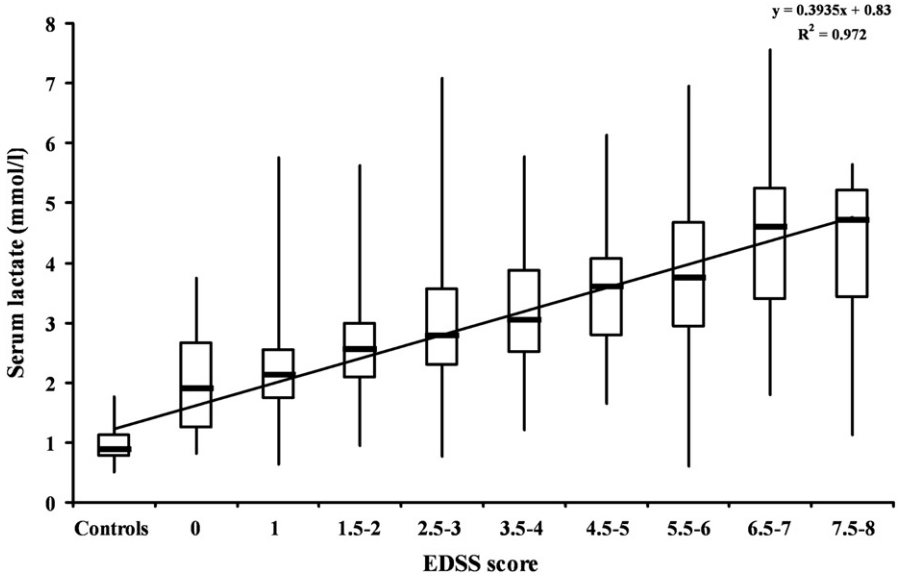

Fig. 4. Box plot showing the linear correlation between the medians of the values of serum lactate recorded in controls and those determined in MS patients divided on EDSS scores. Values in controls refer to 76 serum lactate randomly selected in the values of the 625 healthy subjects. To plot this graph, patients with non-integer EDSS scores were grouped with those of the nearest upper class, as indicated in the figure. The value of the correlation coefficient ($R^2 = 0.972$; $p < 0.0001$) indicated a statistically significant linear correlation.

MS subtype (RR, SP, PP), thereby opening new perspectives for an easy, low cost, low invasive monitoring of disease progression.

In the last decades, also thanks to the development of new imaging techniques such as ^{1}H and ^{31}P MRS, several studies have clearly demonstrated significant metabolic changes in MS lesions and in normal appearing white matter suggesting that changes in important metabolic functions may be implicated in the disease progression. In particular, decrease in NAA [32–34], decrease in the total ^{31}P peak integrals and decrease in phosphodiesters/total ^{31}P [35], increase in total creatine [33,36], and change in the activity of CPK-BB [13,14,34] are all suggestive of altered mitochondrial functions and neuronal energy state. In accordance to these findings, we found that MS patients showed significant alterations of parameters indicative of impaired energy metabolism, such as uric acid, hypoxanthine, xanthine, and creatinine, not only in the CSF but also in serum/plasma [18–20].

According to this strong indication that MS is characterized by metabolic imbalance due to compromised mitochondrial functions, it is conceivable to expect that MS may cause an increase in neuronal lactate production through compensatory mechanisms. Data referring to possible changes in lactate in MS patients are controversial, since either increase [22–24] or decrease [25] in CSF lactate has been reported. To compound the uncertainty about changes of lactate in relation to MS, it should be remembered that both elevated [26] and no change [27] of brain lactate have been reported. In addition, data available in literature have mainly been obtained in relatively restricted cohorts of MS patients [22–25]. However, when lactate was found elevated in the CSF of MS patients [22–24] no correlation with the disease progression on EDSS or with the clinical subtype (RR, SP, PP) has been demonstrated, thus raising questions as to the usefulness of monitoring this molecule in MS. Moreover, no systematic studies aimed at measuring lactate in serum/plasma of MS patients have been performed. Recently, it has been reported that levels of circulating lactate of patients with RR-MS were not different from those found in controls, even though the results of this study were obtained in a very small number of subjects ($n = 16$, for both controls and patients) characterized by a low value of EDSS [37].

The results reported in the present study, demonstrating nearly 3-times higher values of circulating lactate in MS patients than in control healthy subjects, allow hypothesizing that either hyperglycolysis of sclerotic plaques or metabolism of activated inflammatory cells might give raise to overproduction of lactate, which only transiently remains in the CSF and rapidly diffuses into the blood stream. This sequence of events might explain not only the conflicting data of literature [22–27], but also why the increase in CSF lactate was not found to be correlated with EDSS or MS clinical subtype [22–27]. It is certainly possible that lactate in MS is not constantly overproduced but that it is subjected to fluctuations; this would render detecting significant differences difficult with respect to controls when assaying lactate in the nervous tissue or in the CSF. To clarify this issue, it should be important to perform longitudinal studies. It is conceivable that most of the exceeding lactate, intermittently or constantly, flows from the CSF to the blood, thus explaining the significant differences recorded in the concentration of serum lactate, even when MS patients were divided into the RR, SP and PP clinical subtypes (Fig. 2). In fact, we found that the SP and PP subgroups, characterized by higher mean EDSS scores (Table 1) due to higher neurodegenerative component leading to extensive neuroaxonal damage [38], had higher circulating lactate than the RR-MS subgroup (Fig. 2).

In our cohort of 613 MS patients, when plotting the concentrations of circulating lactate as a function of the EDSS scores, a linear correlation has been obtained (Figs. 3 and 4), notwithstanding the presence of some outliers particularly evident in the EDSS 2.5, 6 and 8. Medians of 0.88 mmol/l serum for the control group and of 1.90 (EDSS 0), 2.12 (EDSS 1), 2.58 (EDSS 1.5–2), 2.78 (EDSS 2.5–3), 3.04 (EDSS 3.5–4), 3.60 (EDSS 4.5–5), 3.75 (EDSS 5.5–6), 4.61 (EDSS 6.5–7) and 4.71 (EDSS 7.5–8) for the MS patients divided on EDSS scores (Fig. 4) strongly support the hypothesis of a metabolic impairment associated with the progression of the disease. However, it can be postulated that neither inflammation nor hyperglycolysis at the sclerotic plaques is sufficient to cause such a remarkable increase (up to 5 times the median value of controls) in the concentrations of circulating lactate. According to previous data, MS patients showed an abnormal intramuscular component of fatigue [39] and, those with mild disability, evidenced an increased cost of walking [40,41]. Both findings suggest the potential muscular involvement in MS, possibly caused by a metabolic imbalance of myocytes, and let to assume that part of the elevated circulating lactate detected in our cohort of MS patients is of muscular origin. It is certainly important to observe that not all MS patients had serum lactate higher than that found in controls. A consistent 16.5% of them had values of circulating lactate falling within the range of variability of control healthy subjects (Fig. 1).

It is reasonable to suppose that the increase of lactate may trigger (or may be the result of) a vicious circle in which the compensatory product of mitochondrial malfunctioning (lactate) may contribute to worsen mitochondrial activity itself. In fact, the intracellular acidification caused by increased lactate production may cause dramatic adverse effects on various cell functions, including mitochondrial functions [42].

One important additional observation that can be drawn from the results reported in this study is certainly related to the potential use of serum lactate determination to follow the disease progression in MS patients, as well as to monitor the eventual benefits of a pharmacological treatment. In spite of the very many efforts devoted to find new reliable biomarkers correlated with MS progression [43–47], to date clinicians are still waiting to know what should be assayed in order to have objective parameters, easily measurable, with which predicting the evolution of the disease or following the efficacy of drug administration. It may also be affirmed that clinicians are also waiting to know where this potential biomarker should be assayed: (post mortem) brain tissue, CSF, blood, and extracellular fluid. At present, most of the proposed biomarkers are expensive in terms of cost of analysis (MRS, MRI, immunogenic profile, CD lymphocyte profile, antibodies detection, etc.), of invasiveness (lumbar puncture), and of still uncertain reliability.

Detection of serum lactate is characterized by a very low cost, limited invasiveness, high automation, high reproducibility: the present results, indicating high correlation with the MS progression and MS clinical subtypes, suggest that lactate has all the characteristics of one of the best candidate to become one of the biomarkers of choice to follow MS progression and drug efficacy. It may be easily hypothesized that a frequent serum lactate determination would be of great help for physicians in knowing how an MS patient responds to a given therapy and how the disease is progressing. Also, improvement in the quality of results obtainable by the use of the so called “lactometer” for the self-made lactate measurement might allow an even more frequent measurement of this new potential biomarker.

5. Acknowledgements

This work is dedicated to the memory of Professor Anna Paola Batocchi who had recently passed away. She dedicated her life to study multiple sclerosis and autoimmune diseases of the central nervous system. Without her initial input and idea, this study would have never been carried out.

This work has been funded in part by research funds of the Catholic University of Rome and the University of Catania.

References

[1] E. Miller, Multiple sclerosis, Adv. Exp. Med. Biol. (724) (2012) 222–238.
[2] D. Brassat, When does multiple sclerosis start? Three case reports and a review of the literature, Rev. Neurol. (Paris) 168 (2012) 846–851.
[3] P. Ehling, S. Bittner, T. Budde, H. Wiendl, S.G. Meuth, Ion channels in autoimmune neurodegeneration, FEBS Lett. 585 (2011) 3836–3842.

[4] C. Stadelmann, Multiple sclerosis as a neurodegenerative disease: pathology, mechanisms and therapeutic implications, Curr. Opin. Neurol. 24 (2011) 224–229.
[5] A. Seven, M. Aslan, S. Incir, A. Altıntaş, Evaluation of oxidative and nitrosative stress in relapsing remitting multiple sclerosis: effect of corticosteroid therapy, Folia Neuropathol. 51 (2013) 58–64.
[6] A. Fiorini, T. Koudriavtseva, E. Bucaj, R. Coccia, C. Foppoli, A. Giorgi, M.E. Schininà, F. Di Domenico, F. De Marco, M. Perluigi, Involvement of oxidative stress in occurrence of relapses in multiple sclerosis: the spectrum of oxidatively modified serum proteins detected by proteomics and redox proteomics analysis, PLoS One 8 (2013) e65184.
[7] L. Haider, M.T. Fischer, J.M. Frischer, J. Bauer, R. Höftberger, G. Botond, H. Esterbauer, C.J. Binder, J.L. Witztudim, H. Lassmann, Oxidative damage in multiple sclerosis lesions, Brain 134 (2011) 1914–1924.
[8] K.G. Su, G. Banker, D. Bourdette, M. Forte, Axonal degeneration in multiple sclerosis: the mitochondrial hypothesis, Curr. Neurol. Neurosci. Rep. 9 (2009) 411–417.
[9] K. Su, D. Bourdette, M. Forte, Mitochondrial dysfunction and neurodegeneration in multiple sclerosis, Front. Physiol. 4 (169) (2013) 1–10.
[10] M. Cambron, M. D'Haeseleer, G. Laureys, R. Clinckers, J. Debruyne, J. De Keyser, White-matter astrocytes, axonal energy metabolism, and axonal degeneration in multiple sclerosis, J. Cereb. Blood Flow Metab. 32 (2012) 413–424.
[11] B.D. Trapp, P.K. Stys, Virtual hypoxia and chronic necrosis of demyelinated axons in multiple sclerosis, Lancet Neurol. 8 (2009) 280–291.
[12] E. Hattingen, J. Magerkurth, U. Pilatus, A. Hübers, M. Wahl, U. Ziemann, Combined ^{1}H and ^{31}P spectroscopy provides new insights into the pathobiochemistry of brain damage in multiple sclerosis, NMR Biomed. 24 (2011) 536–546.
[13] C. Tur, C.A. Wheeler-Kingshott, D.R. Altmann, D.H. Miller, A.J. Thompson, O. Ciccarelli, Spatial variability and changes of metabolite concentrations in the cortico-spinal tract in multiple sclerosis using coronal CSI, Hum. Brain Mapp. (2012), http://dx.doi.org/10.1002/hbm.22229: 1-12.
[14] C. Steen, N. Wilczak, J.M. Hoogduin, M. Koch, J. De Keyser, Reduced creatine kinase B activity in multiple sclerosis normal appearing white matter, PLoS One 5 (2010) e10811.
[15] A. Pandit, J. Vadnal, S. Houston, E. Freeman, J. McDonough, Impaired regulation of electron transport chain subunit genes by nuclear respiratory factor 2 in multiple sclerosis, J. Neurol. Sci. 279 (2009) 14–20.
[16] R. Dutta, J. McDonough, X. Yin, J. Peterson, A. Chang, T. Torres, T. Gudz, W.B. Macklin, D.A. Lewis, R.J. Fox, R. Rudick, K. Mirnics, B.D. Trapp, Mitochondrial dysfunction as a cause of axonal degeneration in multiple sclerosis patients, Ann. Neurol. 59 (2006) 478–489.
[17] H.E. Andrews, P.P. Nichols, D. Bates, D.M. Turnbull, Mitochondrial dysfunction plays a key role in progressive axonal loss in multiple sclerosis, Med. Hypotheses 64 (2005) 669–677.
[18] A.M. Amorini, A. Petzold, B. Tavazzi, J. Eikelenboom, G. Keir, A. Belli, G. Giovannoni, V. Di Pietro, C. Polman, S. D'Urso, R. Vagnozzi, B. Utidehaag, G. Lazzarino, Increase of uric acid and purine compounds in biological fluids of multiple sclerosis patients, Clin. Biochem. 42 (2009) 1001–1006.
[19] G. Lazzarino, A.M. Amorini, M.J. Eikelenboom, J. Killestein, A. Belli, V. Di Pietro, B. Tavazzi, F. Barkhof, C.H. Polman, B.M. Uitdehaag, A. Petzold, Cerebrospinal fluid ATP metabolites in multiple sclerosis, Mult. Scler. 16 (2010) 549–554.
[20] B. Tavazzi, A.P. Batocchi, A.M. Amorini, V. Nociti, S. D'Urso, S. Longo, S. Gullotta, M. Picardi, G. Lazzarino, Serum metabolic profile in multiple sclerosis patients, Mult. Scler. Int. 2011 (167156) (2011) 1–8.
[21] H. Langemann, A. Kabiersch, J. Newcombe, Measurement of low-molecular-weight antioxidants, uric acid, tyrosine and tryptophan in plaques and white matter from patients with multiple sclerosis, Eur. Neurol. 32 (1992) 248–252.
[22] W.T. Regenold, P. Phatak, M.J. Makley, R.D. Stone, M.A. Kling, Cerebrospinal fluid evidence of increased extra-mitochondrial glucose metabolism implicates mitochondrial dysfunction in multiple sclerosis disease progression, J. Neurol. Sci. 275 (2008) 106–112.
[23] I.L. Simone, F. Federico, M. Trojano, C. Tortorella, M. Liguori, P. Giannini, E. Picciola, G. Natile, P. Livrea, High resolution proton MR spectroscopy of cerebrospinal fluid in MS patients. Comparison with biochemical changes in demyelinating plaques, J. Neurol. Sci. 144 (1996) 182–190.
[24] N.W. Lutz, A. Viola, I. Malikova, S. Confort-Gouny, B. Audoin, J.P. Ranjeva, J. Pelletier, P.J. Cozzone, Inflammatory multiple-sclerosis plaques generate characteristic metabolic profiles in cerebrospinal fluid, PLoS One 2 (2007) e595.
[25] M.A. Fonalledas Perelló, J.V. Politi, M.A. Dallo Lizarraga, R.S. Cardona, The cerebrospinal fluid lactate is decreased in early stages of multiple sclerosis, P. R. Health Sci. J. 27 (2008) 171–174.
[26] W. Zaaraoui, A. Rico, B. Audoin, F. Reuter, I. Malikova, E. Soulier, P. Viout, Y. Le Fur, S. Confort-Gouny, P.J. Cozzone, J. Pellettier, J.P. Ranjeva, Unfolding the long-term pathophysiological processes following an acute inflammatory demyelinating lesion of multiple sclerosis, Magn. Reson. Imaging 28 (2010) 477–486.
[27] M.F. Schocke, T. Berger, S.R. Felber, C. Wolf, F. Deisenhammer, C. Kremser, K. Seppi, F. T. Aichner, Serial contrast-enhanced magnetic resonance imaging and spectroscopic imaging of acute multiple sclerosis lesions under high-dose methylprednisolone therapy, Neuroimage 20 (2003) 1253–1263.
[28] C.H. Polman, S.C. Reingold, G. Edan, M. Filippi, H.P. Hartung, L. Kappos, F.D. Lublin, L.M. Metz, H.F. McFarland, P.W. O'Connor, M. Sandberg-Wollheim, A.J. Thompson, B.G. Weinshenker, J.S. Wolinskj, Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria", Ann. Neurol. 58 (2005) 840–846.
[29] J.F. Kurtzke, Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS), Neurology 33 (1983) 1444–1452.
[30] F.D. Lublin, S.C. Reingold, Defining the clinical course of multiple sclerosis: results of an international survey National Multiple Sclerosis Society (USA) Advisory Committee on clinical trials of new agents in multiple sclerosis, Neurology 46 (1996) 907–911.
[31] J.D. Artiss, R.E. Karcher, K.T. Cavanagh, S.L. Collins, V.J. Peterson, S. Varma, B. Zak, A liquid-stable reagent for lactic acid levels. Application to the Hitachi 911 and Beckman CX7, Am. J. Clin. Pathol. 114 (2000) 139–143.
[32] M.L. Stromillo, A. Giorgio, F. Rossi, M. Battaglini, B. Hakiki, G. Malentacchi, M. Santangelo, C. Gasperini, M.L. Bartolozzi, E. Portaccio, M.P. Amato, N. De Stefano, Brain metabolic changes suggestive of axonal damage in radiologically isolated syndrome, Neurology 80 (2013) 2090–2094.
[33] I.I. Kirov, A. Tal, J.S. Babb, J. Herbert, O. Gonen, Serial proton MR spectroscopy of gray and white matter in relapsing–remitting MS, Neurology 80 (2013) 39–46.
[34] C. Steen, M. D'haeseleer, J.M. Hoogduin, Y. Fierens, M. Cambron, J.P. Mostert, D.J. Heersema, M.W. Koch, J. De Keyser, Cerebral white matter blood flow and energy metabolism in multiple sclerosis, Mult. Scler. 19 (2013) 1282–1289.
[35] C.A. Husted, D.S. Goodin, J.W. Hugg, A.A. Maudsley, J.S. Tsuruda, S.H. de Bie, G. Fein, G.B. Matson, M.W. Weiner, Biochemical alterations in multiple sclerosis lesions and normal-appearing white matter detected by in vivo ^{31}P and ^{1}H spectroscopic imaging, Ann. Neurol. 36 (1994) 157–165.
[36] M. Bagory, F. Durand-Dubief, D. Ibarrola, J.C. Comte, F. Cotton, C. Confavreux, D. Sappey-Marinier, Implementation of an absolute brain 1H-MRS quantification method to assess different tissue alterations in multiple sclerosis, IEEE Trans. Biomed. Eng. 59 (2012) 2687–2694.
[37] A. Mähler, J. Steiniger, M. Bock, A.U. Brandt, V. Haas, M. Boschmann, F. Paul, Is metabolic flexibility altered in multiple sclerosis patients? PLoS One 7 (2012) e43675.
[38] W. Brück, C. Stadelmann, Inflammation and degeneration in multiple sclerosis, Neurol. Sci. 24 (Suppl. 5) (2003) S265–S267.
[39] K.R. Sharma, J. Kent-Braun, M.A. Mynhier, M.W. Weiner, R.G. Miller, Evidence of an abnormal intramuscular component of fatigue in multiple sclerosis, Muscle Nerve 18 (1995) 1403-1111.
[40] N.F. Taylor, K.J. Dodd, D. Prasad, S. Denisenko, Progressive resistance exercise for people with multiple sclerosis, Disabil. Rehabil. 28 (2006) 1119–1126.
[41] M. Franceschini, A. Rampello, F. Bovolenta, M. Aiello, P. Tzani, A. Chetta, Cost of walking, exertional dyspnoea and fatigue in individuals with multiple sclerosis not requiring assistive devices, J. Rehabil. Med. 42 (2010) 719–723.
[42] R.A. Robergs, F. Ghiasvand, D. Parker, Biochemistry of exercise-induced metabolic acidosis, Am. J. Physiol. Regul. Integr. Comp. Physiol. 287 (2004) R502–R516.
[43] I. Dujmovic, Cerebrospinal fluid and blood biomarkers of neuroaxonal damage in multiple sclerosis, Mult Scler Int (2011), http://dx.doi.org/10.1155/2011/767083.
[44] U. Ziemann, M. Wahl, E. Hattingen, H. Tumani, Development of biomarkers for multiple sclerosis as a neurodegenerative disorder, Prog. Neurobiol. 95 (2011) 670–685.
[45] V. Pravica, D. Popadic, E. Savic, M. Markovic, J. Drulovic, M. Mostarica-Stojkovic, Single nucleotide polymorphisms in multiple sclerosis: disease susceptibility and treatment response biomarkers, Immunol. Res. 52 (2012) 42–52.
[46] V. Singh, R.Q. Hintzen, T.M. Luider, M.P. Stoop, Proteomics technologies for biomarker discovery in multiple sclerosis, J. Neuroimmunol. 248 (2012) 40–47.
[47] T. Tourdias, V. Dousset, Neuroinflammatory imaging biomarkers: relevance to multiple sclerosis and its therapy, Neurotherapeutics 10 (2013) 111–123