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Correlation of deglutition in subacute ischemic stroke patients with peripheral blood adaptive immunity: Essential amino acid improvement

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Abstract

We aimed to document in stroke patients peripheral blood immune cell profiles, their relations with neuro-functional tests, and any possible influence of supplemented essential amino acids (EAAs) may have on both the immune system and the relationship of the latter with neuro-function.

Forty-two dysphagic stroke patients (27 men; 71±9 years) underwent bio-humoral measurements, neuro-functional tests, including Functional Independence Measure (FIM) and Dysphagia Outcome and Severity Scale (DOSS), and were randomized to receive EAAs 8 g/d (EAA group) or isocaloric maltodextrin (placebo group).

At discharge all measurements were repeated 38±1 days after randomization.

At admission, total white cell (TWC), neutrophil (N), and lymphocyte (Lymph) counts were normal and the N/Lymph ratio was higher than normal values (<3.0). At discharge, both TWC and N decreased while Lymph increased significantly. As a result, the N/Lymph ratio significantly decreased (P <0.001) returning to normal levels. Absolute Lymph counts and Lymph % TWC correlated positively with DOSS (r = +0.235, P = 0.04 and r = +0.224, P = 0.05, respectively), negatively with C-reactive protein natural logarithm (ln CRP) (P = 0.02 and P = 0.0001, respectively), which is an inflammation marker. N correlated positively with ln CRP (P = 0.001) and had a slight negative association with FIM (P = 0.07). The N/Lymph ratio was inversely related to FIM (r = −0.262, P = 0.02) and DOSS (r = −0.279, P = 0.01). Finally, FIM correlated with DOSS (r = +0.35, P = 0.05).

For the regression analysis, the overtime changes of Lymph % TWC correlated significantly with DOSS (P = 0.01). There was a positive correlation between Lymph % TWC and DOSS for the entire stroke population (P = 0.015). While this correlation was not important for the placebo group (P = 0.27), it was significant in the EAA subgroup (P = 0.018).

In the sub-acute stroke stage, there may be slight alterations of peripheral blood immune cells. Lymph cells are associated with improved neuro-function tests with evidence that this association is enhanced by supplementing EAAs.

Keywords

dysphagia, essential amino acids, immune system, sub-acute stroke

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Introduction

Acute cerebral ischemia initiates adrenergic- and corticosteroid-mediated[1] inflammatory and immunological responses, both locally in ischemic lesion areas and in the extra-cerebral periphery.[2] In the periphery system, immunological changes contribute to post-stroke immune-depression,[3] a major factor in the development of infection as well as increased mortality risk.[4] Alterations of both T cell proliferation and activity, are associated with the severity of cerebral ischemia[4] and play a role in more frequent human infarct.[5]

In contrast to acute and early post-stroke phases, the profile of peripheral blood immune cells during the sub-acute stage of the disease (3 weeks to 3 months after the acute event) is still not known. It is conceivable that the peripheral immune system may be altered during sub-acute ischemic strokes. Targeting possible changes of the peripheral blood immune cells during sub-acute strokes could be clinically relevant in preventing infection and could potentially improve neurological outcomes.[1]

This study, conducted on sub-acute ischemic stroke patients with dysphagia, has three main objectives. First, it helps understand the profile of circulating lymphocytes (Lymph), neutrophils (N), and the N/Lymph ratio, given that a high N/Lymph ratio may inhibit the immune system[6] and could reduce the possibility of, and/or prolong the time of neurological repair. In clinical settings, the N/Lymph ratio is prognostic in advanced gastric cancer, independent of the absolute N and Lymph counts.[7]

The second objective of the study was to relate the Lymph and N/Lymph ratio to patient retrieval of physical disability and swallowing changes. The third objective was to find whether essential amino acid (EAAs) supplementation improves both Lymph and neuro-function. The rationale here was that EAAs are physiologically important in maintaining normal innate and adaptive immune competence.[8] This is to ensure both the production of cytokines during inflammation[9] and the structure of the pattern-recognition receptors of toll-like receptors,[10,11] which function as immune-system receptors. Moreover, EAAs promote protein synthesis even during acute inflammation[12] and induce protein synthesis for the remodeling of all tissues/organs, including the brain.

Patients and methods

Population

Forty-two dysphagic patients, consecutively admitted after ischemic strokes, to our Rehabilitation Institute (Rehab) (Nervi, Genova, Italy), were enrolled within 37±12 days of their acute event. The patients came from the following origins: stroke units (14.3%); homes (61.9%); and neurological settings (23.8%). None of the patients were on steroid therapy, or had cancer or nephrotic syndrome. All of these factors constituted exclusion criteria as they may affect the reactants of the acute-phase response. Vascular cerebral insult topography was carried out by computed tomography (CT) or magnetic resonance imaging (MRI).

The damaged stroke areas were classified in relation to the location of the ischemic obstruction as PACI (partial anterior circulation infarction; 45.2%), TACI (total anterior circulation infarction; 30.95%), or POCI (posterior circulation infarction; 23.8%). Written informed consent was obtained from the participants or whenever relevant, from their caregivers, after the nature of the study had been fully explained. The study was approved by the Institutional scientific and ethics committees.

Procedures

Within the first 3 days of admission, the following baseline variables were measured:

a) anthropometric characteristics: body weight (BW, kg) found using a mechanical weight lifter; height (m), calculated from knee height.[13] Body mass index was calculated as kg/m^2. Actual BW was referred to habitual (pre-event) BW. Actual/habitual BW $\leqslant$95% was considered a significant loss of BW;
b) bio-humoral variables: (1) routine variables, including serum protein electrophoresis. We calculated the peripheral blood N/Lymph ratio, which in our laboratory ranges between 1 to 3 for healthy individuals; (2) biomarkers of body inflammatory status: C-reactive protein (CRP; normal value < 0.8 mg/dl, determined by an immune-turbidimetric method); erythrocyte sedimentation rate (ESR: normal range, 2–20 mm at the first hour); (3) acute phase reactants: positive proteins (α-1 globulin system: normal range,

210–350 mg/dl; haptoglobin: 30–200 mg/dl; fibrinogen: normal range, 230–550 mg/dl); negative proteins (albumin: normal range, 4.02–4.76 g/dl; prealbumin: normal range, 18–30 mg/dl; and transferrin: normal range, 202–364 mg/dl);

c) functional status: this was evaluated using the Functional Independence Measure (FIM) as reported elsewhere;[14]
d) dysphagia: all patients were admitted with a diagnosis of dysphagia. The presence of dysphagia was controlled by our group using a video fluoroscopy examination. The severity of dysphagia was evaluated using the Dysphagia Outcome and Severity Scale (DOSS), a 7-point scale developed to systematically rate the functional severity of dysphagia.[15] Score range was 1–7, where level 1 denotes severe dysphagia, level 2 moderately severe dysphagia, level 3 moderate dysphagia, level 4 mild-to-moderate dysphagia, level 5 mild dysphagia, level 6 within functional limit/modified independence, and level 7 normal under all situations.

At admission, 21.4% of the patients were on a modified diet, whereas 78.6% were on nasogastric- or percutaneous endoscopic gastrostomy tubes.

Patient randomization. After completing these procedures, the patients were randomized to receive either essential amino acids (EAAs; EAA group, n = 21), or placebo (maltodextrin; placebo group, n = 21). A randomization list was generated using SAS statistical software (SAS Institute, Cary, NC, USA). A and B identified the blinded treatment. The list was made available both to the physician (BE) and to the hospital pharmacists. The physician sequentially allocated patients to treatment A or B according to the randomization list. The first author (AR) who interpreted all results was blinded to the patients' allocation. The experimental group (EAA group) received 8 g/d of EAAs 4 g in the morning + 4 g in the afternoon diluted in half a glass of water. The individual package of EAAs contained: L-leucine 1250 mg, L-lysine 650 mg, L-isoleucine 625 mg, L-valine 625 mg, L-threonine 350 mg, L-cysteine 150 mg, L-histidine 150 mg, L-phenylalanine 100 mg, L-methionine 50 mg, L-tyrosine 30 mg, L-tryp-tophan 20 mg. The placebo group was given an isocaloric formula containing maltodextrin. We used maltodextrin as the placebo instead of isonitrogenous non-essential amino acids because the latter, even under physiological conditions, is inferior to EAAs in promoting protein synthesis. Maltodextrin as a placebo would have been a more appropriate choice since this carbohydrate, like EAAs, potentially exerts an immunological effect.[14] An imbalanced immunological effect between placebo and EAAs would have also potentially caused an imbalanced development of infection.

For patients on artificial nutrition, the placebo or EAAs supplementation was administered through a feeding tube. In the patients on a modified diet, EAAs were given in the form of a jellified mixture. Treatment (EAAs or maltodextrin) lasted 35 days. At 38 ± 1 days from admission to rehab, the variables from (a) to (d) were all repeated.

Rehabilitation therapy. All patients followed the center rehabilitative protocol, which consisted of performing passive, active and active-assistive range-of-motion exercise coordination, assistive ambulation with devices or support. The duration of the treatment by the same therapist was 60 min a day for 5 days a week. Moreover, all patients underwent speech and occupational therapy.

Statistical analysis

All variables were analyzed, reporting means and standard deviation for quantitative variables and distribution frequencies for qualitative variables. A Chi-squared test was used for categorical variables. Subsequently, CRP was transformed into natural logarithmic values (ln CRP). Any differences in variables between baseline and discharge values for the entire population were tested by means of a paired Student's *t*-test. The relation between circulating Lymph, N/Lymph ratio, and the neurofunction test during Rehab were also studied applying a simple correlation analysis.

The patient population was divided into a group which improved DOSS by at least 1 score and a group with stable DOSS. Baseline differences of the variables between these groups were tested using a unpaired *t*-test. Repeated analysis of variance measurements were used to assess any differences over time between the two groups of patients. Linear multiple regression analyses were performed in order to find high association variables

with DOSS in the two patient subgroups. Baseline differences in the variables of patients on EAAs and on placebo were tested by unpaired *t*-test and repeated measurement analysis of variance was used to evaluate any differences in trends overtime. Here again, linear multiple regression analyses were carried out in order to indicate variables with high association with DOSS. Statistical significance was set at $P < 0.05$.

Results

Patient population

The study results showed that at baseline, the patients had normal body weight (BW) (BMI = 23.7±2.8 kg/m^2), however with an average weight loss of 5.3% compared to pre-stroke BW. The subjects had severe loss of physical capacity (FIM: –74% of normal value, on average) and deglutition ability (DOSS: –71%, on average). Moderate to severe dysphagia (DOSS ⩽3) was found in 38% of subjects. Mild systemic inflammation was also present (average CRP levels 2.5-fold higher than normal value). The inflammation was associated with reduced levels of circulating negative proteins (albumin, prealbumin, transferrin) of the acute phase response and with increased serum concentrations of the positive ones (alpha-1 globulin system, haptoglobin, fibrinogen) as well as with blood glucose at upper limit of the normal values.

The patients were daily provided the following: energy 20.6±1.8 kcal/kg, protein 0.971±0.19 g/kg, carbohydrates 2.2±0.4 g/kg, and lipids 0.847±0.18 g/kg.

At discharge, BW further decreased (average decrease: –1.5 kg, $P < 0.02$). This was compatible with a significant increase in both physical disability (average FIM increase: +69%, $P < 0.001$) and dysphagia (average DOSS increase: +1.19 score; $P < 0.001$). Improved dysphagia was found in 30 patients (71.4%) (16 in placebo and 14 in EAA subjects) whereas stable dysphagia was found in 28.6% patients.

Inflammation was still present but was associated with significantly reduced serum levels of the anti-protease system (alpha-1 globulin from 311 mg/dl to 282 mg/dl on average; $P = 0.031$), haptoglobin ($P < 0.001$) and increased serum concentrations of circulating negative proteins of acute phase response. Plasma glucose significantly diminished and normalized ($P = 0.008$). During rehab, both patients who improved and those who did not improve dysphagia had similar numbers of infection episodes (1.8±0.4 vs. 1.6±0.5, respectively; ns).

At discharge, the baseline patients' nutritional intakes did not significantly change.

Circulating Lymph, N counts, and N/Lymph ratio

At baseline, the patients had normal total white cell (TWC), Lymph, and N counts, notwithstanding systemic inflammation, However, N/Lymph ratio was higher (3.76±2.07) than the normal value of our laboratory (⩽3). At discharge, there were significant reductions of base TWC ($P = 0.004$) and N ($P = 0.001$) counts, whereas the Lymph count increased ($P = 0.02$). As a result, the N/Lymph ratio significantly decreased to 2.43±1.3 ($P < 0.001$) and was normalized.

Relationships between circulating Lymph, N/Lymph ratio, and neuro-function tests during Rehab

Absolute lymph counts and % Lymph correlated positively with DOSS ($r = +0.235$, $P = 0.04$ and $r = +0.224$, $P = 0.05$, respectively) and negatively with the inflammation marker ln CRP ($r = -0.265$, $P = 0.02$ and $r = -0.484$, $P = 0.0001$, respectively). N counts were positively linked to ln CRP ($r = +0.37$, $P = 0.001$) and there was a slight negative association with physical ability (FIM, $r = -0.20$, $P = 0.07$). No correlation was found between N and DOSS. There was a strong negative lymph and N correlation ($r = -0.926$, $P < 0.001$). The N/Lymph ratio was inversely related to physical capacity ($r = -0.262$, $P = 0.02$) and deglutition ability ($r = -0.279$, $P = 0.01$), but was positively associated with ln CRP ($r = +0.514$, $P = 0.0001$). The FIM and DOSS ($r = +0.35$, $P < 0.05$) correlated positively.

In order to understand the relationship between the over-time changes of circulating immune cells and dysphagia better, we stratified the entire stroke population into a first group which after Rehab, improved dysphagia (n = 30 subjects) and a second group which did not improve (n = 12 subjects). Table 1 shows the changes of some of the variables of these groups.

Table 1. Over-time changes of some variables of subjects with improved dysphagia and subjects with no improved dysphagia.

Variables	Overtime changes of dysphagia		*P* value
	Improvement (n = 30)	No improvement (n = 12)	
Lymphocytes (% TWC)	+10.35±11.55	−2.1±12.69	0.004
ESR 1st h (mm)	−14.26±45.92	+19.27±28.79	0.033
C-reactive protein (CRP) (mg/dl)	−1.22±2.9	+0.61±2.29	0.05
Prealbumin (mg/dl)	+2.22±7.49	−3.17±8.07	0.05
FIM score	+26.7±19.84	+13.17±15.56	0.04

Values are expressed as mean ± standard deviation. Statistical analysis: Unpaired Student *t* test. Level of significance set at $P < 0.05$.

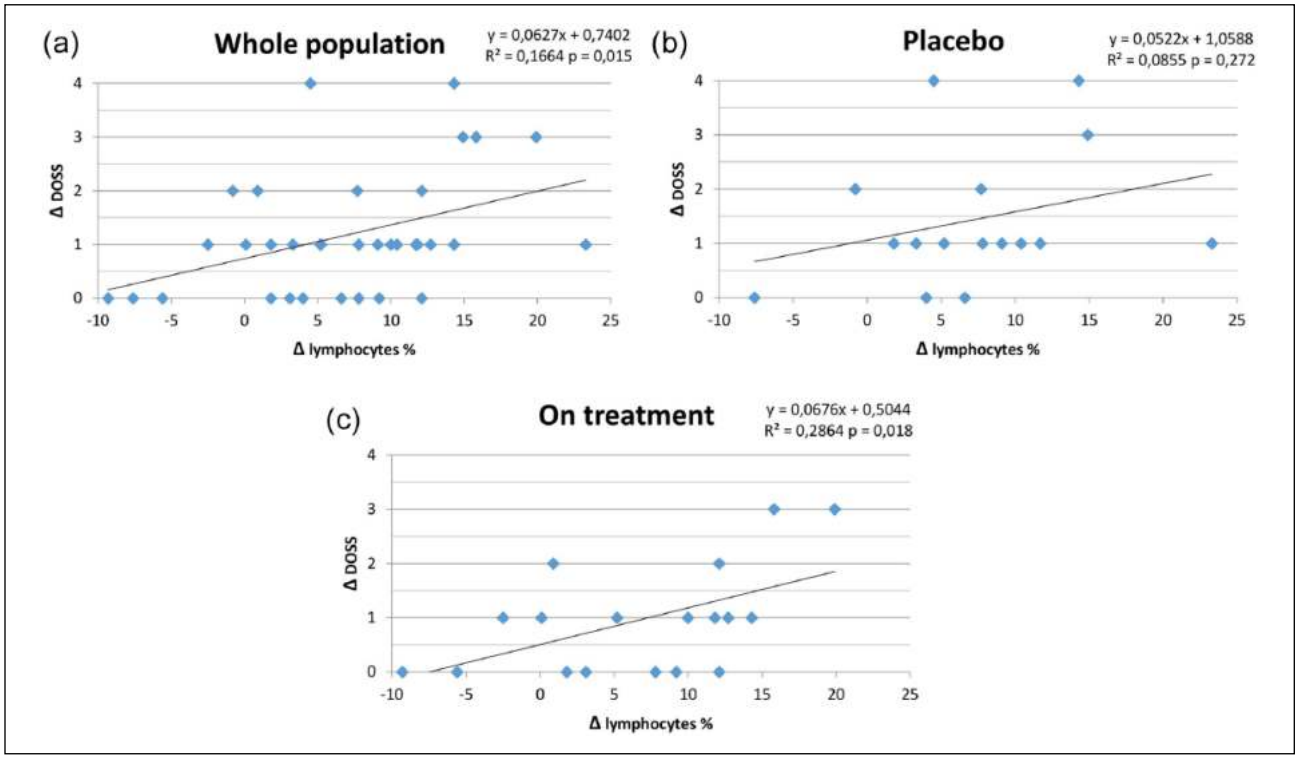

Figure 1. Relationship between over-time changes of peripheral blood lymphocytes as % total white cells and deglutition ability (DOSS) in all stroke population (a), in subjects on placebo (b), and on essential amino acid treatment (c).
Note: the number of dots appearing is lower than the real number of study patients because of overlapping values in some cases.

These variables were simultaneously tested in a logistical regression model showing that only % Lymph was significantly associated with improved swallowing capacity ($P = 0.01$). Coherent with this finding, the over-time changes in % Lymph and DOSS were positively correlated ($P = 0.015$; Figure 1a).

Effects of EAA supplementation on Lymph and dysphagia

At baseline, EAA and placebo groups were similar for all variables considered, except for serum alpha-1 globulin concentrations which were higher in the placebo than the EAA group ($P < 0.02$) (Table 2). During the rehabilitation period, the over-time changes of all variables was similar between the two groups of patients except for the alpha-1 globulin, which diminished for placebo patients (interaction $P = 0.01$) and for N/Lymph ratio which decreased more in the EAA group (interaction $P = 0.04$).

In both groups, improvements of dysphagia were positively linked to improvements in % Lymph (Figure 1b and c) but the association was clearer in the EAA group, suggesting that the association

Table 2. Baseline differences in the variables between placebo and EAA groups.

Variables	Placebo	EAAs	*P* value
Actual body weight (kg)	65.63±15.61	65.17±13.39	1.0
ESR 1st h (mm)	59.88±32.0	38.54±35.7	0.08
Hemoglobin (g/dl)	12.32±1.61	12.41±2.07	1.0
Urea (mg/dl)	42.9±24.8	44.78±33.97	0.9
Creatinine (mg/dl)	0.89±0.31	0.99±0.28	0.7
Glucose (mg/dl)	108.3±17.3	110.3±23.4	0.8
C-reactive protein (CRP) (mg/dl)	2.21±2.66	1.86±2.23	0.4
ln (CRP)	0.28±1.09	−0.10±1.23	0.6
Fibrinogen (mg/dl)	467.1±121.5	405.75±81.01	0.5
Haptoglobin (mg/dl)	306±138.8	237.6±121.28	0.4
α1 globulin (mg/dl)	373.3±143.6	244.5±87.4	0.02
Albumin (g/dl)	2.83±0.39	2.93±0.62	0.8
Prealbumin (mg/dl)	17.5±6.3	18.04±4.29	0.9
Transferrin (mg/dl)	181.1±27.99	176.0±36.57	0.8
Total white cells (TWC) (n°/mm^3)	7664.3±1814.5	7033.5±2464.4	0.4
Neutrophils (% TWC)	66.8±6.9	65.09±13.08	0.8
Lymphocytes (% TWC)	20.1±5.8	23.11±10.89	0.7
Neutrophil/Lymph ratio	3.68±1.45	3.82±1.2	0.9
DOSS score	2.53±1.39	2.26±1.48	0.8
FIM score	32.58±15.98	33.39±19.46	0.9

Values are expressed as mean ± standard deviation. Statistical analysis: Unpaired Student *t* test. Level of significance set at $P < 0.05$.

observed in the entire stroke population (Figure 1a) can mainly be ascribed to the EAA treatment.

Discussion

The study results show that during the sub-acute stage of ischemic strokes, patients had normal peripheral blood Lymph and N counts but high N/Lymph ratio, which normalized after rehab. Furthermore, the study showed that increased peripheral blood % Lymph was significantly associated with improved dysphagia disability and that this relationship may be potentiated by supplementing patients with essential amino acids (EAAs).

The normal peripheral blood Lymph and N counts in patients may be due to reduced corticosteroid production after reduced inflammation.[16] In contrast to the acute stage of stroke,[1,5] the improvement of the adaptive immune system during the sub-acute phase, not only is not detrimental but also may even foster neuro-regeneration.[1] This is indicated here by association of increased blood Lymphs with patient improved clinical-metabolic status and by the positive correlations between the time courses of % Lymph and the retrieval of deglutition ability and physical performance.

This study showed that Lymph cells may support neuronal repair. It is possible that anti-inflammatory intrleukine-10 (IL-10) and transforming growth factor β (TGFβ) secreted from helper T cells may favor tissue repair [1] during the sub-acute stroke phase.

The study showed that EAAs were associated with a significantly reduced blood N/Lymph ratio and improved the relation between the time courses of % Lymph and DOSS. As a result, EAAs may potentially influence both blood immunity and neuro-repair processes. EAAs promote these processes in virtue of several mechanisms. First, EAAs can induce protein synthesis[17–19] in immune cells for Lymph proliferation and duplication.[8] Second, EAA-induced protein synthesis may directly improve the deglutition capacity by affecting the mechanisms underlying normal deglutition, such as inter-neuronal activity and/or deglutition center and/or peripheral neuromuscular function of deglutition.[20] Third, EAA-induced body anabolic status is of paramount importance for brain remodeling and function. Finally, EAAs may influence brain repair/regeneration by inducing hormone insulin like growth factor-1 (IGF-1).[21–23]

Limitations

We recognize that this study has several limitations which will require appropriate investigation. T-Lymph subtypes, particularly regulatory T cells, the anti-inflammatory IL-10, and TGFβ, have not

been determined. This determination may be important to understand the relationship between peripheral immunity and neuro-repair function better in the sub-acute stage of stroke. In addition the functional activity of T cells should also be addressed. A key role in neuro-regeneration may be played by increased plasma and brain contents of IGF-1 after EAA stimulation. The knowledge of circulating IGF-1 levels could contribute to explain the association of supplemented EAAs with the retrieval of brain function including the improvement of dysphagia.

Given the relatively small sample size, we preferred not to investigate possible differences in the variables linked to the patient's genre. It would be interesting to investigate the relationship between peripheral immunity and neuro-function at an earlier post-stroke stage than that considered here, in order to highlight whether there is an optimal time for to influence the retrieval of dysphagia disability with EAA supplementation.

We also need to find whether EAA doses higher than 8 g/d exert further benefits to patients' retrieval of neuro-dysfunction. The knowledge of body composition changes could help the physician to understand which patient has more potential of improving dysphagia.

Finally, the reduction of patients' body weight during rehab indicated that energy 20.6 kcal/kg is not sufficient to maintain body weight in sub-acute strokes.

Clinical implications

This study highlights the importance in sub-acute stroke individuals of monitoring blood N/Lymph ratio over time. Strictly linked to this factor is preventing infection which may be of fundamental importance to avoid the aggravation of patient muscle hypercatabolism[24] which could also affect deglutition musculature. The N/Lymph ratio may be more useful than CRP in understanding the courses of clinical- metabolic- functional status.

The study also shows that protein provision of approximately 1 g/kg/d, may not be enough to exert the best benefit for patient neuro-function retrieval and suggests considering nitrogen intake given as EAAs rather than as proteins. This factor is supported by two considerations. First, we eat proteins but we mainly use their EAAs for protein synthesis. Second, it is EAAs not proteins which pass through the blood brain barrier. Clinically, the use of natural substrates such as EAAs to improve dysphagia should not be underestimated since there are no efficacious therapy, including neuromuscular electrical stimulation[25] at present, to reduce the severity of dysphagia.

Conclusions

The current study shows that in sub-acute stroke subjects peripheral blood adaptive immunity is associated with the retrieval of neuro-function abilities including deglutition. The association may be enhanced by providing patients a supplementation with essential amino acids.

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