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ABSTRACT

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EFFICACY OF PHYSIOTHERAPY INTERVENTIONS ON IMMUNE MODULATION IN CHILDREN WITH CEREBRAL PALSY: A FOCUS ON INFLAMMATORY MARKER REDUCTION

Introduction. Children with cerebral palsy benefit from a wide range of rehabilitation techniques. However, little is known about the impact on the body's pathophysiological processes. Gaining a deeper insight into these processes would assist in creating personalized counseling for patients and enable more targeted rehabilitation.

Aim. This study aims to evaluate the impact of a 30-day rehabilitation program on the serum cytokine profile of children with cerebral palsy by identifying CP-independent biomarkers, assessing rehabilitation-induced changes, and analyzing their role in inflammatory and immune response processes.

Materials and Methods. This study included 15 healthy children (6.42 ± 2.76 years) and 14 children with cerebral palsy (5.10 ± 2.45 years). The rehabilitation program for the children with CP was scheduled for five sessions per week (Saturdays and Sundays were days off) for 6 weeks (42 days total), resulting in 30-day rehabilitation. The sera proteome was analyzed using the Proteome Profiler XL Human Cytokine Array Kit. The relative levels of a predetermined set of 105 cytokines, chemokines, and growth factors were measured. First, pooled sera samples from 14 children with cerebral palsy were compared to 15 healthy children. Next, markers with no difference from healthy children were followed before and after 30-day rehabilitation.

Results. 14 children with cerebral palsy compared to 15 age-matched healthy children showed a relatively same level of 97 sera markers, i.e. CP-independent markers. Complement component C5/C5a, VCAM-1, PECAM-1, IL-8, and adiponectin, i.e. 5 out of 97 markers showed a rehabilitation-induced level decline after 30-day rehabilitation.

Conclusions. The investigation of the sera proteome of children with cerebral palsy showed a declining trend in levels of inflammatory

markers, such as complement component C5/C5a, VCAM-1, PECAM-1, IL-8, and adiponectin, after 30-day rehabilitation.

Keywords: Marker, sera, cytokine, rehabilitation, children, cerebral palsy.

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РЕЗЮМЕ

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ВПЛИВ ФІЗІОТЕРАПЕВТИЧНИХ ВТРУЧАНЬ НА ІМУНОМОДУЛЯЦІЮ У ДІТЕЙ З ДЦП: ДОСЛІДЖЕННЯ РОЛІ ЗАПАЛЮВАЛЬНИХ МАРКЕРІВ

Вступ. Діти з церебральним паралічем отримують користь від широкого спектра реабілітаційних методик. Однак вплив цих методик на патофізіологічні процеси організму залишається недостатньо вивченим. Глибше розуміння цих процесів сприятиме розробці персоналізованих рекомендацій для пацієнтів і забезпечить більш цілеспрямовану реабілітацію.

Мета. Це дослідження має на меті оцінити вплив 30-денної реабілітаційної програми на профіль цитокінів у сироватці дітей з церебральним паралічем шляхом ідентифікації незалежних від церебрального паралічу біомаркерів, оцінки змін, викликаних реабілітацією, та аналізу їх ролі в запальних і імунних процесах.

Матеріали і методи. У дослідженні взяли участь 15 здорових дітей (6,42 ± 2,76 років) та 14 дітей з церебральним паралічем (5,10 ± 2,45 років). Реабілітаційна програма для дітей з церебральним паралічем передбачала п’ять занять на тиждень (субота та неділя – вихідні) протягом 6 тижнів (загалом 42 дні), що становило 30 реабілітаційних днів. Сироватковий протеом аналізували за допомогою набору Proteome Profiler XL Human Cytokine Array Kit. Визначали відносні рівні 105 цитокінів, хемокінів та факторів росту. Спочатку зразки сироватки 14 дітей з церебральним паралічем порівнювали із зразками 15 здорових дітей. Далі проводили аналіз маркерів, рівень яких не відрізнявся від рівня у здорових дітей, до і після 30-денної реабілітації.

Результати. Порівняно з 15 здоровими дітьми відповідного віку, 14 дітей з церебральним паралічем мали відносно однаковий рівень 97 маркерів у сироватці. П’ять із 97 маркерів продемонстрували зниження рівня після 30-денної реабілітації.

Висновки. Дослідження сироваткового протеому дітей з церебральним паралічем показало тенденцію до зниження рівня запальних маркерів, таких як компонент комплементу C5/C5a, VCAM-1, PECAM-1, IL-8 і адипонектин, після 30-денної реабілітації.

Ключові слова: маркер, сироватка, цитокін, реабілітація, діти, церебральний параліч.

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ABBREVIATIONS

CP – cerebral palsy; IL-8 – interleukin 8; PECAM-1 – platelet endothelial cell adhesion molecule, also known as cluster of differentiation 31 (CD31); VCAM-1 – vascular cell adhesion molecule 1

INTRODUCTION

Cerebral palsy (CP) is a clinical descriptor that has undergone many transformations since 1861, with the most recent definition provided in 2007 [1]. CP is a conventionally sporadic phenotype caused by non-progressive brain lesions and presented predominantly by spastic motor form with significant heterogeneity in the degree of movement restriction and complications [2]. CP develops in 1.5–2.5 children per 1000 live births, which results in 17 million individuals globally [3].

Much research has been focused on predicting the condition [4], [5] including molecular pathways and genes associated with predisposition plasma markers of brain hypoxia [6], urine metabolic predictors of neurological outcome after perinatal brain damage [7], and epigenetic markers of blood cell reprogramming in twins discordant for CP development [8].

In contrast, little is known about the effect of rehabilitation on the immune system in children with CP. Peterson et al [9] highlighted systemic blood-related alterations due to spasticity. These speculations were supported later on by cytokine study of sera [10], B-cell distinct transcriptional profile [11], and elevated levels of pro-inflammatory markers such as TNF-α and IL-6 [12]. Furthermore, Zareen et al [13] showed elevated levels of pro-inflammatory markers like erythropoietin with reduced responses to endotoxin. Che and Shi [14] identified several differentially expressed genes, such as IL-1β, IL-6, and TNF, related to immune response, neurogenesis, and inflammation that may play a role in physical activity and rehabilitation for children with CP.

Despite several studies on immune alterations, the effects of rehabilitation on cytokine expression in children with CP remain largely unexplored. This gap in understanding highlights the need for further research in this area. In healthy adults, the link between muscle function and blood alterations was revealed by the investigation of leukocyte epigenetic modification due to training, including altered production of growth factors and cytokines as essential mediators of signaling pathways [15].

The aim of this study was to screen for CP-independet cytokines, chemokines, and growth factors, i.e. levels of signaling molecules that do not differ between healthy children and children with CP. Therefore, we screened 105 serum biomarkers and found that 97 did not show any baseline differences between the healthy and CP groups. We also examined the dynamics of these signaling molecules. After a 30-day rehabilitation period, five of the 97 markers decreased, while the children demonstrated improvements in motor function. Functional analysis indicated that changes in the levels of complement component C5/C5a, VCAM-1, PECAM-1, IL-8, and adiponectin were linked to inflammatory responses and immune regulation. This suggests that rehabilitation may induce immune modulation**.**

MATERIAL AND METHODS

Subjects

A total of 14 children with the spastic form of CP and 15 healthy children were enrolled in the study. To validate our investigation, we performed a sample size calculation based on prior research. Wu [10] found that plasma TNF-α levels were significantly higher in 54 children with cerebral palsy (CP) at 21.4 ± 6.2 pg/ml, compared to 11.2 ± 4.1 pg/ml in 28 healthy controls. To achieve an 80% power and a 5% significance level (two-sided), our study required at least 6 participants per group. This analysis was conducted using G*Power software (version 3.1.9.4) [16]. To address the heterogeneity in the CP group, we strategically increased our sample size to 14 children, strengthening the reliability of our findings. All subjects were native-born Ukrainians.

We included healthy children as a control group to establish baseline levels of cytokines, chemokines, and growth factors. This helped us determine if immune alterations in children with CP are specific to the disease or part of normal variations. The healthy children participated in regular physical education classes at school or preschool. They did not undergo specialized athletic training, ensuring their activity levels remained within a moderate range. Children with clinically confirmed spastic CP (Table 1) were recruited from the Sumy Regional Center for Social Rehabilitation of Children with Disabilities and the Department of Physical Education and Sports of Sumy State University, Ukraine. The spastic form of CP was diagnosed according to the definition, classification, and diagnostic criteria recommended by the guidelines of the Paediatric Stroke Working Group at the Royal College of Physicians [17] and the New York State Department of Health [18], as well as the unified clinical protocol for primary, secondary (specialized), and tertiary (highly specialized) medical care and medical rehabilitation "Cerebral Palsy and Other Organic Brain Lesions in Children Accompanied by Motor Impairments" [19]. Caregivers of the children denied any medication intake by the children.

The inclusion criteria were 1) a diagnosis of spastic CP, 2) age 1.5–10 years, and 3) stable health. Exclusion criteria were 1) a history of epilepsy or fever in the preceding two months, 2) inflammatory response syndrome, 3) diseases with a pro-inflammatory profile, including progressive amyotrophy, encephalomyelitis, severe malnutrition, myasthenia gravis, epilepsy, vision/hearing disorders, or other severe pediatric diseases, 4) other endocrine disorders, metabolic

disorders, autoimmune diseases, or genetic diseases, and 5) clinical symptoms of acute and/or chronic infection. The group of healthy children consisted of volunteer participants who were first-degree relatives (siblings). These children were recruited to ensure genetic and environmental similarities, minimizing potential confounding factors. As required for studies involving human subjects, all caregivers of the participants completed a signed informed consent form before enrollment in the study. All experiments carried out in this study were approved by the ethical committees at Sumy State University and complied with the rules and regulations of the Ukrainian Ministry of Health and the Directive 2001/20/EC of the European Parliament and of the Council of 4 April 2001.

Table 1. Characteristics of children with CP (n = 14) before the rehabilitation

Age, years	*Gender*	*Topographical distribution*	*GMFCS*	*GMFM, %*
2.00	F	Tetraparesis	V	7.75
2.17	M	Diparesis	II	46.32
2.58	M	Tetraparesis	V	10.84
2.58	F	Tetraparesis	V	10.12
3.58	M	Diparesis	I	76.44
4.00	M	Diparesis	V	11.78
5.00	M	Diparesis	I	87.19
5.00	F	Hemiparesis	I	98.89
5.50	M	Double hemiparesis	V	6.22
6.00	F	Diparesis	III	50.34
7.00	F	Diparesis	I	68.81
8.00	M	Hemiparesis	I	95.08
9.00	F	Double hemiparesis	IV	49.76
9.00	F	Diparesis	III	40.42

Sample collection and preparation

This study proceeded in two stages – motor performance assessment and blood sample analysis before and after the 30-day rehabilitation of the children with CP. Blood samples from healthy children were collected once. The rehabilitation program included various therapies to enhance motor coordination and physical performance. These therapies comprised physical exercises, medical massage, Voit therapy, and Bobath therapy, all designed to improve task-specific skills. The program also focused on improving muscle strength and overall fitness. It was conducted at the Sumy Regional Center for Social Rehabilitation of Children with Disabilities and the Department of Physical Education and Sports at Sumy State University in Ukraine. It was scheduled for five sessions per week (Saturdays and Sundays were days off) for 6 weeks (42 days total). This rehabilitation duration aligns with prior research demonstrating that a 4-week structured training intervention can induce leukocyte epigenetic reprogramming [15, 20], as well as reduced adverse health outcomes [3]. Motor improvement was calculated as the GMFM-88 score [21] before rehabilitation subtracted from the GMFM-88 score after rehabilitation in percentages (% gain in motor function = % GMFM-88 score after rehabilitation − % GMFM-88 score before rehabilitation).

During medical examination prior to blood collection no pathological findings were observed. Children and their caregivers indicated no complaints, and body temperature values were within the normal range. Children and their caregivers reported no sleep disturbances prior to blood collection. Blood samples were collected from fasting subjects on Mondays between 8:45 and 9:10 in the morning (to control for diurnal fluctuations in immunologic markers) and at least 65 hours after the last rehabilitation session (in case the last session was scheduled in the afternoon on Friday). The study was performed from November to March.

Serum was prepared by centrifugation at 1000 × g and stored at −20°C until analysis for cytokines, chemokines, and growth factors.

Laboratory testing results

Immunological characteristics of healthy children and children with CP were compared based on our previous study [12] (Table 2, 3).

Table 2. Immunological characteristics of children with CP (n = 14) and healthy controls (n = 15) adjusted for age in a linear regression analysis

Immune system components	*Healthy children*	*Children with CP*	*P*
CD3	2530.48	3142.46	0.0030 **
CD4	1555.88	1944.52	0.0015 **
CD8	854.04	1023.30	0.0462 *
IgA	2.13	2.03	0.4707

Note: The levels of immune system components correspond to the coefficient in the linear regression model, i.e. the predicted value of the independent variable; ∗P < .05; ∗∗P < .01

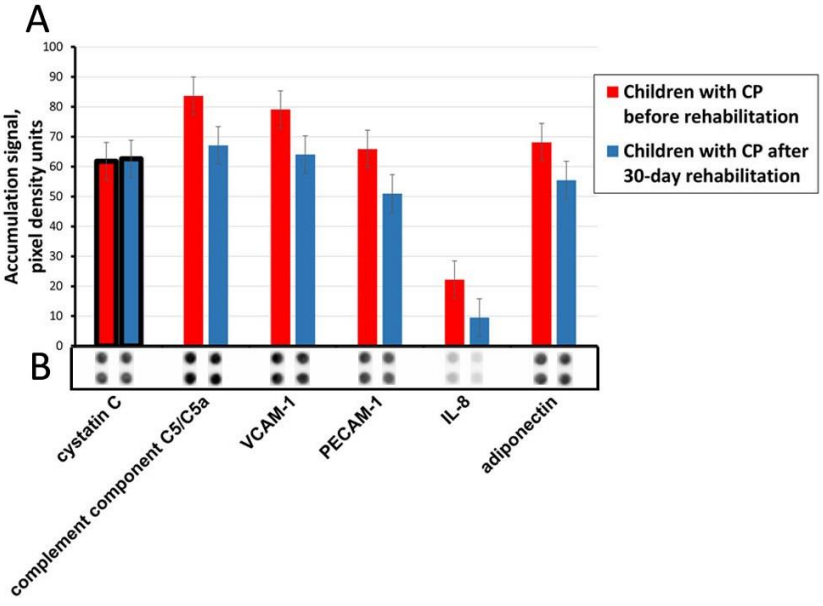
Table 3. Effect of rehabilitation in children with CP after 30-day rehabilitation

Outcome variable	*Before rehabilitation*	*After 30-day rehabilitation*	*P_Value*
$CD3^+$ T-cells, cells/μL	2520.21	2602.07	0.7326
$CD4^+$ T-cells, cells/μL	1474.50	1488.29	0.9217
$CD8^+$ T-cells, cells/μL	864.07	965.71	0.3295
IgA, mg/mL	2.01	1.76	0.0003 ***
GMFM-88 score, %	47.14	50.85	0.00005 ***

Note: The level corresponds to the coefficient in the linear mixed effect model, i.e. the predicted value of the outcome variable for the first fixed factor in the model; ∗∗P < .01; ∗∗∗P < .001, when compared to the level before rehabilitation

A proteomic chip generated data on pooled sera samples (15 healthy and 14 children with CP) to select markers with no correlation with CP. Next, expression levels of sera markers in 14 children with CP were compared before and after 30-day rehabilitation markers to follow rehabilitation efficacy.

We used the Proteome Profiler XL Human Cytokine Array Kit (R&D Systems, Minneapolis, MN), adapted according to the manufacturer's instructions to allow semi-quantification with Bio-Rad-Image-Lab-Software-6.0.1-Windows by chemiluminescence detection in Bio Rad machine with optimal exposure mode. The average signal (pixel density) of the pair of duplicate spots representing each marker was normalized to the mean of 6 positive control references on each membrane. Calibration standard deviations (SD) were established based on SD of positive reference spots for each membrane. The accumulation signal of measure analytes was expressed in pixel density units (Figure 1A, Tables 4a, 5).



Cystatin C was chosen as a negative control marker. The relative levels of serum markers are expressed in pixel density units (Table 5).

(A) The dynamics of relative levels of serum markers in children with CP before and after 30-day rehabilitation (sorted according to effect size in ascending order). Calibration standard deviations (SD) were established based on SD of positive reference spots for each membrane.

(B) The pairs of spots represent the accumulation of chemiluminescent signal for each marker on the two Proteome Profiler XL Human Cytokine Arrays with pooled sera from 14 children with CP before and after 30-day rehabilitation

Figure 1. CP-independent cytokines in children with CP before and after 30-day rehabilitation

Table 4a. Comparison of relative levels of cytokines, chemokines, and growth factors in healthy children vs. children with CP before the rehabilitation

Cytokines, chemokines, and growth factors	*Healthy children, pixel density units*	*Children with CP before rehabilitation, pixel density units*	*Cohen's D*	*Effect size*
TFF3	11.57	11.59	0.00	Very small
Kallikrein 3	3.22	3.24	0.00	Very small
IL-23	1.34	1.39	0.01	Very small
FGF-19	15.19	15.25	0.01	Very small
IL-24	1.85	1.79	0.01	Very small
IL-15	2.30	2.23	0.01	Very small
IL-31	1.90	1.99	0.01	Very small
IL-1β	2.41	2.29	0.01	Very small
IL-33	2.20	2.06	0.01	Very small
FGF-7	2.69	2.86	0.02	Very small
M-CSF	3.20	3.04	0.02	Very small
IL-16	2.61	2.42	0.02	Very small
IFN-γ	3.37	3.14	0.02	Very small
IL-2	4.28	4.54	0.03	Very small
IL-1ra	3.22	3.51	0.03	Very small
Relaxin-2	3.95	3.65	0.03	Very small
Cripto-1	3.00	3.32	0.03	Very small
IL-6	5.01	4.69	0.03	Very small
RAGE	8.17	8.51	0.03	Very small
HGF	4.74	4.37	0.04	Very small
LIF	1.39	1.76	0.04	Very small
IL-11	4.86	5.25	0.04	Very small
IL-27	2.85	3.25	0.04	Very small
MIG-1α/MIP-1β	2.04	2.45	0.04	Very small
I-TAC	3.43	3.85	0.04	Very small
MIF	13.43	13.00	0.04	Very small
MCP-3	1.74	2.17	0.05	Very small
GM-CSF	4.78	4.34	0.05	Very small
IL-3	2.69	2.23	0.05	Very small
Leptin	4.99	4.52	0.05	Very small
IL-34	1.97	2.45	0.05	Very small
MIG	3.45	2.96	0.05	Very small
MIP-3α	2.18	2.72	0.06	Very small
IL-13	2.74	2.18	0.06	Very small
IGFBP-2	68.31	67.72	0.06	Very small
IL-10	3.45	4.04	0.06	Very small
IL-32	2.92	2.33	0.06	Very small
TGF-α	2.75	3.35	0.06	Very small
TNF-α	2.67	3.28	0.06	Very small
FGF basic	4.10	4.71	0.06	Very small
CD30	6.53	7.19	0.07	Very small
G-CSF	3.03	3.70	0.07	Very small
IL-5	1.79	0.99	0.08	Very small
IL-1α	3.51	2.69	0.08	Very small
Pentraxin 3	6.07	6.93	0.09	Very small
GROα	2.86	3.74	0.09	Very small
IL-12 p70	3.01	2.12	0.09	Very small
PF4	59.29	60.21	0.10	Very small
Angiopoietin-2	7.72	8.72	0.10	Very small
IL-22	5.22	6.24	0.11	Very small
Complement Factor D	26.07	27.19	0.12	Very small
Complement Component C5/C5a	82.53	83.68	0.12	Very small
Flt-3 Ligand	4.44	5.65	0.12	Very small

Table 4a Continued

Cytokines, chemokines, and growth factors	*Healthy children, pixel density units*	*Children with CP before rehabilitation, pixel density units*	*Cohen's D*	*Effect size*
Myeloperoxidase	4.90	6.15	0.13	Very small
IL-19	1.36	2.63	0.13	Very small
Fas Ligand	6.22	4.81	0.15	Very small
IL-4	4.49	5.95	0.15	Very small
Emmprin	24.83	26.53	0.18	Very small
IL-17A	18.45	20.17	0.18	Very small
VEGF	2.42	4.28	0.19	Very small
Cystatin C	59.79	61.77	0.20	Small
CD40 ligand	6.57	8.78	0.23	Small
IP-10	6.37	8.72	0.24	Small
ICAM-1	23.37	26.09	0.28	Small
MCP-1	4.60	7.65	0.32	Small
SDF-1α	9.41	12.51	0.32	Small
TfR	7.27	10.55	0.34	Small
IGFBP-3	16.38	13.02	0.35	Small
uPAR	9.78	13.30	0.36	Small
BDNF	20.64	24.84	0.44	Small
Endoglin	60.19	55.33	0.50	Medium
CD14	31.41	26.25	0.53	Medium
GDF-15	11.50	16.94	0.56	Medium
ST2	8.76	14.23	0.57	Medium
Apolipoprotein A-I	55.61	62.71	0.73	Medium
MMP-9	38.29	45.73	0.77	Medium
Thrombospondin-1	27.91	35.55	0.79	Medium
Vitamin D BP	49.90	57.60	0.80	Medium
Resistin	8.02	16.26	0.85	Large
TIM-3	39.54	49.15	0.99	Large
Growth Hormone	19.28	29.55	1.06	Large
IL-18 Bpa	55.79	66.52	1.11	Large
Chitinase 3-like1	53.24	64.31	1.15	Large
VCAM-1	67.93	79.08	1.15	Large
RANTES	31.26	42.42	1.15	Large
Osteopontin	22.16	33.36	1.16	Large
SHBG	67.01	79.03	1.24	Very large
Lipocalin-2	22.49	35.03	1.30	Very large
DPPIV	133.16	119.35	1.43	Very large
CD31	49.60	65.90	1.69	Very large
MIP-3β	19.33	2.96	1.69	Very large
PDGF-AB/PDGF-BB	30.76	47.31	1.71	Very large
IL-8	4.89	22.21	1.79	Very large
ENA-78	24.75	42.87	1.88	Very large
Adiponectin	49.60	68.13	1.92	Very large
Angiopoietin-1	22.72	41.54	1.95	Very large
Dkk-1	12.04	31.03	1.97	Very large
C-reactive Protein	109.61	130.90	2.20	Huge
BAFF	18.99	43.19	2.50	Huge
TARC	7.06	32.65	2.65	Huge
Angiogenin	118.73	149.10	3.14	Huge
Serpin E1	113.89	144.95	3.21	Huge
RBP-4	149.17	117.24	3.30	Huge
EGF	40.33	79.54	4.06	Huge
PDGF-AA	40.60	105.83	6.75	Huge

Table 5. Comparison of relative levels of cytokines, chemokines, and growth factors in children with CP before the rehabilitation vs. children with CP after the rehabilitation

Cytokines, chemokines, and growth factors	*Children with CP before rehabilitation, pixel density units*	*Children with CP after rehabilitation, pixel density units*	*Cohen's D*	*Effect size*
Cystatin C	61.77	62.57	0.13	Very small
Complement Component C5/C5a	**83.68**	**67.12**	**2.64**	**Huge**
VCAM-1	**79.08**	**64.06**	**2.40**	**Huge**
CD31	**65.90**	**51.02**	**2.37**	**Huge**
IL-8	**22.21**	**9.52**	**2.02**	**Huge**
Adiponectin	**68.13**	**55.48**	**2.02**	**Huge**
IGFBP-2	67.72	55.75	1.91	Very large
TIM-3	49.15	37.87	1.80	Very large
ENA-78	42.87	32.28	1.69	Very large
DPPIV	119.35	108.97	1.66	Very large
PDGF-AB/PDGF-BB	47.31	38.16	1.46	Very large
Osteopontin	33.36	24.95	1.34	Very large
MMP-9	45.73	37.88	1.25	Very large
PF4	60.21	52.96	1.16	Large
Thrombospondin-1	35.55	29.08	1.03	Large
Growth Hormone	29.55	23.62	0.95	Large
Angiopoietin-1	41.54	35.74	0.93	Large
Chitinase 3-like1	64.31	59.31	0.80	Medium
Dkk-1	31.03	26.06	0.79	Medium
IL-17A	20.17	15.44	0.75	Medium
Resistin	16.26	11.67	0.73	Medium
Vitamin D BP	57.60	53.30	0.69	Medium
BDNF	24.84	20.67	0.67	Medium
ICAM-1	26.09	21.94	0.66	Medium
TfR	10.55	6.56	0.64	Medium
SDF-1α	12.51	8.66	0.62	Medium
IL-18 Bpa	66.52	62.83	0.59	Medium
RANTES	42.42	38.98	0.55	Medium
GDF-15	16.94	13.62	0.53	Medium
TNF-α	3.28	6.53	0.52	Medium
ST2	14.23	11.08	0.50	Medium
uPAR	13.30	10.52	0.44	Small
Endoglin	55.33	52.76	0.41	Small
IP-10	8.72	6.35	0.38	Small
TFF3	11.59	9.29	0.37	Small
VEGF	4.28	2.18	0.33	Small
Emmprin	26.53	24.49	0.33	Small
CD40 ligand	8.78	6.84	0.31	Small
Pentraxin 3	6.93	5.33	0.26	Small
Lipocalin-2	35.03	36.52	0.24	Small
IL-11	5.25	3.77	0.23	Small
CD14	26.25	24.78	0.23	Small
RAGE	8.51	7.20	0.21	Small
I-TAC	3.85	2.56	0.21	Small
Complement Factor D	27.19	25.92	0.20	Small
Myeloperoxidase	6.15	4.90	0.20	Very small
MCP-1	7.65	6.44	0.19	Very small
G-CSF	3.70	2.57	0.18	Very small
IL-10	4.04	2.91	0.18	Very small
MIG-1α/MIP-1β	2.45	1.33	0.18	Very small
FGF basic	4.71	3.63	0.17	Very small
Relaxin-2	3.65	2.73	0.15	Very small

Table 5 Continued

Cytokines, chemokines, and growth factors	*Children with CP before rehabilitation, pixel density units*	*Children with CP after rehabilitation, pixel density units*	*Cohen's D*	*Effect size*
FGF-19	15.25	14.35	0.14	Very small
MIF	13.00	12.16	0.13	Very small
MIP-3α	2.72	1.88	0.13	Very small
TGF-α	3.35	2.54	0.13	Very small
IL-22	6.24	5.43	0.13	Very small
IL-27	3.25	2.52	0.12	Very small
IL-33	2.06	1.35	0.11	Very small
IL-19	2.63	1.95	0.11	Very small
Fas Ligand	4.81	4.16	0.10	Very small
Flt-3 Ligand	5.65	5.02	0.10	Very small
IL-6	4.69	4.12	0.09	Very small
IL-1α	2.69	2.14	0.09	Very small
IL-2	4.54	3.99	0.09	Very small
MIP-3β	2.96	2.43	0.09	Very small
IL-23	1.39	1.87	0.08	Very small
MCP-3	2.17	1.71	0.07	Very small
MIG	2.96	2.50	0.07	Very small
IL-31	1.99	1.54	0.07	Very small
IGFBP-3	13.02	12.57	0.07	Very small
IFN-γ	3.14	2.71	0.07	Very small
M-CSF	3.04	2.62	0.07	Very small
IL-3	2.23	2.64	0.07	Very small
IL-32	2.33	1.93	0.06	Very small
IL-4	5.95	5.56	0.06	Very small
CD30	7.19	6.81	0.06	Very small
IL-1ra	3.51	3.88	0.06	Very small
GROα	3.74	3.41	0.05	Very small
IL-5	0.99	0.68	0.05	Very small
Kallikrein 3	3.24	2.96	0.05	Very small
Cripto-1	3.32	3.58	0.04	Very small
IL-13	2.18	1.93	0.04	Very small
LIF	1.76	1.53	0.04	Very small
IL-34	2.45	2.22	0.04	Very small
Apolipoprotein A-I	62.71	62.93	0.04	Very small
IL-24	1.79	1.58	0.03	Very small
IL-1β	2.29	2.08	0.03	Very small
Leptin	4.52	4.31	0.03	Very small
SHBG	79.03	78.83	0.03	Very small
FGF-7	2.86	2.75	0.02	Very small
IL-12 p70	2.12	2.03	0.02	Very small
GM-CSF	4.34	4.42	0.01	Very small
IL-16	2.42	2.36	0.01	Very small
IL-15	2.23	2.21	0.00	Very small
Angiopoietin-2	8.72	8.73	0.00	Very small
HGF	4.37	4.36	0.00	Very small

Including cystatin C as a negative control marker was essential due to its stability, its lack of direct involvement in inflammation related to CP, and its role in ensuring consistent measurements over time. As it is primarily linked to renal function and systemic protease inhibition, cystatin C stands apart from inflammatory cytokines and endothelial markers. This distinction allowed us to use it as an internal control, confirming that variations in inflammatory markers – like VCAM-1, PECAM-1, and complement C5/C5a – were genuinely due to rehabilitation effects rather than random biological variations. Furthermore, analyzing immune markers in children is crucial for understanding systemic immune dysregulation associated with CP.

While traditionally viewed as a motor disorder, increasing evidence highlights its connection to persistent low-grade inflammation and altered immune responses.

Levels of cystatin C did not differ between healthy children and children with CP, and there were no changes after 30-day rehabilitation.

Statistical analysis

Comparison of healthy children and children with CP by Fisher's exact test did not show a significant difference according to gender – the odds ratio was 1.14 with the following presentation: 7 (50.00%) males and 7 (50.00%) females in children with CP and 7 (46.67%) males and 8 (53.33%) females in the control group; P = 1. Comparison of healthy children and children with CP by using a robust linear regression model did not show a significant difference according to age (6.42 years in healthy children and 5.10 years in children with CP; P = 0.1869). The comparability of controls and children with CP in terms of immune system components was determined using a robust linear regression model designed using RStudio software [20] (Version 1.3.1073) with the MASS and repmod packages (rlm() and rob. pvals() functions) to account for outliers. The levels of immune system components (the level of CD3+, CD4+, CD8+ T-cells, and the IgA) were chosen as the independent variables. The dependent variables in the model were group (healthy, children with CP) and age (Table 2). Considering the effect of sex on the levels of the components of the immune system, we adjusted the values for both age and sex, but no differences were found (data not shown).

The efficacy of rehabilitation was explored using a linear mixed effect model [22] with the lme4 package (the lmer() function), and gain in motor function was the outcome variable. Age was used as the fixed factor, and severity of motor dysfunction (in terms of GMFCS) was added as a random intercept to account for variation between different severity levels of motor dysfunction (Table 3).

A linear mixed effect model was designed using RStudio with the lme4 and lmerTest packages (the lmer() function) to explore the effects of rehabilitation. Motor performance (GMFM-88 score) and levels of immune system components (the numbers of CD3+, CD4+, and CD8+ T-cells and the levels of IgA) were chosen as the outcome variables. The fixed factor in the model was time point (before rehabilitation, and after 30 days of rehabilitation). Variation between individuals was accounted for in the model via 14 random intercepts associated with children with CP. $P < .05$ was considered significant.

First, we calculated the effect size between the relative levels of the markers on different arrays using the mean of the pair of duplicate spots and the calibration SD of each membrane to verify the difference in marker expression. Next, the effect size was calculated based on Cohen`s D [20] using Microsoft Excel 2019. Markers with huge effect sizes between arrays (Figure 1A, 1B; Table 4a, 4b) were identified.

A Functional Annotation Clustering feature of DAVID 6.8 (The Database for Annotation, Visualization and Integrated Discovery) [24] was used to define biological functions and processes associated with altered levels of cytokines, chemokines, and growth factors using the default settings with medium stringency.

Table 4b. Interpretation for Cohen's D

Cohen's D	*Effect size*
0.01	Very small
0.2	Small
0.5	Medium
0.8	Large
1.2	Very large
2	Huge

RESULTS

The 30-day rehabilitation resulted in a gain in motor function for every child with CP with an average improvement of 5.6% when adjusted for age and GMFCS (a statistical process applied to rates of improvement that allowed us to compare children of different ages and severity of CP). In addition, linear mixed effect regression analysis showed an effect of age on gain in motor function (P = 0.0021) (Fig. 2), i.e. there was greater gain in motor function at earlier age. Similar results were reported in the study by Lee [25]. As reported in our previous study [12] there was statistically significant decrease in levels of IgA and no statistically significant change in levels of CD3+, CD4+, CD8 T-cells (Table 3).

We started screening sera markers via the sample pooling technique. This allowed us to compare the levels of 105 biomarkers between healthy children and children with CP cost-efficiently [26]. As a result, we identified 97 CP-independent biomarkers, i.e., markers that represent no difference between healthy children and children with CP (Table 4a).

Following that, we assessed cytokine changes after a 30-day rehabilitation period. Adiponectin, VCAM-1, complement component C5/C5a, PECAM-1, and IL-8, i.e., five out of the 97 markers (Figure 1A, 1B) exhibited a declining trend after motor improvement. Utilizing the DAVID web tool, we pinpointed relevant biological processes, including the cells and molecules involved in local acute inflammatory response, inflammatory response, and secretion.

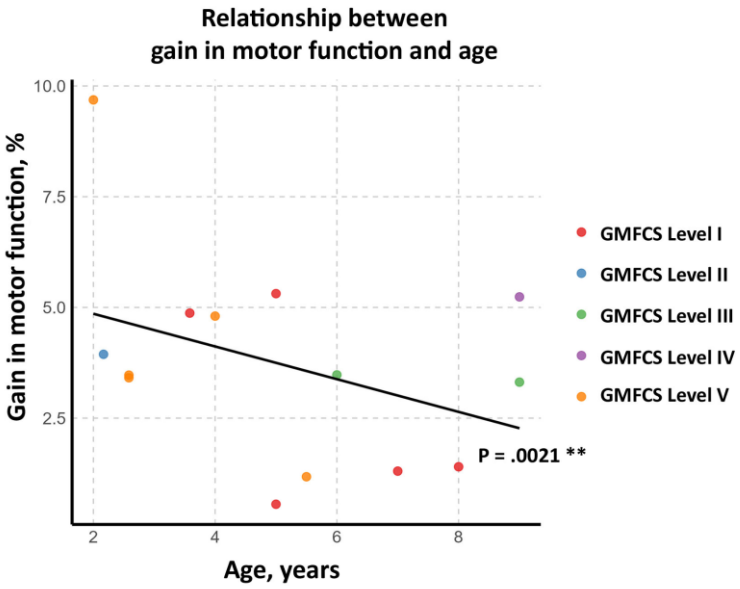


Figure 2. Relationship between gain in motor function and age in children with CP after 30-day rehabilitation
Relationship between gain in motor function and age in children with CP grouped according to severity of motor dysfunction (GMFCS) (the regression line was calculated using results from linear mixed effect regression analysis function lmer())

DISCUSSION

In this study, we showed the effects of rehabilitation on the dynamics of CP-independent signaling molecules in children with cerebral palsy (CP). Our findings showed decreased levels of adiponectin, VCAM-1, complement component C5/C5a, PECAM-1, and IL-8 after 30-day rehabilitation. It is in line with the study by Sharova et al [12], which demonstrated that rehabilitation can reduce immunological markers such as IgA, indicating that rehabilitation may modulate the elevated inflammatory profile observed in children with CP.

Moreover, Kruse et al [27] showed altered responses of CD8+ T-cell levels to exercise in adults with CP compared to typically developing adults. Moreover, Turton et al [28] suggested that cytokine responses to training may depend on individuals' baseline conditions.

Adiponectin is an anti-inflammatory and insulin-sensitizing signal molecule. Reduced plasma adiponectin levels in both healthy and hypertensive rats after exercise training were shown in a study by Kilic-Erkek et al [29]. Numao et al [30] found that only high-intensity exercise significantly decreased adiponectin levels in obese men, while moderate exercise had no notable effect, suggesting an impact of the metabolic state and low-grade inflammation on the response.

Schmidt-Lucke et al [31] demonstrated a biphasic change in levels of VCAM-1 following physical activity. Initially, soluble VCAM-1 level increases immediately after exercise, followed by a significant decline (9%) within one hour, indicating complex multi-phase metabolic regulation.

Our findings also show a decrease in complement component C5/C5a levels following the 30-day rehabilitation program for children with CP. It is in line with existing research indicating a correlation between increased physical fitness and lower levels of circulating complement proteins. Rothschild-Rodriguez et al [32] reported that higher cardiorespiratory fitness in children is associated with reduced levels of C3 and C4, while C5–C9, properdin, and factor B remain unchanged in athletes. In contrast, levels of C1-inhibitor, C3, C4, C5a, and C6 do not change after moderate-intensity cycling or running. These conflicting findings suggest that exercise intensity, muscle damage, and immune cell infiltration all influence complement activation.

PECAM-1 (platelet endothelial cell adhesion molecule-1, CD31) is a transmembrane glycoprotein expressed on cells. It mediates adhesion and is required for leukocyte transmigration. The extracellular domain of PECAM-1 can be found in circulation due to endothelial cell apoptosis, and elevated serum levels of PECAM-1 have been documented in conditions characterized by vascular injury and systemic inflammation, such as myocardial infarction, acute ischemic stroke, and multiple sclerosis [33]. We observed a reduction in PECAM-1 levels after rehabilitation. This aligns with a broader overall reduction in inflammatory signaling, suggesting that physical rehabilitation in children with CP

not only enhances motor outcomes but may also provide vascular-protective immunomodulatory effects.

IL-8 is a chemokine and pro-inflammatory cytokine involved in angiogenesis. Riccieri et al [33] discuss elevated IL-8 levels in relation to chronic inflammatory conditions, as well as endothelial dysfunction. For example, in systemic sclerosis, high circulating levels of IL-8 are linked to internal organ involvement, particularly pulmonary fibrosis. Elevated IL-8 levels were observed alongside increased levels of VEGF, PDGF-BB, and HGF, as well as PECAM-1 and leptin. IL-8 levels decrease in this study may reflect endothelial remodulation.

CONCLUSIONS

In this study, we showed the declining trend in levels of inflammatory markers, such as complement component C5/C5a, VCAM-1, PECAM-1, IL-8, and adiponectin after 30-day rehabilitation. These markers showed no difference between healthy children and children with CP before rehabilitation. Thus, our study suggests that rehabilitation evokes a unique pathophysiological pattern of cytokine response. The identification of biomarkers on an individual level is needed to confirm findings.

PROSPECTS FOR FUTURE RESEARCH

Better understanding the pathophysiological processes behind rehabilitation is a step towards tailoring treatment/rehabilitation for an individual patient.

AUTHOR CONTRIBUTIONS

Sharova O. V.: idea and study design; data collection and analysis; statistical analysis; writing the paper; final approval of the paper.

Smiyan O. I.: study design; critical review; final approval of the paper.

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CONFLICT OF INTEREST

All authors report no biomedical financial interests or potential conflicts of interest.

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