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RELATIONSHIP OF PERIPHERAL LYMPHOCYTE SUBSETS AND SKELETAL MUSCLE MASS INDEX IN SARCOPENIA: A CROSS-SECTIONAL STUDY

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Abstract: *Objectives:* Lymphocytes can affect the proliferation and migration of muscle satellite cells, which may be associated with reduced muscle mass in patients with sarcopenia. The present study aimed to further enrich understandings of the changes of blood lymphocytes and explore the relationship between peripheral lymphocyte subsets and muscle mass in patients with sarcopenia. *Design:* A cross-sectional study. *Setting:* Geriatrics department of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. *Methods:* Eighty-five subjects were enrolled in this study, and were divided into two groups: the sarcopenia group (n=60) and the non-sarcopenia group (n=25). The diagnosis of sarcopenia was based on the diagnostic criteria updated by the Asian Working Group for Sarcopenia (AWGS) in 2014. Complete blood count, peripheral lymphocyte subsets, and body composition of all patients were measured. *Results:* Skeletal muscle mass index (SMI) was negatively correlated with $CD4^+CD28^{null}$ T lymphocytes in peripheral blood in patients with sarcopenia. *Conclusion:* The result of our study may point out the role of $CD4^+CD28^{null}$ T lymphocytes in the pathogenesis of sarcopenia.

Key word: Sarcopenia, lymphocyte subsets, body composition, appendicular skeletal muscle mass, skeletal muscle mass index, $CD4^+CD28^{null}$ T cells.

Introduction

Sarcopenia is a clinical syndrome characterized by continuous loss of skeletal muscle mass, muscle strength or function (1). It usually occurs in the elderly, but it can also occur in an earlier stage of life. Sarcopenia, which increases the risk of falls and fractures, is also closely associated with metabolic diseases and cognitive dysfunction, resulting in serious adverse effects on people's quality of life and inflicting a heavy socio-economic burden (2-5).

A variety of risk factors are involved in the development and progression of sarcopenia, such as inflammation, genetics, nutrition, aging, exercise and other factors (6). Lymphocytes and their secreted proteins play a crucial role in muscle repair and regeneration, considered as a potential control switch for this process (7). T lymphocytes are essential for skeletal muscle regeneration and repair, CD8 + T cells can penetrate damaged muscles, facilitate the secretion of MCP-1 to recruit Gr1high macrophages, thus promoting the proliferation of myoblasts (8). Treg cells respond to specific cytokines that can accumulate in injured muscles and promote muscle growth by releasing growth factors, such as amphiregulin (9). T Lymphocytes can also affect the proliferation and migration of muscle satellite cells (10), which may be associated with reduced muscle mass in patients with sarcopenia. These studies suggest that lymphocytes may be involved in the loss of muscle mass that leads to sarcopenia. Unfortunately, the changes in lymphocyte subsets in patients with sarcopenia are rarely studied.

To further enrich the understanding of the changes of peripheral lymphocyte subsets and explore the relationship between peripheral lymphocyte subsets and muscle mass in patients with sarcopenia, this study focused on the relationship between peripheral T lymphocyte subsets and muscle mass in sarcopenia patients.

Materials and methods

Subjects

According to the Helsinki Declaration, this study was approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (IRB ID: TJ-C20141112).

Eighty-five inpatients were enrolled in our study. All subjects were inpatients of geriatrics department of Tongji hospital from January 2015 to July 2017. Patients with malignant tumors, end-stage diseases, primary or secondary immunodeficiency, severe organ dysfunction, genetically inherited muscular dystrophies, and inability to complete relevant tests were excluded.

All participants provided written informed consent before data collection.

Sarcopenia definition

Considering that all of the subjects were Asian, The diagnosis of sarcopenia was based on the diagnostic criteria updated by the Asian Working Group for Sarcopenia (AWGS) in 2014 (11). The dual-energy X-ray absorption method was used to determine the diagnosis of Sarcopenia when the SMI (Skeletal muscle mass index) was lower than 7.26 Kg/m^2 in males or 5.45 Kg/m^2 in females.

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Table 1
Demographic characteristics, body composition, lymphocyte subsets results of the No Sarcopenia and Sarcopenia groups

Variables	All(n=85)	No Sarcopenia (n=25)	Sarcopenia (n=60)	P
Gender, female (n, %) $	22(0.258)	10(0.400)	12(0.200)	0.037*
Age(years)	58(19)	52(14)	60(26)	0.041
Height(m)	1.659±0.074	1.662±0.729	1.657±0.075	0.791
Weight(kg)	67.933±12.452	74.720±12.107	65.105±11.554	0.001*
BMI(kg/m^2)	24.422(4.046)	25.771(5.815)	23.469(3.157)	0.000*
ASM(kg)	17.236±3.937	19.319±4.078	16.368±3.563	0.001*
SMI(kg/m^2)	6.193±1.048	6.914±0.979	5.892±0.928	0.000*
Percent of fat mass (%)	31.068±5.743	32.39±6.021	30.527±5.582	0.172
Lean / fat ratio	2.195±0.644	2.086±0.553	2.240±0.678	0.319
FM to height^2(kg/m^2)	7.210(2.370)	8.250(3.550)	6.990(2.250)	0.012*
LM to height^2(kg/m^2)	16.379±2.308	17.700±2.492	15.829±2.002	0.000*
CD3+CD4+ #	0.363±0.120	0.370±0.163	0.360±0.097	0.723
CD3+CD8+ #	0.277±0.122	0.264(0.119)	0.257(0.121)	0.307
Treg (%) #	0.023±0.010	0.023±0.012	0.023±0.009	0.712
CD4+CD28+ #	0.341±0.101	0.337±0.103	0.343±0.101	0.778
CD4+CD28null #	0.012(0.019)	0.011(0.021)	0.012(0.018)	0.765
CD8+CD28+#	0.155±0.593	0.158±0.056	0.154±0.060	0.753
CD8+CD28null #	0.930(0.074)	0.101(0.063)	0.090(0.081)	0.576
WBC(×10^9/L)	5.810(1.980)	5.400(1.940)	5.870(2.270)	0.213
NEUT(×10^9/L)	3.140(2.080)	3.080(1.240)	3.145(2.080)	0.276
LYM(×10^9/L)	1.960(0.770)	1.850(0.780)	1.970(2.830)	0.919
MONO(×10^9/L)	0.450(0.240)	0.360(0.230)	0.460(0.200)	0.093
Eosinophil(×10^9/L)	0.140(0.100)	0.120(0.100)	0.150(0.100)	0.927
Basophil(×10^9/L)	0.020(0.020)	0.020(0.030)	0.010(0.020)	0.057
RBC(×10v/L)	4.700(0.960)	4.650(0.960)	4.750(0.930)	0.440
Hb(g/L)	138.000(24.500)	137.000(27.5)	138.500(23.3)	0.572
PLT(×10^9/L)	199.000 (72.000)	193.000 (81.00)	201.500(75.000)	0.232

Data are presented as mean±SD or median (interquartile range), where appropriate; $ Data are presented as number and rate; # the ratio to lymphocytes; *statistically significant difference

Anthropometric parameters

The body weight, height, and body mass index (BMI) of all participants were measured with a digital scale of 0.2 kg and a standard visual range meter of 0.2 cm. Participants took off their socks, shoes, and heavy clothes before measuring. BMI is defined as weight (kg) divided by the square of height (m). All measurements were carried out by trained staff.

Blood sampling

All subjects' blood was taken from the anterior cubital vein. Laboratory tests were evaluated, including complete blood count and lymphocyte subsets.

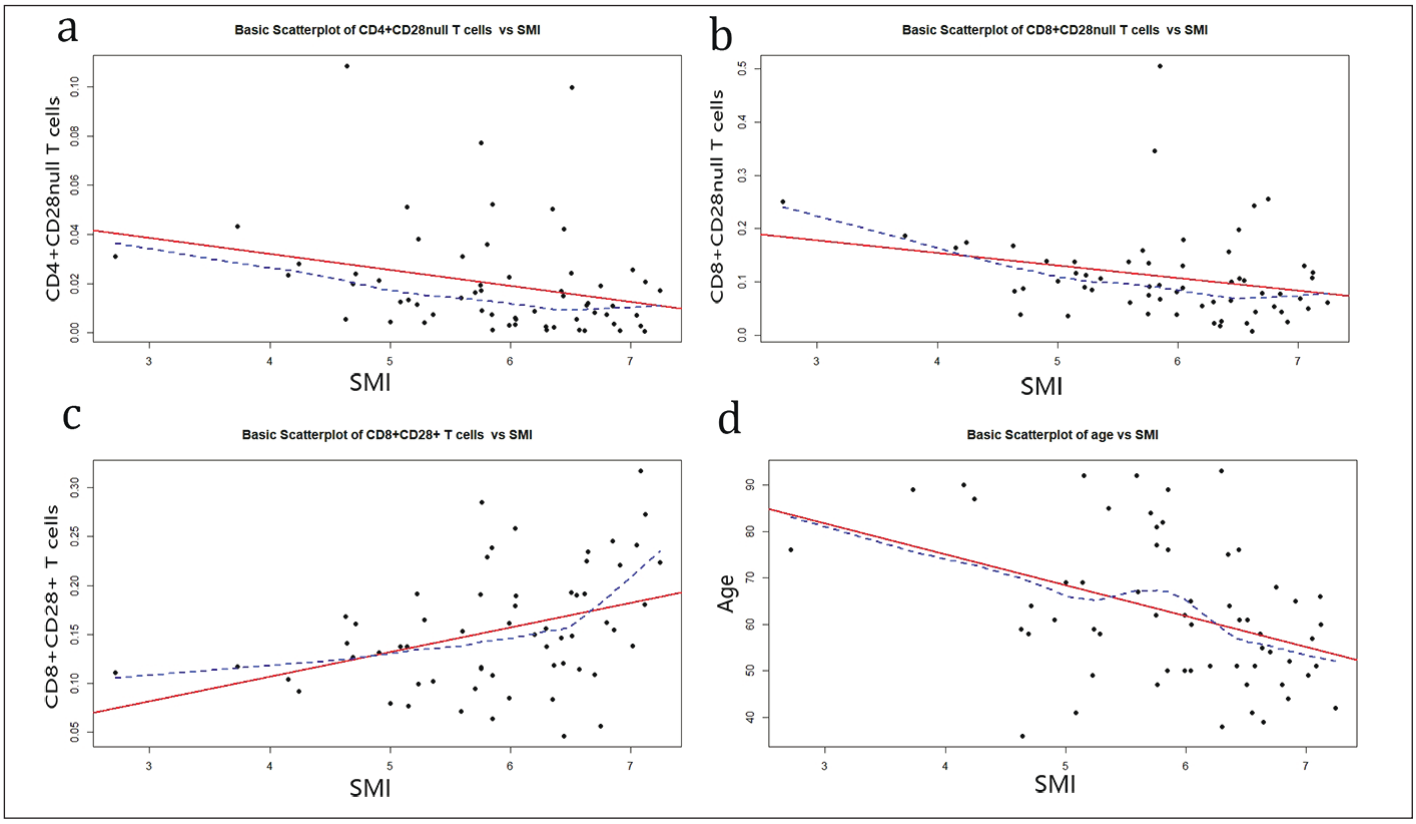
Measurement of lymphocyte subsets

Lymphocyte subsets were analyzed on the same day that the peripheral blood sample was collected.

The main lymphocyte groups were determined by flow cytometry. BD LSRFortessa flow cytometry and Diva software (BD Bioscience) were used to obtain data and calculate the percentage of lymphocyte subsets. In brief, 130μl whole blood was stained with 20μl antibody mixture, which contained the following antibodies: CD3 Pacific Blue, CD127 PE-Cy7, CD28 and CD45 Pacific Orange, CD4 BV711, CD8 APC-H7, CD25 APC, CD45RA FITC, CD56 ECD (BD Biosciences). Stir gently at room temperature and incubate for 25 minutes, then add 2 μl of BD FACSTM dissolved solution. Incubate the tube at room temperature for 15 minutes and place the tube in

Figure 1

Scatterplot of CD4+CD28null T cells, CD8+CD28null T cells CD8+CD28+ T cells and age VS SMI. SMI was negatively correlated with CD4+CD28null T cells, CD8+CD28null T cells and age, and positively correlated with CD8+CD28+ T cells



a centrifuge at 1500 rpm for 5 minutes. Take the supernatant, add BSA (Albumin from bovine serum)-containing PBS (Phosphate Buffered Saline) 2 ml, mix evenly. Then the tube was centrifuged at 1500 rpm for 5 minutes, the supernatant was taken out, and 0.3 ml PBS was added to the supernatant and placed in flow cytometry.

Body Composition

DXA measurements (Hologic QDR 2000 plus, Hologic Inc., Waltham, MA) were used to measure body composition, including body weight, lean mass, fat mass, bone mass, fat distribution, bone distribution and lean distribution. To ensure data quality, standardized region partition method and phantom calibration method are adopted. Appendicular skeletal muscle mass (ASM) was calculated from lean body mass of extremity minus bone mass of extremity; Skeletal muscle mass index (SMI) was calculated as absolute ASM (kg)/height squared (m^2) (12).

Statistical Analysis

Categorical variables were summarized as counts and percentages and continuous variables were summarized as mean ± standard deviation or median (interquartile range). Participant's characteristics were compared using the chi-square test, Student's t-test, and Mann–Whitney U test, where appropriate. Spearman's correlation coefficient was used to evaluate the correlation strength and direction. Linear regression analysis was used to quantify the relationship between two variables. Multiple regression analysis was used when two or more independent variables were assumed to affect the results. Significance was set at <0.05. All statistical tests are performed using IBM SPSS software. (Version 22, Armonk, NY, USA). The scatterplot is drawn by R software (version3.5.2; R Foundation for Statistical Computing, Vienna, Austria).

Result

Eighty-five subjects were enrolled in this study, and were divided into two groups: the sarcopenia group (n=60) and the non-sarcopenia group (n=25). The demographic characteristics, results of body composition and lymphocyte subsets test were listed in Table1.

Correlations between SMI and other parameters in sarcopenia group were listed in Table 2. The level of SMI was negatively correlated with $CD4^+CD28^{null}$ T cells, $CD8^+CD28^{null}$ T cells and age (Figure1), and positively correlated with height, weight, BMI, eosinophil, basophil and $CD8^+CD28^+$ T cells (Figure1).

Multiple linear regression was performed to reveal factors

determining SMI. Stepwise selection was applied with ten significant independent variables: gender, BMI, Age, height, weight, eosinophils, basophils, $CD4^+CD28^{null}$ T cells, $CD8^+CD28^+$ T cells, and $CD8^+CD28^{null}$ T cells. The analysis showed that four variables were significantly associated with SMI: Weight, Gender, Age and CD4+CD28nullT cells. The coefficient of determination R2 was 0.805 (Table 3). Residual diagnosis is consistent with the hypothesis of regression analysis.

Table 2
Spearman's correlations between SMI and the other factors in Sarcopenia

	r	P value
Age	-0.387	0.002*
Gender	-0.676	0.000*
Height	0.629	0.000*
Weight	0.738	0.000*
BMI	0.576	0.000*
CD3+CD4+#	0.020	0.881
CD3+CD8+#	0.096	0.464
Treg#	-0.052	0.691
CD4+CD28+#	0.092	0.486
CD4+CD28null#	-0.370	0.004*
CD8+CD28+#	0.410	0.001*
CD8+CD28null#	-0.304	0.018*
WBC	0.191	0.144
NEUT	0.134	0.309
LYM	0.188	0.151
MONO	0.223	0.087
Eosinophil	0.380	0.003*
Basophil	0.265	0.040*
RBC	0.306	0.017*
Hb	0.534	0.000*
PLT	-0.013	0.919

*statistically significant difference; # the ratio to lymphocytes

Discussion

Lymphocytes are closely related to muscle repair and regeneration (7), thus, we hypothesized that muscle mass loss in patients with sarcopenia was associated with lymphocytes. Skeletal muscle mass index (SMI), calculated as absolute ASM (kg)/height squared (m^2), is a reliable measure of muscle mass. This study focused on the correlation between SMI and lymphocyte subsets in patients with sarcopenia.

The present study demonstrated that SMI was negatively correlated with $CD4^+CD28^{null}$ T cells, $CD8^+CD28^{null}$ T cells and age, and positively correlated with height, weight, BMI, eosinophils, basophils and $CD8^+CD28^+$ T cells in patients with sarcopenia. After adjusting for the effects of gender, age, height, weight and BMI, SMI were still associated with $CD4^+CD28^{null}$ T cells. This suggests that $CD4^+CD28^{null}$ T cells may associated with the loss of muscle mass in patients with sarcopenia.

$CD4^+CD28^{null}$ T cell is a subset of $CD4^+$ T helper 1, characteristically lack CD28, a co-stimulatory receptor important to the activation and function of T cells (13). $CD4^+CD28^{null}$ T cells dilate in some chronic inflammation-related diseases and are almost undetectable in healthy people (14). Currently, how $CD4^+CD28^{null}$ T cells are generated is still controversial, repeated antigen stimulation may be one of the reasons for $CD4^+CD28^{null}$ T cells amplification (15). Compared with $CD4^+CD28^+$ T cells, $CD4^+CD28^{null}$ T cells showed stronger anti-apoptotic ability and higher cytotoxic function (16, 17). Usually, the high frequency of $CD4^+CD28^{null}$ T cells is thought to be associated with poor prognosis of the disease.

In addition to releasing cytotoxic particles containing perforin and granzyme B, $CD28^{null}$ T cells can also produce cytokines such as TNF-α and IFN-γ (18). These inflammatory cytokines are thought to play a role in the pathogenesis of sarcopenia. IFN-γ was reported related to the regenerative capacity of skeletal muscle in animal experiments (19). The study also confirmed that plasma TNF-α levels were associated with ASM after adjusting for the effects of age, height, weight, and diabetes (20). Recently published a prospective cross-sectional cohort study confirmed high TNF-α levels are associated with an increased risk of sarcopenia and the TWEAK/ TNF-α/IL-18/NF-kappa B proinflammatory signaling pathway plays a dominant role in the mechanism for sarcopenia (21). $CD4^+CD28^{null}$ T cells may be associated with sarcopenia through these inflammatory factors.

Previous studies also found that $CD28^{null}$ T cells could be activated following interaction with MHC-expressing muscle fibers and cause muscle fiber damage in patients with dermatomyositis and polymyositis (22). However, whether the loss of muscle mass associated with sarcopenia is related to CD28null T cells has not been reported.

Some limitations exist in this study. First of all, since this design is a cross-sectional study, it is impossible to establish any causal relationship, but this study confirmed the link between SMI and $CD4^+CD28^{null}$ T cells in patients with sarcopenia. Second, the results depend on a single site rather than continuous measurements, so biodiversity and potential laboratory measurement errors can easily affect the accuracy of the data. Third, the relationship between SMI and $CD4^+CD28^{null}$ T cells may be confused by some unmeasured covariance. The last, due to inflammatory markers such as TNF-α were not available, the pathway underlying the association is not clear.

This study reveals that SMI was negatively correlated with $CD4^+CD28^{null}$ T cells in sarcopenia patients. The result of our study may point out the role of $CD4^+CD28^{null}$ T lymphocytes

Table 3

Multiple linear regression modeling (stepwise) for SMI based on Gender, BMI, Age, Height, Weight, Eosinophil, Basophil, CD4+CD28null, CD8+CD28+, CD8+CD28null in Sarcopenia. Coefficient of determination r2 =0.805, multiple correlation coefficient =0.897

Independent variables	Coefficient	Standard error	Standard Coefficient	t	P
Constant	4.430	0.543		8.155	0.000
Weight	0.038	0.006	0.475	6.164	0.000
Gender	-0.950	0.165	-0.425	-5.761	0.000
Age	-0.011	0.004	-0.191	-3.058	0.003
$CD4^{+}CD28^{null}$	-5.151	2.528	-0.123	-2.037	0.046

Variables not included in the model: BMI, Height, CD8+CD28+, CD8+CD28null, Eosinophil, Basophil.

in the pathogenesis of sarcopenia. Further prospective designed studies are necessary to highlight the role of $CD4^{+}CD28^{null}$ T cells in sarcopenia and the contribution of $CD4^{+}CD28^{null}$ T cells to this pathogenesis.

Author Contributions: Hong-lian Zhou, Cun-Tai Zhang, Ting Xu and Shuai-wen Huang designed the study and wrote the manuscript. Hong-lian Zhou and Ting Xu and Shuai-wen Huang performed the statistical analysis, Shuai-wen Huang, Cun-Tai Zhang, Ting Xu and Hong-lian Zhou did the data curation, Hong-lian Zhou and Cun-Tai Zhang supervised the study, Hong-lian Zhou, Cun-Tai Zhang ,Ting Xu and Shuai-wen Huang reviewed the manuscript.

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Ethical standards: This study was approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (IRB ID: TJ-C20141112). All participants provided written informed consent before data collection.

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