JOURNAL OF MICROBIOLOGY, EPIDEMIOLOGY AND IMMUNOBIOLOGY. 2021; 98
DOI: https://doi.org/10.36233/0372-9311-67
ORIGINAL RESEARCHES

Original article
https://doi.org/10.36233/0372-9311-67

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Association of the increased circulating CD62L^{lo}CXCR4^{hi} neutrophil count with carotid atherosclerosis

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Abstract

Introduction. The role of neutrophils in the initiation and progression of atherosclerosis as well as in the development of its complications has received scientific attention only in the recent years. Today, there is growing evidence to support a role of the CXCL12/CXCR4 axis in sustained inflammation during different chronic inflammatory diseases by retaining neutrophils at inflammatory sites.

The aim of the study is to assess the diagnostic and prognostic significance of circulating CD62L^{lo}CXCR4^{hi} neutrophils in patients with carotid atherosclerosis.

Materials and methods. A total of 75 patients (52% of men and 48% of women) aged 40 to 64 years were examined. None of them were diagnosed with atherosclerotic cardiovascular diseases. All the patients underwent carotid artery duplex scanning. The flow cytometry and CD16, CD11b, CD62L, CD182 (CXCR2) and CD184 (CXCR4) conjugated monoclonal antibodies were used for phenotyping and differentiation of neutrophil subpopulations.

Results. Atherosclerotic plaques in carotid arteries were detected in 72% of the patients; most of the patients were diagnosed with stenosis development in more than one of the carotid arteries (CA). The elevated levels of circulating CXCR4^{hi} neutrophils were associated with the levels of total cholesterol (r = 0.377; p = 0.001), low-density lipoprotein (LDL) cholesterol (r = 0.293; p = 0.014) and triglycerides (r = 0.388; p = 0.003). The study revealed direct correlation between the circulating CXCR4^{hi} neutrophil count and the cumulative percentage of CA stenosis (r = 0.300; p = 0.011), including the number of stenosed CA (r = 0.291; p = 0.034). It was also found that CXCR4^{hi} neutrophil counts demonstrated a statistically significant increase along with the increased number of stenosed CA (p = 0.025). The ROC analysis findings show that the elevated CXCR4^{hi} neutrophil counts ≥260 cells/μL made it possible to diagnose stenotic lesion of 4 CAs with a sensitivity of 71.4% and specificity reaching 76.6%.

Conclusion. In patients with carotid atherosclerosis, the increased count of circulating CD62L^{lo}CXCR4^{hi} neutrophils was associated with the increased number of stenosed CAs, while no significant changes were observed in the other examined subpopulations of neutrophil granulocytes. The increased CD62L^{lo}CXCR4^{hi} neutrophil count made it possible to diagnose stenotic lesion of 4 CAs with a sufficient sensitivity and specificity.

Keywords: *atherosclerosis, neutrophils, carotid atherosclerosis, aged neutrophils*

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Ethics Committee of the South-Ural State Medical University.

Funding source. This study was not supported by any external sources of funding.

Conflict of interest. The authors declare no apparent or potential conflicts of interest related to the publication of this article.

For citation: Dolgushin I.I., Genkel V.V., Baturina I.L., Emelyanov I.V., Savochkina A.Y., Shaposhnik I.I. Association of the increased circulating CD62L^{lo}CXCR4^{hi} neutrophil count with carotid atherosclerosis. *Journal of microbiology, epidemiology and immunobiology = Zhurnal mikrobiologii, èpidemiologii i immunobiologii.* 2021;98(2):176–183.
DOI: https://doi.org/10.36233/0372-9311-67

Научная статья
https://doi.org/10.36233/0372-9311-67

Ассоциация увеличения количества циркулирующих CD62L^{lo}CXCR4^{hi}-нейтрофилов с распространённостью каротидного атеросклероза

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Аннотация

Введение. Изучение роли нейтрофилов в инициации и прогрессировании атеросклероза и развитии его осложнений активно ведется лишь на протяжении последних нескольких лет. К настоящему времени имеются данные, свидетельствующие о важной роли оси CXCL12/CXCR4 в поддержании воспаления при различных хронических воспалительных заболеваниях путем задержки нейтрофилов в очагах воспаления.

Цель исследования — установить диагностическую и прогностическую значимость циркулирующих $CD62L^{lo}CXCR4^{hi}$-нейтрофилов у пациентов с каротидным атеросклерозом.

Материалы и методы. Обследовали 75 пациентов (52% мужчин и 48% женщин) в возрасте 40–64 лет без установленных атеросклеротических сердечно-сосудистых заболеваний. Всем пациентам проводили дуплексное сканирование артерий каротидного бассейна. Фенотипирование и дифференцировку субпопуляций нейтрофилов осуществляли методом проточной цитометрии с использованием конъюгатов моноклональных антител CD16, CD11b, CD62L, CD182 (CXCR2) и CD184 (CXCR4).

Результаты. Атеросклеротические бляшки в артериях каротидного бассейна были выявлены у 72% пациентов, при этом у большинства пациентов диагностировано стенозирование более одной сонной артерии (СА). Увеличение количества циркулирующих $CXCR4^{hi}$-нейтрофилов ассоциировалось с уровнями общего холестерина (r = 0,377; p = 0,001), холестерина липопротеинов низкой плотности (r = 0,293; p = 0,014) и триглицеридов (r = 0,388; p = 0,003). Выявлены прямые корреляционные связи между количеством циркулирующих $CXCR4^{hi}$-нейтрофилов и суммарным процентом стенозирования СА (r = 0,300; p = 0,011), а кроме того — с количеством стенозированных СА (r = 0,291; p = 0,034). Отмечено статистически значимое нарастание количества $CXCR4^{hi}$-нейтрофилов по мере увеличения количества стенозированных СА (p = 0,025). По данным ROC-анализа, увеличение количества $CXCR4^{hi}$-нейтрофилов ≥260 кл/мкл позволяло диагностировать стенозирующее поражение 4 СА с чувствительностью 71,4% и специфичностью 76,6%.

Заключение. У пациентов с каротидным атеросклерозом увеличение циркулирующих $CD62L^{lo}CXCR4^{hi}$-нейтрофилов ассоциировалось с увеличением числа стенозированных СА при отсутствии значимых изменений в других оцениваемых субпопуляциях нейтрофильных гранулоцитов. Увеличение количества $CD62L^{lo}CXCR4^{hi}$-нейтрофилов позволяло с достаточной чувствительностью и специфичностью диагностировать стенозирующее поражение 4 СА.

Ключевые слова: *атеросклероз, нейтрофилы, каротидный атеросклероз, стареющие нейтрофилы*

Этическое утверждение. Исследование проводилось при информированном согласии пациентов. Протокол исследования одобрен Этическим комитетом ЮУГМУ.

Источник финансирования. Авторы заявляют об отсутствии внешнего финансирования при проведении исследования.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Для цитирования: Долгушин И.И., Генкель В.В., Батурина И.Л., Емельянов И.В., Савочкина А.Ю., Шапошник И.И. Ассоциация увеличения количества циркулирующих $CD62L^{lo}CXCR4^{hi}$-нейтрофилов с распространенностью каротидного атеросклероза. *Журнал микробиологии, эпидемиологии и иммунобиологии.* 2021;98(2): 176–183.
DOI: https://doi.org/10.36233/0372-9311-67

Introduction

The role of neutrophils in the initiation and progression of atherosclerosis as well as in the development of its complications has received scientific attention only in the recent years [1]. To a large extent, the interest was propelled by new discoveries in the biology of neutrophils, which led to the revision of the traditional beliefs about their functions and the heterogeneity of their population [2]. The discovery of new pathophysiological mechanisms, through which neutrophil granulocytes influence atherogenesis and development of cardiovascular diseases (CVDs), brought them into focus as a therapeutic target and ushered in a number of clinical studies [3, 4].

It was found that as they age, neutrophils alter their phenotype. This process is known as neutrophil aging [5]. The central role in this process is played by the C-X-C motif chemokine ligand (CXCL)12/CXCR4 axis [6]. Another important fact is that aging of neutrophils has a significant impact on their functional characteristics, downregulating the CD62L expression and upregulating the CD11b, TLR4 expression, the capacity to form neutrophil extracellular traps and produce reactive oxygen species [7]. Today, there is growing evidence to support a role of the CXCL12/CXCR4 axis in sustained inflammation during different chronic inflammatory diseases by retaining neutrophils at inflammatory sites [8, 9]. The CXCR4 expression on circulating neutrophils was found to increase during chronic inflammatory diseases, for example, during systemic lupus erythematosus [10]. A number of experimental studies revealed a pro-atherogenic role of CXCL12, a ligand for CXCR4 [11]. Increased CXCL12 expression is also observed in unstable atherosclerotic plaques (ASPs) [12]. The diagnostic and prognostic role of circulating $CXCR4^{hi}$ neutrophils in patients with atherosclerotic CVDs requires further exploration in clinical studies.

The aim of the study is to assess the diagnostic and prognostic significance of circulating $CD62L^{lo}CXCR4^{hi}$ neutrophils in patients with carotid atherosclerosis.

DOI: https://doi.org/10.36233/0372-9311-67

Materials and methods

The study was performed with a total of 75 patients (39 (52%) men and 36 (48%) women) aged 40 to 64 years, without any diagnosed atherosclerotic CVDs, and having had duplex ultrasound examination of their carotid arteries as administered by their primary physician for the purpose of cardiovascular risk (CVR) assessment. All the enrollees signed their informed consent for participation in the study. The study protocol was approved by the Ethics Committee of the South Ural State Medical University.

The following medical conditions were justified reasons for non-inclusion and/or exclusion in accordance with the criteria for participation in the study:

- previously diagnosed atherosclerotic CVDs (a history of cerebrovascular disease, coronary heart disease, peripheral artery disease, coronary or peripheral arterial revascularization);
- severe hepatic and renal dysfunction (the glomerular filtration rate is lower than 30 mL/min/1.73 m^2);
- malignant tumors;
- existing and confirmed chronic inflammatory diseases;
- acute inflammatory or infectious diseases during the last 28 days.

Ultrasonic examination

All the patients underwent carotid artery duplex scanning. The following arteries were examined bilaterally, in a longitudinal and transverse section, along the entire length: common carotid arteries (CCA) with CCA bifurcation, internal carotid arteries (ICAs), external carotid arteries (ECAs) at anterior, lateral and posterior angles. The examination was performed in a B-mode, color-flow mode, pulsed Doppler ultrasonography, power Doppler imaging by using a Samsung Medison EKO7 digital ultrasound multipurpose diagnostic scanner and a linear probe at a frequency of 10 MHz.

The intima-media thickness (IMT) was measured automatically (AutoIMT function), bilaterally, in the distal third of CCA, by 1 cm proximal the CCA bifurcation at the anterior angle. The mean CCA IMT (mIMT) was calculated by using the formula:

$$\text{mIMT} = \frac{(\text{leftCCA IMT} + \text{rightCCA IMT})}{2}.$$

The ASP was deemed as a focal increase in the intima-media thickness by more than 1.5 mm or exceeding the surrounding IMT by 0.5 mm or exceeding the IMT of the adjacent CCA sections by 50% [13]. The stenosing percentage was estimated through planimetric measurements, by using a B-mode, along the diameter in the cross-sectional area of an artery. The stenosis percentage was calculated by using the ECST (The European Carotid Surgery Trial) method [14]. The maximum stenosis percentage in a particular patient and the cumulative percentage of CA stenosis were estimated, when any ASPs narrowing the lumen of arteries were detected. Then, the number of stenosed CCAs and ICAs was estimated (the maximum number — 4).

Laboratory tests

The following laboratory test values were estimated: total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C), triglycerides, glycosylated hemoglobin, creatinine, including the further estimation of the glomerular filtration rate following the CKD-EPI equation, and high-sensitivity C-reactive protein.

The phenotyping and differentiation of neutrophil subpopulations were performed by flow cytometry with a Navios 6/2 flow cytometer (Beckman Coulter) by using conjugated monoclonal antibodies: CD16-PE-Cy7.0 (Invitrogen), CD11b-FITC, CD62L-PE (Beckman Coulter), CD182 (CXCR2)-PE-Cy5.0 (BD Biosciences), CD184 (CXCR4)-PE-CF594 (BD Biosciences). We used whole-blood phenotyping with detection of min 30,000 events.

Statistical analysis

The findings were further analyzed with the help of the IBM SPSS Statistics v.18 software for data statistical analysis. The qualitative variables were described by using absolute and relative ratios (percentages). The quantitative variables were described by using the median (Me) with the interquartile range (Q_{25}–Q_{75}) if the variable was not normally distributed, or the average and standard deviation in case of the normal distribution of the variable. The Spearman rank correlation was used to measure the relationship between the variables. The Mann–Whitney U test was used to assess the significance of differences between two groups. Differences were considered statistically significant at the significance level of 0.05.

The multiple logistic regression analysis was used to identify independent predictors of the dependent variable. The analysis was instrumental in detecting the dependance of the binary categorical variable on a number of other variables, both continuous and categorical. The linear regression was used to assess the dependence of one quantitative variable on another variable. The ROC curve analysis was used to identify threshold values of the studied variables, including the assessment of sensitivity and specificity as well as the estimation of the area under the ROC curve at a confidence interval of 95%.

Results

The clinical profile of the patients is given in **Table 1**.

Following the applicable European guidelines, at the time of enrolment in the study, a very high CVR

was found in 13 (17.3%) patients, while 11 (14.6%) patients had a high CVR, 31 (41.3%) patients were diagnosed with a moderate CVR, and 20 (26.6%) patients had a low CVR [15].

ASPs in carotid arteries were found in 72% patients (**Table 2**). Most of the patients were diagnosed with stenosing of more than one CA.

The findings of the correlation analysis showed that the elevated levels of circulating $CXCR4^{hi}$ neutrophils (**Table 3**) were associated with the levels of TC ($r = 0.377$; $p = 0.001$), LDL-C ($r = 0.293$; $p = 0.014$), and triglycerides ($r = 0.388$; $p = 0.003$). The analysis also revealed a direct correlation between the circulating $CXCR4^{hi}$ neutrophil count and the cumulative percentage of CA stenosis ($r = 0.300$; $p = 0.011$) as well as between the above count and the number of stenosed CA ($r = 0.291$; $p = 0.034$).

The ROC curve analysis was performed to assess the predictive value of the circulating $CD16^{hi}CD11b^{br}CD62L^{lo}CXCR4^{hi}$ neutrophil count regarding the existence of advanced atherosclerosis of carotid arteries, including development of a stenotic lesion of 4 CAs (**Fig. 2**).

The increase in the $CXCR4^{hi}$- neutrophil count reaching ≥260 cells/μL made it possible to diagnose a stenotic lesion of 4 CAs with a sensitivity of 71.4% and specificity of 76.6%. With the threshold value increased to 333 cells/μL, the specificity went up to 90.6%, while the sensitivity went down to 57.1%. The logistic regression analysis showed that with the circulating $CXCR4^{hi}$ neutrophil count increasing to ≥260 cells/μL, the odds ratio for a stenotic lesion of 4 CAs was 14.1 (95% CI 1.6–119; $p = 0.015$) as adjusted for the gender, age, existing type 2 diabetes and arterial hypertension, smoking and LDL-C levels.

Table 1. Clinical characteristics of patients

Parameter	Value
Age, years, Me (Q_{25}–Q_{75})	50,0 (44,5; 56,0)
Body mass index, kg/m², Me (Q_{25}–Q_{75})	27,4 (25,0; 31,2)
Obesity, *n* (%)	23 (30,6)
Abdominal obesity, *n* (%)	45 (60,0)
Smoking, *n* (%)	16 (21,3)
Type 2 diabetes mellitus, *n* (%)	5 (6,66)
Hypertension, *n* (%)	38 (50,6)
Antiplatelets, *n* (%)	9 (12,0)
β-Blockers, *n* (%)	19 (25,3)
Inhibitors of renin-angiotensin system, *n* (%)	19 (25,3)
Diuretics, *n* (%)	8 (10,6)
Statins, *n* (%)	23 (30,6)
Oral hypoglycemic agents, *n* (%)	6 (8,00)
Total cholesterol, Me (Q_{25}–Q_{75})	5,80 (5,14; 6,50)
Low-density lipoproteins cholesterol, Me (Q_{25}–Q_{75})	3,46 (2,96; 4,26)
High-density lipoproteins cholesterol, Me (Q_{25}–Q_{75})	1,40 (1,19; 1,61)
Triglycerides, Me (Q_{25}–Q_{75})	1,33 (1,00; 1,80)
High sensitivity C-reactive protein, mg/l, Me (Q_{25}–Q_{75})	1,40 (0,85; 3,48)
Glycated hemoglobin, %, Me (Q_{25}–Q_{75})	5,67 (5,20; 6,05)
Estimated glomerular filtration rate, ml/min/1.73 m², Me (Q_{25}–Q_{75})	73,0 (61,8; 96,5)

Discussion

Studies of innate and adaptive immune cells participating in atherogenesis tend to focus on monocytes, macrophages, and T lymphocytes. The pivotal role of neutrophils in development of atherosclerosis and its complications is beyond dispute. However, the available data lack consistency; the information is patchy and needs further research *in vivo* [16].

Table 2. Results of carotid duplex ultrasound scanning

Parameter	Value
Mean carotid intima–media thickness, Me (Q_{25}–Q_{75})	0,65 (0,56; 0,71)
Carotid plaque, *n* (%)	54 (72,0)
Maximal carotid stenosis, Me (Q_{25}–Q_{75})	24,0 (0,00; 32,0)
Total carotid stenosis, Me (Q_{25}–Q_{75})	27,0 (0,00; 73,0)
Carotid stenosis ≥50%, *n* (%)	5 (6,66)
Number of carotid arteries with stenosis, *n* (%)	
1	19 (25,3)
2	16 (21,3)
3	12 (16,0)
4	7 (9,33)

Table 3. Differentiation of neutrophils in the studied cohort of patients

Neutrophil phenotype	Value, cells/μl
$CD16^{hi}CD11b^{hi}CD62L^{hi}$ (mature neutrophils)	2666 (2111; 3313)
$CD16^{hi}CD11b^{hi}CD62L^{lo}$ (activated neutrophils)	27,5 (14,0; 46,2)
$CD16^{hi}CD11b^{br}CD62L^{lo}CXCR4^{hi}$ (aging neutrophils)	185 (98,5; 269)

DOI: https://doi.org/10.36233/0372-9311-67

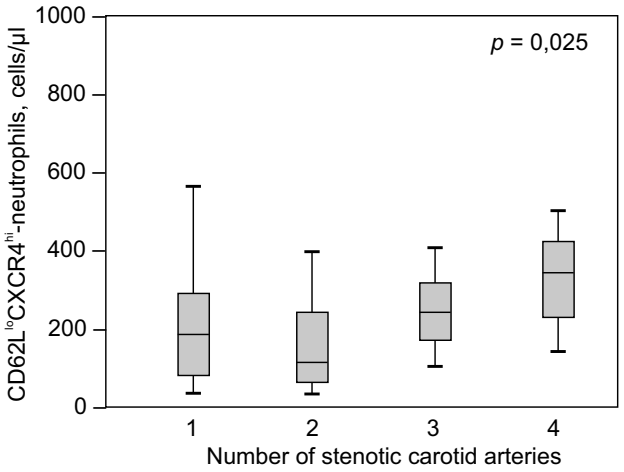

Fig. 1. The circulating CXCR4^{hi} neutrophil count in relation to the number of the affected CAs.

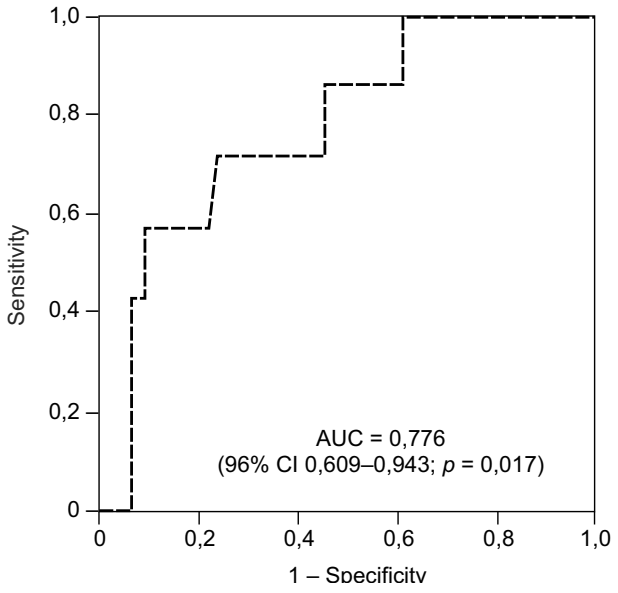

Fig. 2. ROC-curve for CD16^{hi}CD11b^{br}CD62L^{lo}CXCR4^{hi}-neutrophils.

The main findings of the conducted study are as follows:

1) In the group of patients under study, the count of aged neutrophils directly correlated with the levels of TC, LDL-C, and triglycerides;

2) The number of stenosed CAs increased together with the circulating CD62L^{lo}CXCR4^{hi} neutrophil count, while no significant changes were observed in the other studied subpopulations of neutrophil granulocytes;

3) The increased CD62L^{lo}CXCR4^{hi} neutrophil count made it possible to diagnose stenotic lesion of 4 CAs with adequate sensitivity and specificity.

CXCL12, also known as SDF-1 (stromal cell-derived factor-1), is a chemokine playing a critical role in homing of neutrophils, the significance of which in the pathogenesis of atherosclerosis is still fairly unclear [11]. Yet, it has been proved that ASPs have increased CXCL12 expression, and increased serum CXCL12 levels can aggravate the progression of atherosclerosis and directly correlate with the severity of carotid atherosclerosis [17, 18]. As is known, the clearance of circulating CD62L^{lo}CXCR4^{hi} neutrophils occurs primarily during night hours when the CXCL12 concentration in the bone marrow reaches peak levels [19]. The systemic persistent low-grade inflammation, which is observed in atherosclerosis, and the increased concentrations of CXCL12 in the systemic circulation and ASPs may adversely affect the migration of aged neutrophils to the bone marrow, increasing their lifespan and their levels in blood [7]. In its turn, the subpopulation of CD62L^{lo}CXCR4^{hi} neutrophils, which is characterized by the most active migration to inflammation sites and by the ability to form neutrophil extracellular traps and produce reactive oxygen species, has the maximum potential for migration into the subendothelial space, contributing to vascular inflammation and progression of atherosclerosis [8, 19, 20].

In our opinion, the increase in the CD62L^{lo}CXCR4^{hi} neutrophil count along with the increase in the number of CAs suggests an increased intensity of systemic inflammation in patients with advanced polyvascular phenotype of atherosclerosis [21]. It may also indicate the activation of one of the inflammatory signaling pathways — CXCL12/CXCR4. The diagnostic significance of CD62L^{lo}CXCR4^{hi} neutrophils for the CA atherosclerotic disease, which was demonstrated during the study, suggests the possibility of application of the quantitative assessment of the neutrophil population in diagnosis and prognosis of atherosclerotic CVDs.

The performed study has some limitations:

- a small number of patients included in the study;
- non-estimated serum concentrations of CXCL12.

Conclusion

In patients with carotid atherosclerosis, the increased circulating CD62L^{lo}CXCR4^{hi} neutrophil count was associated with the increased number of stenosed CAs. The CD62L^{lo}CXCR4^{hi} neutrophil count of ≥260 cells/μL made it possible to diagnose a stenotic lesion of 4 CAs with a sensitivity of 71.4% and specificity of 76.6%.

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Author contribution. All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published.

The article was submitted 17.07.2020;
accepted for publication 22.01.2021;
published 20.03.2021

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Участие авторов. Все авторы внесли существенный вклад в проведение поисково-аналитической работы и подготовку статьи, прочли и одобрили финальную версию до публикации.

Статья поступила в редакцию 17.07.2020;
принята к публикации 22.01.2021
опубликована 20.03.2021