RAT PERIPHERAL BLOOD LEUCOCYTE REACTION IN THE AGE ASPECT ON THE BACKGROUND OF METABOLIC SYNDROME EXPERIMENTAL MODELING

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Abstract

The purpose of the research is to investigate links between body mass index and changes in the leucocyte formula of peripheral blood in rats of different ages in experimental modeling of nongenetic alimentary obesity with subsequent MS development; to identify possible associations between these indicators in animals depending on age.

Contingent and research methods. Metabolic syndrome was reproduced according to the developed method in rats of different age groups by combining high-fat and high-carbohydrate diet in parallel with pharmacological correction of physiological satiation inhibition.

Results. It is determined that the mechanisms of low gradient chronic inflammation development in adipose tissues on the background of metabolic syndrome differ from classical inflammatory response mechanisms. Weight gain is associated with the activation of various leucocyte forms depending on age and degree of mass increase. Young rats have body mass index associated with the amount of peripheral blood neutrophils and more delayed in

time; adult animals have body mass index associated with eosinophils, starting from the first month of metabolic syndrome development; old animals have body mass index associated with monocytes and lymphocytes, during the whole period of the trial. The dependence of inflammatory reaction symptoms on the age and sex of the animal on the background of obesity is confirmed by the present associative links (peripheral blood cell composition – animal's age - body mass index) and can be determined later, as a marker factor for metabolic syndrome development.

Keywords: leucocyte reaction; chronic low-grade inflammation; metabolic syndrome.

ЛЕЙОЦИТАРНА РЕАКЦІЯ ПЕРИФЕРИЧНОЇ КРОВІ ЩУРІВ У ВІКОВОМУ АСПЕКТІ НА ТЛІ ЕКСПЕРИМЕНТАЛЬНОГО МОДЕЛЮВАННЯ МЕТАБОЛІЧНОГО СИНДРОМУ

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Резюме

Мета роботи - дослідити зв'язок між індексом маси тіла та змінами в лейкоцитарній формулі периферичної крові у щурів різного віку в умовах експериментального моделювання негенетичного аліментарного ожиріння з подальшим розвитком МС. Визначити можливі асоціації між цими показниками у тварин в залежності від віку.

Контингент та методи дослідження. Метаболічний синдром відтворювали згідно розробленої методики у щурів різних вікових груп шляхом комбінації високожирової і високовуглеводної дієти у паралелі із фармакологічною корекцією гальмування фізіологічного насичення тварин.

Результати. Встановлено, що механізми розвитку низькоградієнтного хронічного запалення в жировій тканині на тлі розвитку метаболічного синдрому мають відмінності від класичних механізмів розвитку запальної реакції. Збільшення маси тіла асоційовано із активацією різних форм лейкоцитів в залежності від віку та ступеня збільшення маси тіла. У молодих щурів індекс маси тіла асоційований із кількістю нейтрофілів периферичної крові і більше відстрочений у часі; у дорослих

тварин – із еозинофілами, починаючи із першого місяця розвитку метаболічного синдрому; у старих тварин – із моноцитами та лімфоцитами, на всіх строках експерименту. Спряженість проявів запальної реакції від віку та статі тварини на тлі розвитку ожиріння підтверджується наявністю асоціативних зв'язків (клітинний склад периферичної крові – вік тварини – індекс маси тіла) і може бути визначено в подальшому, як маркерний фактор спрямованості розвитку метаболічного синдрому.

Ключові слова: лейкоцитарна реакція; низькоградієнтне хронічне запалення; метаболічний синдром.

ЛЕЙОЦИТАРНАЯ РЕАКЦИЯ ПЕРИФЕРИЧЕСКОЙ КРОВИ КРЫС В ВОЗРАСТНОМ АСПЕКТЕ НА ФОНЕ ЭКСПЕРИМЕНТАЛЬНОЙ МОДЕЛИ МЕТАБОЛИЧЕСКОГО СИНДРОМА

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Резюме

Цель. установить связь между индексом массы тела и изменениями в лейкоцитарной формуле периферической крови у крыс разного возраста и пола в условиях экспериментального моделирования негенетического алиментарного ожирения с дальнейшим формированием метаболического синдрома. Определить возможные ассоциации между данными показателями у животных в зависимости от возраста.

Контингент и методи исследования. Метаболический синдром воспроизводили согласно разработанной методике: комбинация высокожировой и высокоуглеводной диеты в параллели с фармакологической коррекцией торможения физиологического насыщения животных.

Результаты. Установлено, что механизмы развития низкоуровневого хронического воспаления в жировой ткани на фоне развития метаболического синдрома, имеют отличия от классических механизмов развития воспалительной реакции. Увеличение массы тела ассоциировано с активацией различных форм лейкоцитов в зависимости от возраста и степени увеличения массы тела. У молодых крыс индекс массы тела ассоциирован с количеством нейтрофилов и более

отсроченный во времени; у взрослых животных - с эозинофилами, начиная с первого месяца развития метаболического синдрома; у старых животных - с моноцитами и лимфоцитами, на всех сроках эксперимента. Сопряженность проявлений воспалительной реакции от возраста и пола животного на фоне развития ожирения, подтвержденные наличием ассоциативных связей (клеточный состав периферической крови - возраст животного - индекс массы тела) могут определяться в дальнейшем, как маркерный фактор направленности развития метаболического синдрома.

Ключевые слова: лейкоцитарная реакція; низкоградиентное хроническое воспаление; метаболический синдром.

Introduction

Obesity is a typical pathology form, which is the cornerstone of type 2 diabetes development, various forms of cardiovascular diseases, as well as one of the mechanisms of metabolic syndrome (MS) [1, 2]. In response to obesity, by excessive functional activity, hypertrophied adipocytes indirectly stimulate pro-inflammatory mediators release, activate corresponding pathways in all metabolically active cells, leading to cytokine production increase and attracting immune cells to the extracellular space. Therefore, obesity can be considered as primary (phlogogenic) factor of chronic low-grade inflammation (LGI) development in the body, which is further supported by immune cells and involves other tissues to response [3]. LGI biological role in co-morbid condition development as well as its link with other pathogenetic mechanisms of MS chronicity is currently pending. A lot of authors note that inflammation mechanisms in MS have specific characteristics [4], so studying of inflammation activation mechanisms in adipose tissue in obesity, as a MS component, can help to control the course of MS and determine therapy effectiveness.

Nowadays laboratory, functional, instrumental and other methods are believed to be the main ones for health condition assessment. It is suggested, that their complex form a powerful and effective tool for early diagnosis of many diseases [5]. Obesity is usually diagnosed using anthropometric features measurement by assessing body mass index (BMI), which allows the doctor to classify patients according to obesity degree: from overweight to pathological obesity (World Health Organization) [6]. However, this simple and useful index does not assess metabolic changes associated with obesity, which in turn are prerequisites for insulin resistance development in peripheral tissues [7], as well as LGI, provided by innate and adaptive immune systems activation, causing immunological changes [8]. The issue of early diagnosis and detection of metabolic changes caused by obesity is urgent, and finding

solution is believed to contribute to integrative assessment criteria in patient's MS development. Over the past few years, a term "metabolomics" appeared in literature; this term is used as a useful assessment tool for metabolic changes at cellular and humoral levels, typical among patients with overweight or obesity [9].

Taking into account the abovementioned key points, in this research we assumed that peripheral blood cell composition changes, resulted due to LGI initiation in stimulated adipose tissue, may be systematic, and subsequently may become the basis for initial diagnosis and direction of MS initial stage development. Therefore, the purpose of the research was to investigate the link between peripheral blood leucocyte reaction and body mass index in animals of different ages in experimental modeling of non-genetic alimentary obesity with subsequent MS development, to identify possible associations between these indicators in animals depending on age.

Design, contingent and methods research

The research was carried out using 360 non-linear white rats, bred in KhNMU vivarium. All animals were divided by age into 3 main groups. The first group included 120 young immature animals aged 3-4 months, weighing 170.0±7.8 g. The second group included 120 adult rats aged 5-6 months, weighing 240.0± 14.7 g. The 3rd included 120 old rats (mature, of post-productive period), aged 18 months, weighting 360.0± 21.8 g. The age of the animals was selected according to the table of periodization of the animals' age in comparison with human age [10]. Each group of rats, in turn, was divided into subgroups by the term of MS modeling: K - control group (intact animals); A - experimental group with MS modeling during 1 month; B - MS modeling during 3 months; and the third subgroup C – MS modeling for 6 months. Each group included both males and females (50% to 50%).

MS experimental model was reproduced in rats using high-fat and high-carbohydrate diet combination on the background of pharmacological correction of physiological satiation inhibition, which was reproduced by subcutaneous administration of Betaspan suspension (impaired glucose tolerance) once a week at a dose of 20 μg/kg of body weight during 6 weeks in combination with aurothioglucose (hyperphagia activation) at a dose of 10 μg/kg once a week intraperitoneally during 6 weeks [11].

Anthropometric indicators were determined using commonly used methods. During the trial, rats' body weight and nasal-anal length were measured and basing on these indicators body mass index (BMI) was calculated.

Peripheral blood leucocyte reaction was described basing on determination of TLC and leucocyte formula. TLC was calculated using Goryaev chamber, staining was performed

using acetic acid with gentian violet. To determine the leucocyte formula, blood smears were fixed in methanol and stained with azure-II-eosin. The percentage of leucocytes was converted to absolute number of cells according to TLC [12].

Statistical analysis was performed using the applied programs package SPSS 17.0. The mean and standard error of the mean (M ± SEM) were used to describe continuous variables, complying with normal distribution law. Peripheral blood cell counts and general indices were compared in groups depending on MS modeling duration using ANOVA single-factor model. For descriptive statistics, rats with body weight decreased during the high-calorie food consumption were excluded from further consideration, and the data were adjusted for sex. Conducted analysis of variance showed that inter-group variance was statistically significantly greater than the intra-group variance ($p<0.001$) in subgroups with different diet durations. A posteriori comparison in these subgroups was performed using Bonferoni test.

Linear regression models were used to determine associations between peripheral blood parameters and BMI in each age group. Standardized regression coefficients β were used for comparative analysis of variables differing in mean values and variances. P <0.05 was considered statistically significant.

Animals were withdrawn from the trial under anesthesia using instantaneous decapitation method, which corresponds with international principles of the Declaration of Helsinki "On Humane Treatment of Animals", adopted by the General Assembly of the World Medical Association (2000) and national "General Ethical Principles of Animal Research" (Ukraine, 2001), consistent with the provisions of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 18.03.1986), as well as the Charter of the Ukrainian Association of Bioethics and GLP Standards (1992), standard provisions with issues of ethics of the Ministry of Health of Ukraine No. 66 dated 13.02.2006.

Results

MS modeling during the first month caused some difficulties; it was found that the MS development signs expressivity depended on the age and sex of rats. The increase in BMI of young rats was slower compared to animals of other age groups. BMI of rats of the first group on the 1-st and 3-rd month of the trial increased slowly and fluctuated within excess body weight. External signs of obesity were not obvious. The study showed that not all young rats that received a combined diet for a month, increased their body weight compared to control values (Fig. 1).
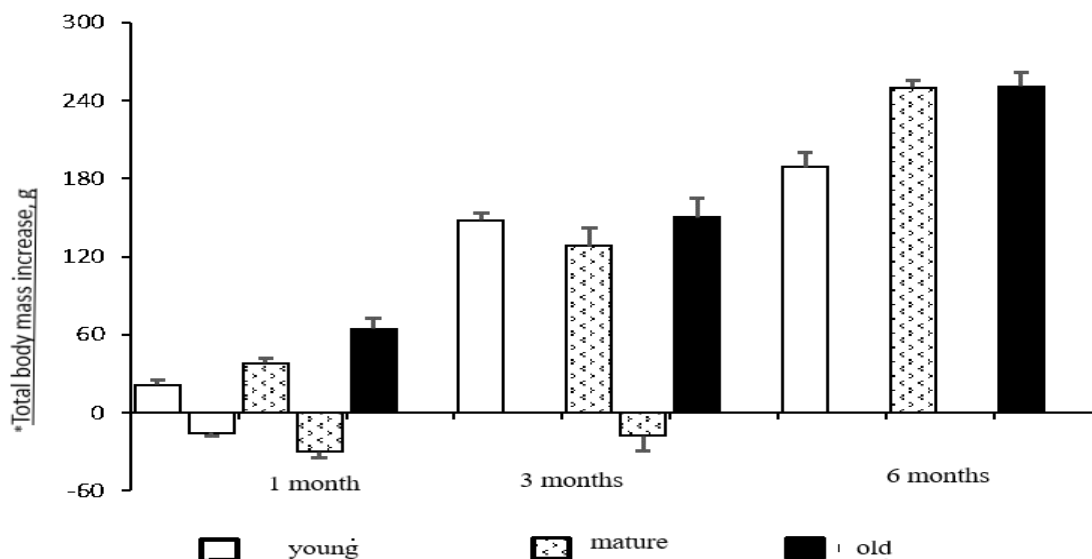
Fig. 1. Rats total body mass increase depending on the diet duration

It is conclusively noted that the indicators of total body weight gain in 25% of young males (12/48) and 27% of females (13/48) did not increase, but on the contrary, decreased compared to control values (Fig. 1). Interestingly, that females weight loss was more pronounced than in males (M±SEM (7.46 ±1.57) g and (21.13±3.18) g, respectively, p<0.001). Compared with other experimental groups during the first month of MS modeling, young rats did not significantly gain weight at all (Table).

This probably indicates a great compensatory ability of metabolic homeostasis maintained by the young organism, which does not depend on the sex of the animal [13]. Young animals' body adapts more favorably to changes in homeostasis and such adaptation primarily occurs due to hyperplasia of adipose tissue cells, at the same time inhibiting the ability of fat cells to perform the endocrine function, as adipose tissue does not produce inflammatory mediators during hyperplastic growth. It has been noted, that during this period, despite the development of microcirculatory disorders and adipose tissue hypoxia, there is an inhibition of phenomenon of "secondary damage", especially in vital organs [14].

Leucocyte formula of rats of the first group on the background of MS modeling during the 1st and 3rd months didn't show any significant changes. Peripheral blood TLC did not differ significantly from control values. Only by the 6th month of MS development there was an evidential increase in peripheral blood TLC: 1.7 times compared with intact animals. It has been noted that the increase in leucocytes amount during the 6th month of the trial occurred due to increase in banded neutrophils, which are considered to be non-specific primary defense mechanism in response to environmental changes, possibly due to their re-distribution

in the circulatory system.

Dependence of changes in the leukocyte response of peripheral blood of rats on body weight on the background of experimental modeling of metabolic syndrome (M±SEM)

Indicators	Control	The duration of the diet		
		1 month	3 month	6 month
Young rats				
BMI, kg/м^2	4,33±0,06	4,27±0,06	7,39±0,17[1,2]	8,62±0,24[1,2,3]
Leukocytes, 10^9/l	5,37±0,21	5,91±0,18	5,79±0,25	9,47±0,54[1,2,3]
Neutrophils stab, 10^9/l	0,09±0,01	0,23±0,02	0,35±0,05[2]	1,27±0,09[1,2,3]
Segmented neutrophils, 10^9/l	0,35±0,02	0,69±0,07[1]	0,70±0,07[1]	1,29±0,09[1,2,3]
Lymphocytes, 10^9/l	4,42±0,16	4,45±0,12	3,97±0,15	6,04±0,41[1,2,3]
Monocytes, 10^9/l	0,34±0,02	0,41±0,02	0,56±0,06[1]	0,66±0,06[1,2]
Eosinophils, 10^9/l	0,17±0,02	0,13±0,01[1]	0,21±0,02[1]	0,21±0,02[2]
Mature rats				
BMI, kg/м^2	5,15±0,38[a]	6,42±0,90[1,a]	8,00±2,40[1,2]	11,41±1,91[1,2,3,a]
Leukocytes, 10^9/l	5,28±0,13	8,06±0,50[a]	16,54±1,43[1,2,a]	15,57±1,01[1,2,a]
Neutrophils stab, 10^9/l	0,14±0,01	0,65±0,13[a]	1,87±0,21[1,2,a]	1,58±0,15[1,2]
Segmented neutrophils, 10^9/l	0,39±0,03	0,88±0,07	1,65±0,10[1,2,a]	0,94±0,10[1,3]
Lymphocytes, 10^9/l	4,20±0,10	5,70±0,35[a,b]	11,32±0,91[1,2,a]	10,63±0,60[1,2,a]
Monocytes, 10^9/l	0,45±,01	0,56±0,05	1,17±0,12[1,2,a]	1,63±0,15[1,2,3,a]
Eosinophils, 10^9/l	0,11±0,01	0,27±0,04	0,53±0,08[1,2,a]	0,80±0,08[1,2,3,a]
Old rats				
BMI, kg/м^2	5,22±0,68[a]	8,15±1,23[1,a,b]	9,95±1,59[1,2,a,b]	12,60±2,26[1,2,3,a,b]
Leukocytes, 10^9/l	5,15±0,15	14,06±0,60[a,b]	16,96±0,81[a]	24,86±1,28[a,b]
Neutrophils stab, 10^9/l	0,18±0,01[a]	1,55±0,14[1,a,b]	1,89±0,15[1,a]	2,27±0,21[1,2,a,b]
Segmented neutrophils, 10^9/l	0,31±0,02	1,49±0,10[1,a,b]	1,70±0,11[1,a]	1,95±0,20[1,a,b]
Lymphocytes, 10^9/l	3,78±0,12	8,03±0,20[1,a,b]	10,53±0,36[1,2,a]	15,63±0,68[1,2,3,a,b]
Monocytes, 10^9/l	0,73±0,05[a,b]	1,59±0,18[1,a,d]	1,63±0,15[1,a,b]	3,45±0,28[1,2,3,a,b]
Eosinophils, 10^9/l	0,16±0,01	1,41±0,13[1,a,b]	1,22±0,12[1,a,b]	1,56±0,17[1,2,3,a,b]

Note: [1] – the significance of differences in control; [2] – the significance of differences in relation to the 1-month diet; [3] – the significance of differences in relation to the 3-month diet; [a] – the significance of differences in relation to young animals; [b] – the significance of differences relative to mature animals.

The body mass of rats of the second group (adult rats) increased significantly after the 1st month of MS modeling both in terms of control values compared to the first group. The diet in group 2A was ineffective in only 16% of males (8/48), and in group 2B in 4.16% of females (2/48). Throughout the trial, adult rats lost almost 11.97% of their body mass (31.75 [20.25-44.25] g) (Table).

Leucocytes amount significantly increased in all experimental subgroups of group 2. Thus, in group 2B there was maximum increase in peripheral blood TLC compared to control values, without significant further dynamics (Table). Increase in leucocytes content was connected largely with eosinophilic granulocytes. The strongest association of BMI was noted with eosinophils (Fig. 2). To a lesser extent, the increase in TLC was connected with monocytes-macrophages, also, in group 2A-2B there was no association of BMI with segmental neutrophils found.

In group 3 (old rats), MS modeling was the most effective: there was an increase in the absolute number of monocytes in control group compared with groups 2K and 3K, despite total leucocyte count remained relatively constant.

After 1 month of trial old rats showed a significant increase in body weight and severe leukocytosis due to both granulocytes and mononuclear cells (Table). In this group, lymphocytosis was significantly more pronounced than in 1A and 2A. Activation of monocytes and eosinophils was observed in group 3B-3C against the background of developed lymphocytosis. Obvious associative links with BMI with changes in lymphocytic, monocytic, and (to a lesser extent) eosinophilic reactions have been determined (Fig. 2). It should be noted that in this age group the leucocyte formula differed from the first and second groups in control.

Discussion

Thus, the results obtained show that the rats' BMI increase, which occurs against the background of MS development, is an active inflammatory reaction stimulator, the severity of which depends more on the age of the animal and less on its sex. It is established that body mass increase correlates with an increase in blood inflammatory response in the group of young animals by the 6th month of MS modeling, but these rates are lower than those of mature and old rats. This provides a basis for considering adipose tissue activity (caused by obesity) and impaired lipid metabolism (release of excess free fatty acids into the blood) to be the initial link in LGI development in adipose tissue, which shows its differences from the classical development of inflammation depending on animals' age [15].
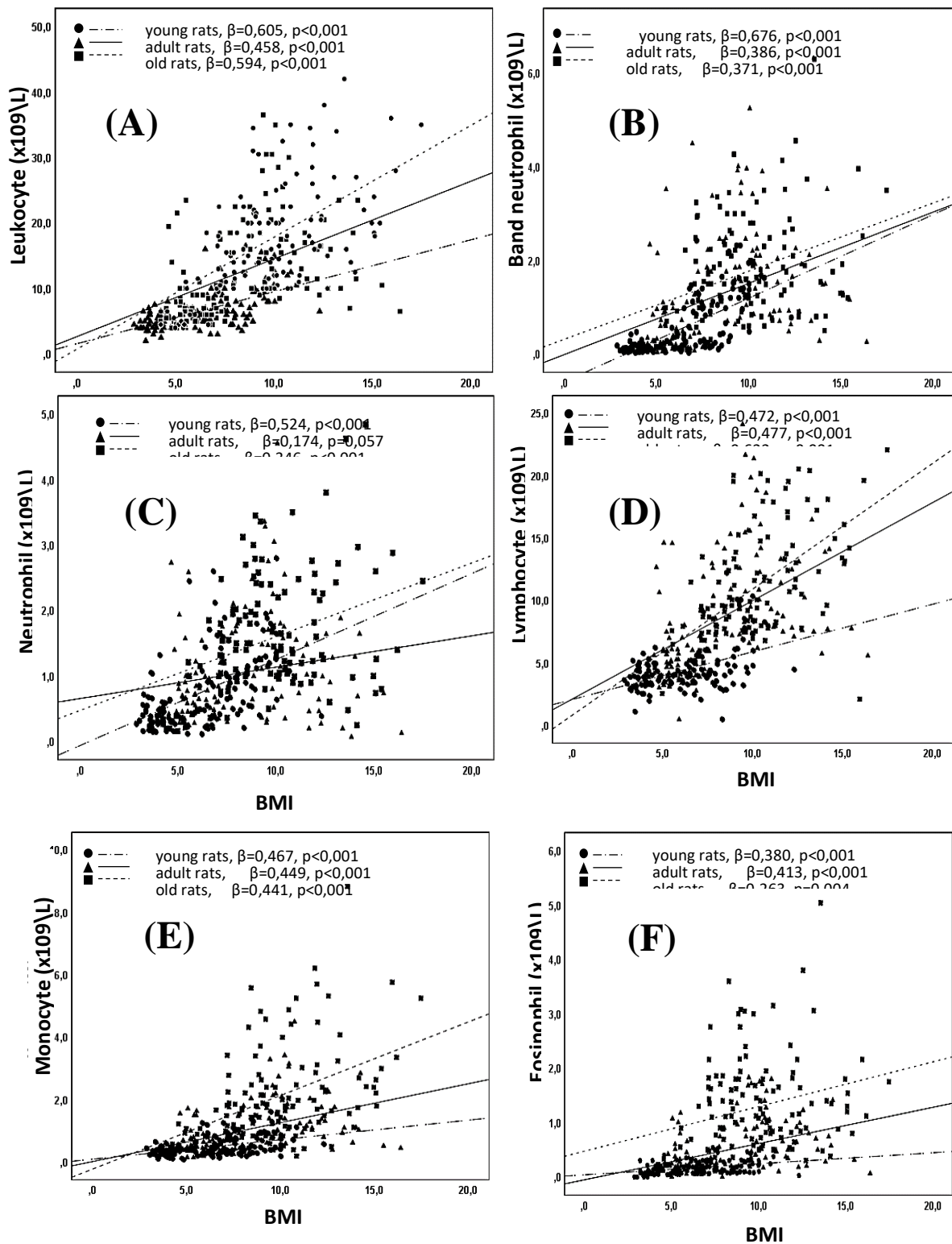
Fig. 2. The ratio of body mass index (BMI) to the content of leukocytes (A), rod neurons (B) and segmental (C), lymphocytes (D), monocytes (E) and eosinophils (F) of peripheral blood of rats depending on age. β is a standardized linear regression coefficient.

It is known that leucocyte amount in peripheral blood at inflammation can show what happens in the blood system as a whole, namely the link between the leucocyte outwandering from the blood to inflammation site and their entry from the bone marrow into the blood [16]. Our data show that changes in peripheral blood leucocyte response, developed on the background of alimentary obesity, have a number of characteristic features, compared with the classical development of inflammation, as well as certain associative relationships with animals' age. Thus, the group of young animals show the largest correlation coefficients between BMI and total amount of neutrophils (Fig. 2). The second group of rats shows the largest associations with eosinophils, which, to some extent, may be consistent with data on synthetic activity enhancement of eosinophils involved in inflammation site, which, unlike neutrophils, have the ability to further synthesis [17] and the possibility of autoimmune component connection in LGI development (Fig. 2). It should be noted that despite the relatively constant total amount of leucocytes in control for all age groups, the monocyte count was associated with age the most. It was observed that BMI of older animals is most associated with monocytes and lymphocytes, as a consequence of the severity of the monocyte reaction depending on age.

Data obtained indicate the importance of establishing intercellular relationships at early stages of LGI development, which are supported by the predominance of the release of anti- or pro-inflammatory mediators, both by activated blood cells and functionally active adipose tissue cells. This makes it possible to identify additional criteria for LGI development and to foresee the direction of secondary systemic damage. The obtained data can help to develop new criteria for diagnostics, monitoring, further development, identification of prognostic risks of MS occurrence and effectiveness of MS therapy depending on age.

Conclusions:

1. Dependence of inflammatory reactions on animal's age and sex in alimentary obesity development with subsequent formation of MS has been established, which is confirmed by the presence of associative links (body mass index - peripheral blood cell composition - age of the animal)

2. Associations between changes in BMI and separate peripheral blood cells activity depending on age have been established. BMI of young rats is associated with neutrophils and more delayed; BMI of adults is associated with eosinophils, starting from the first month of MS development; for older animals it is associated with monocytes and lymphocytes, at all stages of trial.

3. The obtained data on the ratio of BMI to changes in peripheral blood qualitative composition, taking into account the age of the animal, which occurs against the background of MS development can be used as primary monitoring and can be considered as one of the criteria for determining LGI in adipose tissue and as a prognostic factor in MS probability.

Conflicts of interest: authors have no conflict of interest to declare

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