

The Activity of IL-6 and TNF- α in Adipose Tissue and Peripheral Blood in horses Suffering from Equine Metabolic Syndrome (EMS)

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Summary

Equine metabolic syndrome (EMS) is a metabolic disorder characterized by excessive obesity and/or regional adiposity, insulin resistance (IR) and prior or current laminitis. In the course of the EMS, an important role exert systemic inflammation caused by excessive expression of proinflammatory cytokines. The aim of the current investigation was to examine relationships between expression IL-6 and TNF- α in adipose tissue and their concentration in peripheral blood in horses with EMS. On the basis of range of research procedures, horses were divided into two groups: group A (EMS horses, n=8) and group B (healthy horses with overweight, n=8). The concentration of the proinflammatory cytokines in the peripheral blood and their expression in the adipose tissue were determined. The results of the EMS group showed numerous macrophages and lymphocytes infiltration and increased diameters of adipocytes ($P<0.05$). The concentration of TNF- α in the serum of insulin-resistant horses was statistically higher compared to the healthy individuals. No significant differences were observed between the EMS horses and the control horses in the concentration of IL-6 in the serum. Moreover, our research revealed expression of investigated cytokines in adipose tissue. The results shows a higher expression of TNF- α in the EMS groups. What is more, macrophages infiltration were observed. In the case of IL-6, were detected the similar arrangement of this cytokines in adipocytes between both test groups. The study indicates the importance of pro-inflammatory proteins TNF- α in the equine metabolic syndrome.

Keywords: Equine Metabolic Syndrome, TNF- α , IL-6, Insulin Resistance, Obesity

Atlarda Metabolik Sendrom (EMS) Sancısında, Adipoz Dokuda ve Periferal Kanda IL-6 ve TNF- α nın Aktivitesi

Özet

Atların metabolik sendrom (EMS)'u aşırı obezite ve/veya bölgelik yağılanması ile karakterize, insülin direnci (IR), ileri derecede veya aniden oluşan laminitis ile karakterize metabolik bir hastalıktır. EMS derslerinde, proinflamatuar sitokinlerin aşırı ekspresyonu ile oluşan sistemik inflamasyon uygulamada önemli rol oynadığı anlatılmaktadır. Bu çalışmanın amacı, adipoz dokuda sentezlenen ve periferal kanda bulunan, IL-6 ve TNF- α 'nın EMS'li atlarda oluşan ilişkilerini incelemektir. Araştırmanın ana hedefine göre atlar esas olarak, iki gruba ayrıldı: Grup A (EMS atlar, n = 8) ve grup B (kilolu sağlıklı atlar, n = 8). Periferal kanda proinflamatuar sitokinlerinin konsantrasyonu ve bunların adipoz dokularındaki etkisi belirlenmiştir. EMS grubunun sonuçları, pek çok makrofaj ve lenfosit infiltrasyonu ve adipositlerin çaplarında artış ($P<0.05$ gösterdi). İnsüline dirençli atların serumunda TNF- α konsantrasyonu sağlıklı bireylere göre kıyaslandığında istatistiksel olarak yüksek sonuçlar görüldü. Serumındaki IL-6'nın konsantrasyonunu EMS'li atlarda ve kontrol grubu atlar arasında anlamlı farklar gözlandı. Ayrıca, araştırmada incelenen yağ dokularda sitokinlerin ekspresyonu saptandı. Sonuçlarda EMS'li grupta TNF- α nın daha yüksek anlamı olduğu görüldü. En önemlisi makrofaj infiltrasyonu gözlandı. IL-6'nın olgusunda, adipositlerde sitokinlerin benzer durumu her iki grupta tespit edildi. Çalışma atların metabolik sendromunda pro-inflamatuar proteinlerden TNF- α 'nın önemini ortaya çıkardı.

Anahtar sözcükler: Atların Metabolik Sendromu, TNF- α , IL-6, İnsülin Direnci, Obezite

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INTRODUCTION

Recently, the obesity is becoming an increasing problem of civilization, affecting both humans and animals, and thus it becomes the subject of intense research. This issue is investigated on two main levels affecting the carbohydrate and lipid metabolism: genetic and environmental predisposition such as physical activity and nutrition/diet [1-4].

The obesity occurrence and high insulin serum level are two of the few clinical symptoms allowing to diagnose disease termed metabolic syndrome (*MetS-Metabolic Syndrome, Syndrome X*). In the field of veterinary medicine, the concept of the equine metabolic syndrome was proposed for the first time in 2002 by Johnson et al. [5]. In 2010, the American College of Veterinary Internal Medicine, finally defined and officially recognized the Equine Metabolic Syndrome (EMS) as a disease entity [6]. The EMS is defined as a metabolic disorder that includes chronic/acute laminitis, frequent low grade laminitis, hyperinsulinemia, pathologic obesity and insulin resistance (IR). Additional features characterizing the EMS are dyslipidemia, fluctuating levels of blood adipokines and systemic inflammation [6,7]. Equine metabolic syndrome most commonly affects primitive breeds of horses, i.e., ponies and/or heavy horses, as well as those with the relatively low demand for selective nutrients (Quarter Horse, Arabian, Morgans) [8-13]. Genetic predisposition and environmental factors, such as overfeeding and lack of the exercises, contribute to initial physiological insulin resistance and subsequent chronic insulin resistance. Genetic predisposition to pathologic obesity may be caused by a specific gene mutation, i.e., melanocortin-4 receptor, which is responsible for the regulation of feed intake and insulin sensitivity [14]. Persistently high blood glucose level results in the reduction or loss of the sensitivity of cell membranes to the action of insulin. Furthermore, insulin resistance increases the risk of laminitis by stimulating the synthesis of endothelin-1 (ET-1), and activation of the sympathetic nervous system, which results in the narrowing of blood vessels. Disturbance of blood circulation within the hoof capsule is the cause of excessive accumulation of fluid in the interior, and in consequence, detachment of the hoof capsule from the coffin bone [15,16].

In horses, the symptoms of excessive obesity include regional adiposity as well as irregular fat distribution at the base of the tail area, around eyes and at the base of the neck, what is called in the literature as "cresty neck" [13,17].

Pathological adiposity in EMS individuals is strongly associated with insulin resistance, and thus adipose tissue play an important role in the course of this disease. Additionally, adipose tissue is not only an energy reservoir, but also a source of various pro-inflammatory cytokines [13,18-20]. These includes mainly tumor necrosis factor

alpha (TNF- α) and interleukin 6 (IL-6). The TNF- α is a protein secreted by adipocytes and stromal vascular cells (primarily macrophages). The role of this protein in the pathogenesis of obesity and insulin resistance involves: (i) inhibition of the activity of genes that regulate lipid and glucose metabolism, as well as (ii) reduction of secretion of adipokines with specific anti-hyperglycemic properties. In the liver, TNF- α inhibits the expression of genes closely related to the transport of glucose into the cells [21]. Studies conducted in humans indicate a strong correlation between the TNF- α expression in adipose tissue of obese individuals and the level of hyperinsulinemia [19,22]. Another cytokine, in addition to TNF- α , playing a significant role in the EMS is IL-6, a molecule responsible for initialization and regulation of acute inflammatory response. This cytokine is also responsible for the inhibition of insulin receptor expression and the reduction of adipogenesis process. Moreover, IL-6 reduces the secretion of adiponectin, peptide hormone that has anti-inflammatory properties [22-24].

Currently, diagnosis of equine metabolic syndrome, especially at early stages of the disease development is challenging for clinicians. The diagnostic procedures are still under development and require further improvements. There is increasing interest in the towards significance of the enteroinsular axis in the pathophysiology of EMS, but still the dynamic tests, mainly the combined glucose-insulin test, are one of the main possibility of EMS diagnosis [11]. However, additional information about cytokines contribution to the physiological path of EMS is required, thus acquiring this knowledge is crucial.

The aim of this study was to determine the levels of TNF- α and IL-6 in the serum and adipose tissue derived from EMS and healthy horses. Our objective was to correlate the information about cytokine levels in the serum with their distribution in the fat tissue. We hypothesize that both information are relevant for the EMS diagnostic and can contribute to the proper assessment of animals' physiological condition.

MATERIAL and METHODS

Ethical Approval

This research was approved by the II Local Ethical Committee (No. 64/2008 and individual agreement for experiments conducted on animals No. 21/2009), localized at the Wrocław University of Environmental and Life Sciences, Wrocław, Poland.

Experimental Horses

Sixteen heavy draft horses (Silesian breed and Haflingers), different gender, age ranging from 8-14 years were chosen for this experiment. All horses were qualified on the basis of their medical history and detail medical interview with the owners. The horses included into the

study came from the Upper Silesia region in Poland. All individuals had free access to water, sandy paddock and performed work of moderate intensity. Body weight was measured in all individuals using mobile, electronic Equine Bosh Scale (Bosh, Germany). None of the qualified mares was pregnant. Horses from the investigated group had a history of laminitis, with clearly visible deformities of the hoof. Moreover, horses qualified into the study were characterized by regional adiposity and exhibited general obesity. Body condition score (BCS) was measured in the all horses using a scoring system of 1 (emaciated) through 9 (extremely obese) to estimate fat deposition and was based on the system developed by Henneke et al.^[25]. Horses, which were characterized by obesity and extreme obesity (8-9) qualified for the experimental group. The other horses with the assessment of 6-7 were assigned to the control group^[25]. All investigated individuals underwent following clinical examinations: (i) measurements resting insulin level in the serum, (ii) measurements of resting glucose level (iii), the Combined Glucose-Insulin Test (CGIT test). Before performing CGIT, all horses had limited access to Timothy hay in amounts 4 kg for sixteen hours. First, blood was collected from the jugular vein for the determination of resting level glucose and insulin. Next, 50% dextrose solution (150 mg/kg bw) was injected intravenously immediately followed by application of insulin bolus (IV 0.10 U/kg bw). For the determination of glucose level by means of glucometer (Glucosens 1040), blood samples were collected at 1, 5, 15, 25, 35, 45, 60, 75, 90, 105, 120, 135 and 150 minutes after administration of a bolus. In total, blood samples were collected for 2.5 h^[13,26,27]. Sterile technique was maintained throughout the collection process. On the basis of aforementioned procedures and the results of the study, horses were divided into two groups: group A (EMS horses, n=8) and group B (healthy horses, n=8).

Clinical Examination

All experimental animals were clinically examined by two independent veterinarians. Horses were evaluated for gait and hoof capsule using X-ray photography. Additional visual examination and palpation was performed in order to identify subclinical or clinical signs of laminitis.

Blood Samples Collection and ELISA Tests

Blood was collected from the jugular vein from all examined animals (16 individuals), into tubes with the granulate and the accelerator in an amount of 10 ml, once from each horse. Then, this material was centrifuged, preserved in liquid nitrogen and transported to the laboratory. Blood insulin level was determined using the Equine ELISA Test (BioVendor, Czech Republic), for which blood samples were centrifuged and serum was removed. Next, IL- 6 and TNF- α were detected using an equine IL6 ELISA immunoassay kit (Genorise Scientific, US) and TNF- α (Genorise Scientific, US), according to the manufacturers

instructions. Sterile technique was maintained throughout the collection process.

Histochemistry and Immunohistochemistry Examination of Fat Tissue

Adipose tissue sample (2 g) from the base of the mane were collected from each horse under local anesthesia (2% Lignocainum, Polfa S.A., Poland). The histological material was preserved in 10% buffered formalin for 24 h.

Next, 5 μ m-thick sections were obtained on a Microm HM 340E microtome (Zeiss, Germany) and placed on histological slides. Samples were subsequently deparaffinized with xylene, ethanol (decreasing concentrations from 100 to 70 %) and washed with distilled water. Slides were stained with hematoxylin (Shandon™, Thermo Scientific US) for 8 minutes, rinsed in running tap water for 10 minutes and stained with eosin (Shandon™, Thermo Scientific US) for 5 min. Sections were dehydrated by washing with ethanol (increasing concentrations from 70 to 100%), followed by xylene and sealed with DPX mounting medium (AquaMed, Poland). Analysis was performed using light microscope (Axio Imager A1, Zeiss) ^[28].

The 3 μ m-thick tissue sections were cut, dewaxed and rehydrated. Immunoperoxidase cell labeling was performed using polyclonal antibodies against IL-6 (Genorise Scientific, USA) and TNF- α (R&D Systems, Germany). Heat-induced antigen retrieval was performed as follows: slides were incubated in the target retrieval solution pH=9.0 (Dako, Denmark) for 20 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and slides were washed with TBS (tris-buffered saline) for 5 min each. Tissue samples were labeled with antibody solutions. Primary antibodies were incubated for 20 min at 20°C. Detection was performed with EnVision™ Systems (Dako, Denmark). Sections were counterstained with Mayer's hematoxylin for 1 min, dehydrated and sealed. Analysis was carried out with optical microscope (Axio Imager A1, Zeiss Germany).

Morphometric Examination

Morphometric analysis of adipose tissue was performed with an Axio Imager light microscope (Zeiss, Germany) using 10 fields of view for each individual (each slide). Photographic documentation was compiled using the Axio Camera (Zeiss, Germany).

Statistical Analysis

Verification of data normality was performed using Shapiro-Wilk test, whereas the Levene's test was used to assess the equality of variances. Depending on the results of data validation, comparison analysis was performed using parametric or non-parametric statistical tests, i.e., Student's t test or Mann-Whitney U test, respectively. The P values lower than 0.05 were considered to be significant.

All statistical calculations were performed using the STATISTICA 7.0 software (StatSoft, Inc., Statistica for Windows, Tulsa, OK, USA).

RESULTS

Clinical Picture of Investigated Horses

A clinical examination of experimental animals revealed a significant degree of obesity and regional adiposity. The average body weight of horses in group A (EMS horses) was 709 kg, while in group B (control horses), the body weight was 674 kg (*Table 1*). Horses from EMS group had unusual distribution of body fat, particularly around the base of the tail, eyes and at the base of the mane. Despite increased body weight of horses within group B, no symptoms of pathological adiposity were noticed. Horses assigned to group A were characterized by elevated levels of rest insulin (ranging from 60 to 100 μ U/ml). Moreover the results of CGIT test were positive. Horses assigned to group B, showed normal rest insulin level (ranging from 5 to 20 μ U/ml) and the CGIT test gave negative result.

The Morphology, Morphometry, Immunohistochemistry and Immunoassays of Adipose Tissue

Adipose tissue collected from horses within group A had a dark brown color. By contrast, adipose tissue obtained from horses of group B was of pale straw color. Morphological examination of adipose tissue in group A, revealed a slight degree of mononuclear cells, abundant macrophages and lymphocytes infiltration (*Fig. 1B*). In addition, histological examination of adipose tissue in group A exhibited a slight fibrosis (*Fig. 1B*). In group B, no inflammatory cells infiltration was observed (*Fig. 1A*). Adipose tissue in group B had proper histological picture (*Fig. 1A*).

Table 1. Body weight of individual horses qualified for the experimental and the control groups

Tablo 1. Deneysel ve kontrol grubundaki atların bireysel vücut ağırlıklarını kualifikasiyonu

Patient	Horses with EMS (Group A)	Healthy Horses (Group B)
1	710	625
2	684	686
3	712	680
4	680	692
5	730	720
6	710	679
7	728	645
8	721	664
X	709	674

The data of morphometric measurements of adipose tissue derived from insulin-resistant horses (group A) and the control (group B) were analyzed statistically. The results of comparative analysis showed that the average diameter of adipocytes forming the tissue obtained from group A was larger than of adipocytes in group B (*Fig. 2*).

The immunohistochemical analysis performed to detect IL-6 distribution showed similar arrangement of this cytokine in adipocytes' membrane both in EMS and healthy horses (*Fig. 1C, D*). The level of IL-6 in the serum was higher in group B, although statistical analysis showed no significant differences in the serum concentration of IL-6 between both test groups. The mean concentration of serum IL-6 in the group A was noted at the level of 1.893 ± 0.0073 μ g/mL, while in group B, the average level of IL-6 was 1.888 ± 0.0079 μ g/mL (*Fig. 3*).

The analysis of TNF- α distribution showed that in adipocytes of both investigated groups this cytokine localized primarily in the nucleus (*Fig. 1E,F*). The level of TNF- α in the serum samples was higher in group A. In this group 1.9 ± 0.02 units of TNF- α were detected, while in group B it amounted to 1.2 ± 0.02 units. The observed differences were statistically significant (*Fig. 4*).

DISCUSSION

The frequency of the obesity in animals is increasing, posing a serious problem in veterinary medicine. Similarly as in the human population, the obesity in animals is considered as a medical condition of epidemic character [5,29]. The problem of obesity is especially common for small animals, i.e., dogs and cats, but it also affects other domestic animals including heavy horses (mainly draft horses). The World Horse Welfare estimates that the obesity frequency in the United Kingdom is reported in 50% of the population of pleasure riding horses [30,31]. Background for the development of horses obesity is similar to that in humans and results mainly from improper diet, physical inactivity, but also genetic predispositions. High-carbohydrate diet provides large dose of energy, which is very rarely balanced with the physical activity. The obesity affects animals by decreasing its performance, reproductive success and longevity. Moreover, in the case of horses, prolonged and untreated obesity increases the risk of development of metabolic diseases such as equine metabolic syndrome (EMS), strongly associated with insulin resistance (IR), laminitis and hyperinsulinemia. Considering these facts, the obesity in horses leads to life-threatening conditions [3,5,29,30]. The obesity in horses is often neglected by the owners, and sometimes it is difficult to determine by the veterinary clinicians. Various methods have been applied to evaluate symptoms of obesity or obesity-related diseases such as EMS. However, veterinarians are still seeking for a save diagnostic tool, helping recognize early signs of metabolic disorders,

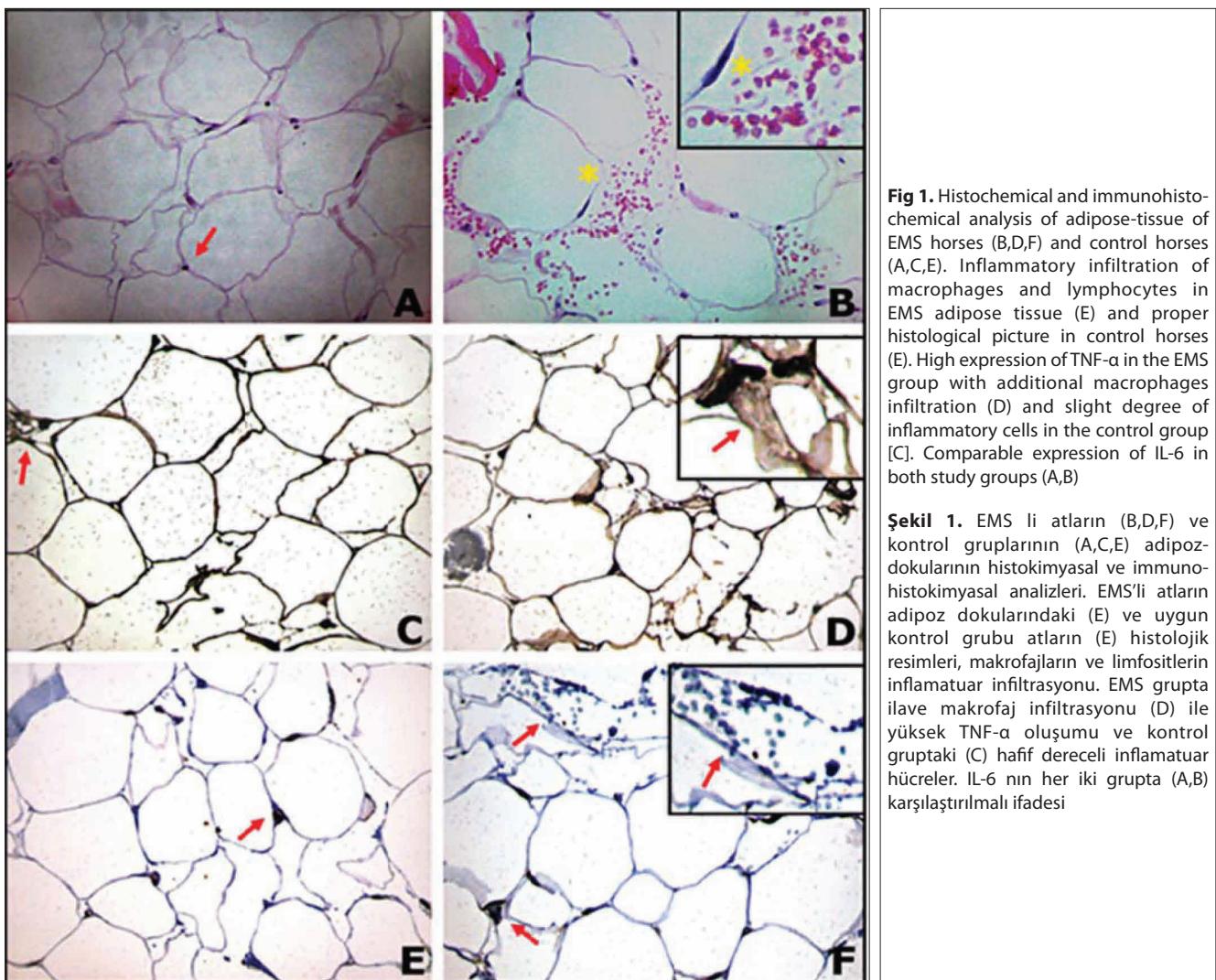


Fig 1. Histochemical and immunohistochemical analysis of adipose-tissue of EMS horses (B,D,F) and control horses (A,C,E). Inflammatory infiltration of macrophages and lymphocytes in EMS adipose tissue (E) and proper histological picture in control horses (E). High expression of TNF- α in the EMS group with additional macrophages infiltration (D) and slight degree of inflammatory cells in the control group [C]. Comparable expression of IL-6 in both study groups (A,B)

Şekil 1. EMS li atların (B,D,F) ve kontrol gruplarının (A,C,E) adipodokularının histokimyasal ve immunohistokimyasal analizleri. EMS'li atların adipoz dokularındaki (E) ve uygun kontrol grubu atların (E) histolojik resimleri, makrofajların ve limfositlerin inflamatuar infiltrasyonu. EMS grupta ilave makrofaj infiltrasyonu (D) ile yüksek TNF- α oluşumu ve kontrol gruptaki (C) hafif dereceli inflamatuar hücreler. IL-6 nin her iki grupta (A,B) karşılaştırılmalı ifadesi

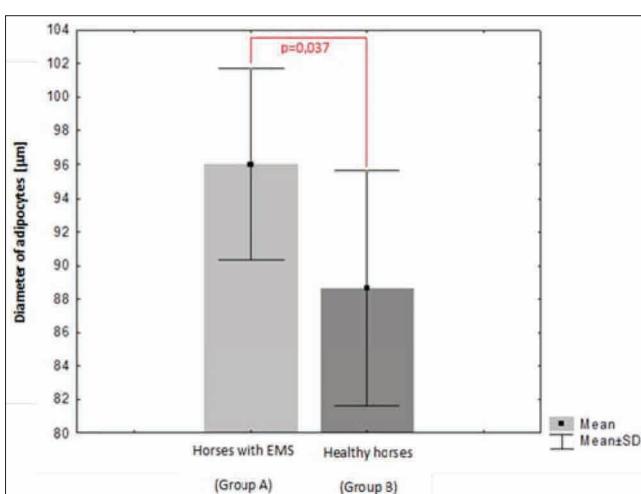


Fig 2. Statistical comparison analysis of adipocytes diameter in both groups investigated. Results were consider significant at $P<0.05$

Şekil 2. Her iki grupta adipozit çaplarının istatistiksel olarak karşılaştırılmalı analizi. Sonuçlar anlamlı bulundu $P<0.05$

especially when recommended methods, such as combined insulin test might be dangerous for animals' life [11,32].

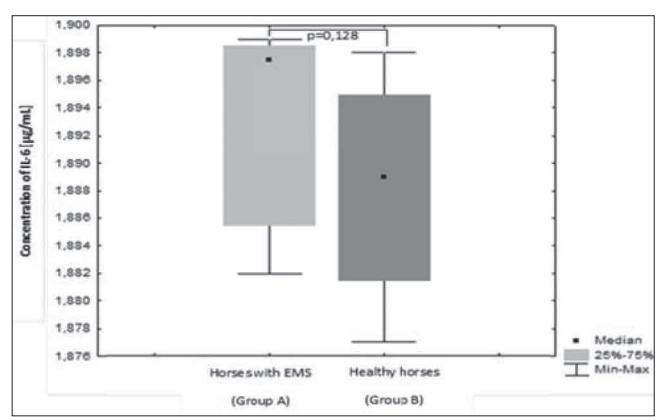


Fig 3. Comparison of IL-6 level in the serum of EMS and healthy horses
Şekil 3. EMS'li ve sağlıklı atlarda IL-6 seviyelerinin karşılaştırılması

Some physical characteristic of animals are crucial when diagnosing obesity. Obese horses are distinguished by the accumulation of adipose tissue especially in the tail base, area surrounding eyes and the neck region [29,32]. As it was reported previously, the obesity might correlates with the morphology of adipocytes [33]. This observation confirms

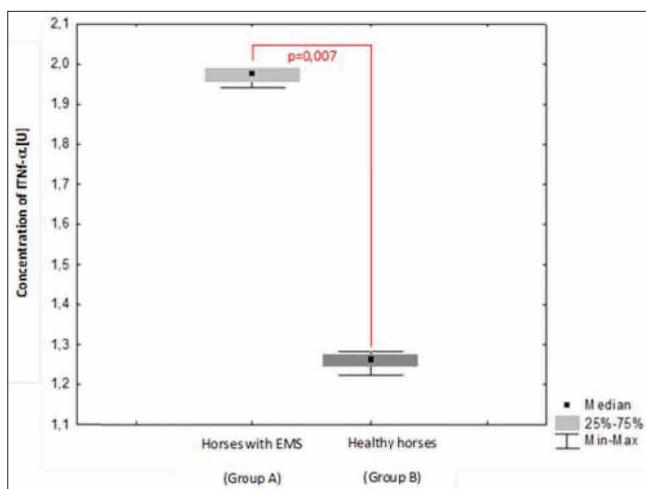


Fig 4. Comparison of TNF- α level in the serum of EMS and healthy horses

Şekil 4. EMS'li ve sağlıklı atlarda TNF- α seviyesinin karşılaştırılması

our findings where we noticed the higher adipocytes size average in EMS horses, compared to the healthy individuals with observed overweight. Due to the fact that the adipose tissue is a complex structure and may act in an endocrine manner, the disturbance of adipocytes homeostasis may have negative effect on the organism. The adipokines might act not only in an endocrine manner but also, in an autocrine and paracrine fashion, therefore, their activity might lead to adverse metabolic consequences and may give different clinical picture of disease. The body mass increasing contribute to dysfunction of secretion of adipokines, which influence on inflammation and glycemic function [34-36]. Furthermore, the hypertrophic adipocytes secrete a variety of factors that foster both peripheral and hepatic resistance to insulin. Among the adipokines, the raised activity of TNF- α and IL-6 might play a key role in the development of insulin resistance and might lead in consequence to the development of EMS [7]. Moreover the subinflammatory state, induced by obesity, can be accelerated by the macrophages infiltrating of the adipose tissue, - which additionally promotes a chronic condition [35,36]. Our research showed abundant infiltration of macrophages and lymphocytes in adipose tissue of EMS horses whereas healthy individuals did not exhibited this phenomenon. Taking under consideration that the IL-6 and TNF- α are involved in the development of insulin resistance, we decided to investigate if there are correlation between the level of both cytokines in the serum and their distribution in in adipose-tissue. We found the elevated level of both cytokines in the serum of EMS horses (group A). However, a statistical analysis reviled only significant differences in the concentration of TNF- α between the groups. Immunohistochemical analysis showed intracellular localization of the TNF- α in adipocytes of EMS as well as in control horses. Uysal et al. [37] showed that the deletion of TNF- α 1 or TNF- α 2 receptor results in a significantly improved insulin sensitivity in the

diet-induced obesity in mice but also in leptin-deficient ob/ob mice [37]. Other research reported that TNF- α may act as pivotal mediator in the insuline resistance as high TNF- α expression in human fat tissue correlated positively with BMI, percentage of body fat, and hyperinsulinemia, whereas weight loss caused a decrease of TNF- α level [38]. Based on our results, we argue that in BSC-related insulin resistance, the TNF- α may play a key role. Our results correlates with the data obtained by other research groups in animal experimental models as well as in human patients [7]. The role of IL-6 in the insulin resistance is not clear because of conflicting data. However, some research has revealed an relationship between IL-expression in the mares obesity and their age [7,39]. With the increase of BCS in mares under 20 years of age there was a decrease of IL-6. However, in human, it seems that high level of IL-6, which is typical for chronic inflammation states, is associated with the obesity and type 2 diabetes [7]. They, in turn, can result in insulin resistance and temporarily elevated level of this cytokine not affecting glucose homeostasis [40]. Our research showed similar arrangement of IL-6 distribution in adipocytes in both study groups and no significant differences in the serum concentration of IL-6 both in EMS and healthy horses. The researched horses were younger than fifteen years of age and older than seven years of age. Thus, our results might suggest the effect of age on activity IL-6 in adipose tissue and peripheral blood in obesity horses. However, the results emphasize the need for further studies to explain the role IL-6 in the course of the pathophysiology EMS. In the present research we showed correlation between the level of TNF- α in serum as well as in adipose tissue in EMS horses. We state that this information's might be used as an auxiliary tool in the process of equine metabolic syndrome diagnosis.

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