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Are Proteins Such as MMP2, IGF1, IL-13, and IL-1ra Valuable as Markers of Fitness Status in Racehorses? A Pilot Study

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Abstract: In a recent study, in which more than a thousand racehorses were evaluated, three potential blood markers were selected. It was concluded that insulin-like growth factor 1 (IGF1), interleukin-1 receptor antagonist (IL-1ra), and matrix metalloproteinase 2 (MMP2) may enable the early detection of horses at risk of injuries. However, in other studies, it was suggested that cytokine concentrations indicate the athlete's status better than mRNA expression in blood cells. Thus, the purpose of this study was to evaluate changes in IGF1, MMP-2, and novel markers such as interleukin-13 (IL-13) after exercise in horses at different fitness levels as well as after different intensities of exercise. ELISA tests were performed on thirty-one racehorses [n = 31], who were divided into an inexperienced [beginner] group [n = 20] and an experienced [advanced] group [n = 6]. In addition, differences between race and training session were evaluated to see the influence of different intensities of exercise. Blood samples were taken before and after exercise. The basal IGF1 concentration was lower in an inexperienced group ($p < 0.01$) as well as IL-13 ($p < 0.05$) in comparison to the experienced group. There were no differences between pre- and post-exercise samples, changes in multiples or between training, and racing exercises. In conclusion, the basal values of some cytokines may appear to be more beneficial in forecasting horse fitness level.

Keywords: inflammatory response; myokines; exercise; injury; cytokines

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1. Introduction

Inadequate conditioning or sudden increases in workload might strain the musculoskeletal system, resulting in the weakening of locomotor system structures or overtraining. This may contribute to the production of fatigue fractures due to its high intensity and accumulation of microtrauma. Injuries in horses, such as fractures or tendon/ligament injuries, can have severe consequences, including euthanasia due to the inability to recover [1]. It was documented that the prevalence of a fatigue injury like bucked shins syndrome of the third metacarpal bone affects 30 to 70% of Thoroughbred horses in the first months of race training [2]. It is important to note that musculoskeletal injuries in horses often result from a combination of several factors. Proper management, regular veterinary care, appropriate conditioning, and a balanced approach to training can help minimize the risk of musculoskeletal fatigue injuries in horses. Also, detecting the point of balance between inflammatory processes and repair mechanisms that may represent an appropriate or an inappropriate adaptive response is beneficial for horses' health and welfare management. It should be recognized that this point is likely to be different for each horse, and not only because there will be differences in training strategies.

Clinical monitoring of fitness and performance in athletes can be supported by the analysis of various blood parameters. These biomarkers provide vital information about the horse's physiological status, training adaptability, and overall health [3,4]. However, there is no single suitable biomarker that would allow for the detection of fatigue disorders at the

early stage of their formation. In a recent study, in which more than a thousand racehorses were evaluated, three potential blood markers were selected [5]. It was suggested that the insulin-like growth factor 1 (IGF1), interleukin-1 receptor antagonist (IL-1ra), and matrix metalloproteinase 2 (MMP2) mRNA expression may enable the early detection of horses at risk of injuries. However, it was suggested that in exercise physiology, peptide concentrations indicate the athlete's status better than mRNA expression in blood cells [6,7]. This is explained by the transcriptional and post-transcriptional regulation of mRNA expression and degradation, which may have an influence on protein production. Thus, there is a lack of studies connected with the evaluation of IGF1, MMP2, and IL-1ra changes in protein level during different types of exercise load in racehorses.

In addition, interleukin-13 (IL-13) blood concentration changes in humans as well as mice were proposed as a useful marker for endurance training, published in a very recent study in "Science" [8]. IL-13 is not as extensively studied in the context of exercise as other cytokines because it is primarily known for its involvement in allergic and inflammatory responses [9,10]. An increase in IL-13 concentration in response to exercise has been reported, particularly in aerobic exercise in humans, contributing to the resolution of exercise-induced inflammation and promoting tissue repair and remodeling. Additionally, IL-13 influences the improvement in endurance capacity by enhanced mitochondrial respiration and fatty acid utilization by the working muscle [8]. In our previous study, we evaluated the changes in this cytokine level in comparison to commonly accepted anti-inflammatory markers (IL-1ra and IL-10) in endurance and racehorses [9]. We confirmed that there was an increase in the IL-13 serum concentration after the 100 km ride in endurance horses and higher IL-13 basal serum concentration, as well as a decrease in IL-13 after exercise in the untrained group of racehorses. However, there was no comparison between different types of exertion (training vs. race). We also decided to compare the changes in the novel cytokines values with commonly accepted anti-inflammatory markers such as IL-1ra [11], which can attenuate the production of pro-inflammatory cytokines and limit the extent of inflammation associated with exercise-induced muscle damage.

Several developing load-sensitive parameters have been identified that potentially improve future athlete load monitoring in humans. However, such studies are still rare in horses. Thus, the aim of this study was to evaluate the usefulness of IGF1, MMP-2, and IL-13 at the protein level as "readiness to run" markers in racehorses under different exercise loads for the first time.

2. Materials and Methods

2.1. Animals

For this study, in total, thirty-one (n = 31) healthy Arabian (n = 23) and Thoroughbred (n = 8) racehorses (approx. 450–550 kg), both stallions and mares (70:30) aged 2–5 years old, were selected. All racehorses were trained by one trainer and housed in the same stable on straw under the same environmental conditions. They were fed with the standard diet designed for racehorses (oats 5.5 kg/horse, meadow hay 7.5 kg/horse, and special concentrate for performance horses). The concentrated feed and roughage were given three times per day.

Horses were examined during training sessions and races under similar environmental conditions. The training sessions were on sand for 800 m at a speed of about 800 m/min. During the training session, horses were divided into the inexperienced (beginner) group (n = 20) and the experienced (advanced) group (n = 6). The selection of those groups was based on their experience in racing. Horses for which it was the beginning of their first training season were included in the inexperienced group, whereas animals for which it was their second or further training season were included in the experienced group. Because we did not notice any differences between the results obtained from Arabians and Thoroughbreds, both were analyzed together.

Additionally, a third group was created to include horses exercising during a race (1600–2200 m) on grass. This group consisted of ten horses (n = 10). In this group, horses

at different fitness levels were examined together just to compare the differences between workload intensity (training vs. race). The limited number of horses in this group related to difficulties in obtaining blood samples after races.

All horses were examined by veterinary practitioners, and no visible health disturbances were observed. Heart rate, mucous membranes (color and moisture), dehydration (measured as the time it takes for a pinched skin to fold over the point of the shoulder and flatten), and regularity of gait were evaluated. Additionally, a basal morphological and biochemical blood examination before and after exercise was performed, and no pathologies were observed.

2.2. Samples

The blood samples were collected before the start of exercise (1 h before feeding), immediately after exercise, and at 30 min at the end of the physical effort as a part of standard veterinary diagnostic procedures. Thus, no approval of the Local Commission for Ethics in Animal Experiments was required, according to the Polish legal regulations and the European directive EU/2010/6. However, for this study, only samples obtained before and 30 min after exercise were examined because changes in the cytokine serum concentration occur at least 30 min after exercise according to previous studies [5,7,9]. We are aware that the sampling limitations may have influenced the results by omitting the later increase in the cytokine concentration. However, as this is a pilot study, we wanted to check if using the routine fitness monitoring time scheme is sufficient for measuring changes in the mentioned cytokines.

Peripheral blood was gathered from the jugular vein into sterile K2-ethylenediaminetetraacetic acid (K2-EDTA) tubes for hematological tests and sterile dry tubes for serum analyses using the BD Vaccutainer system (BD, USA). The tubes were centrifuged (3000× *g*, 15 min) and serum was isolated and stored at −80 °C for further analyses.

2.3. Procedures

To determine the selected cytokines concentration of IGF1, IL-6, MMP2, IL-1ra, and IL-13 the available immunoenzymatic commercial assay dedicated to equine species was used (ELK Biotechnology Co., Ltd., Wuhan, China), which was performed according to the manufacturer's instructions [9]. Intra-assay precision (precision within an assay): CV% < 8%; inter-assay precision (precision between assays): CV% < 10%). The absorbance was measured via a Multiscan Reader (Labsystem, Helsinki, Finland) using the Genesis V3.00 software program.

2.4. Statistical Analysis

Statistical analysis was conducted using the OriginPro 2022 statistics package (OriginLab Corporation, Northampton, MA, USA). To assess the variability in cytokine levels, either a one-way ANOVA or a one-way repeated measures ANOVA was employed. For comparing specific groups, a post hoc pairwise comparison was conducted using the Bonferroni test. The significance level was set at $p < 0.05$.

If the data deviated from the assumption of normality, the non-parametric Friedman ANOVA test was utilized. Subsequently, the Wilcoxon–Nemenyi–McDonald–Thompson test was employed for a post hoc analysis of the aforementioned test. The significance level for these tests was set at 0.05.

Before conducting the analysis, outlier detection procedures were performed, which involved utilizing either Linear Regression or Grubbs' test. This step was taken to ensure the integrity of the data. According to the results of this analysis, any points were removed.

3. Results

The basal IGF1 serum concentration was increased in the more advanced group ($p = 0.00791$) (Figure 1a); however, there was no difference between the fold change in the

pre- and post-exercise samples (difference between these two measurements) as well as between the training and race effort (Figure 1b,c).

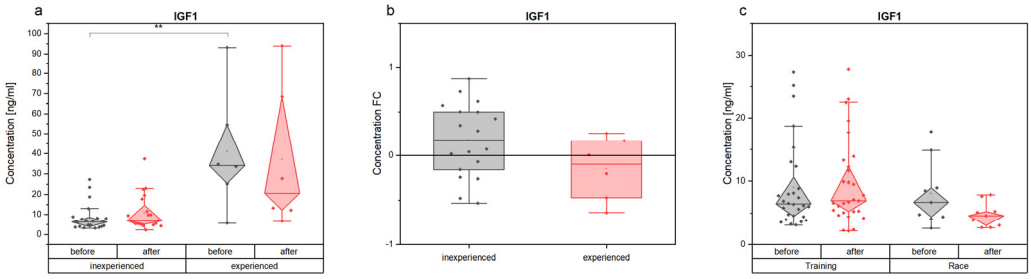

Figure 1. The concentration of IGF1 [ng/mL] before and after exercise for both experienced groups (**a**), the fold change in response to inexperienced and experienced groups (**b**), and the concentration change before and after two types of activity: training and race (**c**). In the box plots, the upper whisker represents the maximum value; the upper line Q3 (**upper quartile**); the center line the median; the lower line Q1 (**lower quartile**); and the lower whisker represents the minimum value. Significance levels are: ** $p < 0.01$.

It was no difference in the IL-6, IL-1ra, and MMP2 serum concentration between the pre- and post-exercise samples, fold change, as well as between the training and race effort or between the beginner and more advanced groups (Figures 2a–c, 3a–c and 4a–c). The basal IL-13 was increased in the more advanced group ($p < 0.05$) (Figure 5a), whereas there were no changes between the training and race effort between the beginner and more advanced groups (Figure 5b,c).

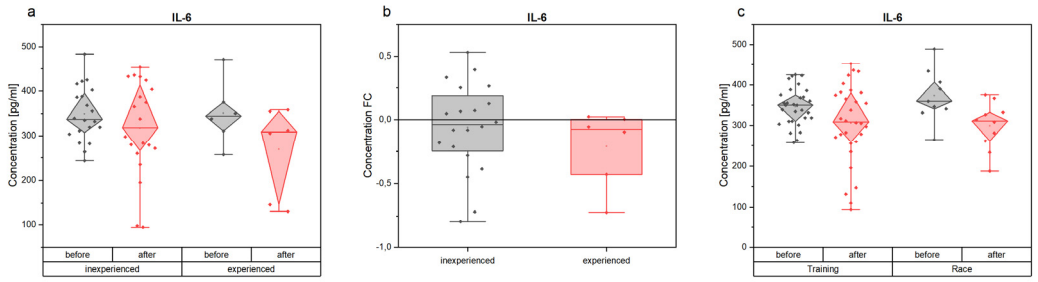

Figure 2. The concentration of IL-6 [pg/mL] before and after exercise for both experienced groups (**a**), the fold change in response to inexperienced and experienced groups (**b**), and the concentration change before and after two types of activity: training and race (**c**). In the box plots, the upper whisker represents the maximum value; the upper line Q3 (**upper quartile**); the center line the median; the lower line Q1 (**lower quartile**); and the lower whisker represents the minimum value.

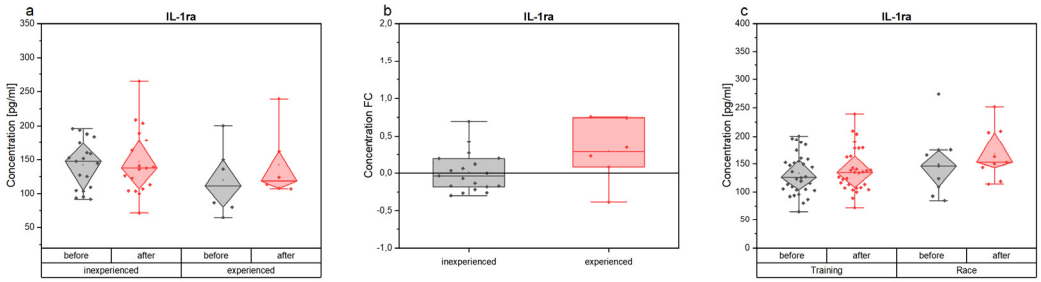

Figure 3. The concentration of IL-1ra [pg/mL] before and after exercise for both experienced groups (**a**), the fold change in response to inexperienced and experienced groups (**b**), and the concentration change before and after two types of activity: training and race (**c**). In the box plots, the upper whisker represents the maximum value; the upper line Q3 (**upper quartile**); the center line the median; the lower line Q1 (**lower quartile**); and the lower whisker represents the minimum value.

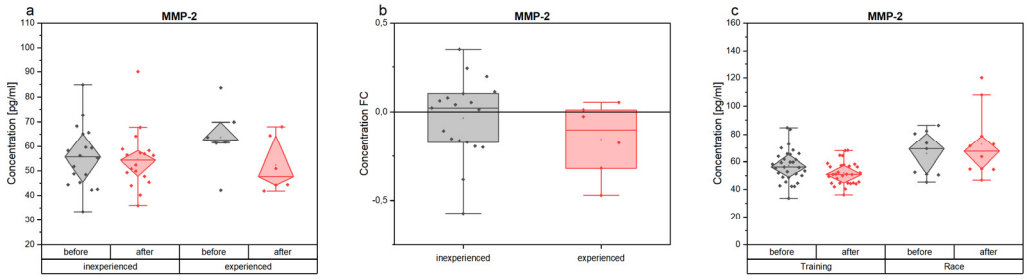

Figure 4. The concentration of MMP-2 [pg/mL] before and after exercise for both experienced groups (**a**), the fold change in response to inexperienced and experienced groups (**b**), and the concentration change before and after two types of activity: training and race (**c**). In the box plots, the upper whisker represents the maximum value; the upper line Q3 (**upper quartile**); the center line the median; the lower line Q1 (**lower quartile**); and the lower whisker represents the minimum value.

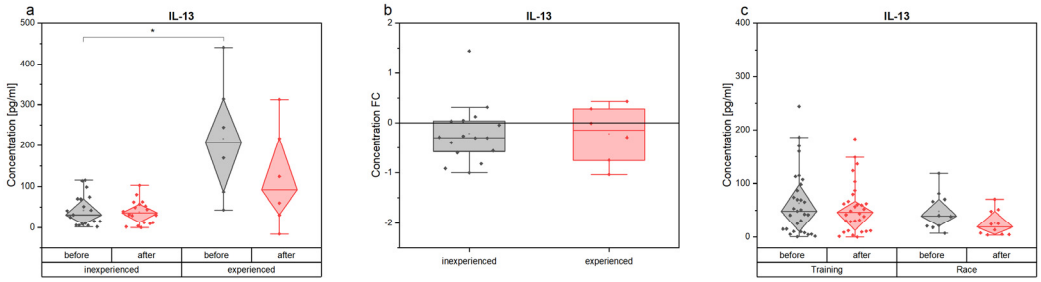

Figure 5. The concentration of IL-13 [pg/mL] before and after exercise for both experienced groups (**a**), the fold change in response to inexperienced and experienced groups (**b**), and the concentration change before and after two types of activity: training and race (**c**). In the box plots, the upper whisker represents the maximum value; the upper line Q3 (**upper quartile**); the center line the median; the lower line Q1 (**lower quartile**); and the lower whisker represents the minimum value. Significance levels are: * $p < 0.05$.

4. Discussion

Consequently, there were no changes in the post-exercise concentration on the studied parameters in our investigation. Even acute orthopedic injury, such as intra-articular lipopolysaccharide (LPS) injection, in synovitis model studies does not change the cytokine gene expression until 4–6 h post-injection [12]. As well, systemic LPS administration has been shown to not result in a significant increase in the pro-inflammatory cytokines mRNA expression until 2 h post-injection [13]. In addition, it was demonstrated that such changes do not occur shortly after exercise [7,14]. Thus, basal values seem to be more useful in helping evaluating horses' performance [5]. The choice between using the basal (pre-exercise) values and post-exercise values of biomarkers for fitness monitoring depends on the specific goals, context, and the biomarkers in question [15]. There are advantages and limitations to each approach. Some biomarkers, like resting heart rate, are more relevant to basal measurements, while others, like lactate levels or heart rate recovery, are more pertinent to post-exercise measurements [15]. Ultimately, an effective fitness monitoring program may include a combination of both basal and post-exercise measurements. Basal values provide a reference point for overall health and stability, while post-exercise values offer insights into acute performance, recovery, and training adaptations. Thus, the specific parameters and monitoring approach should align with the goals and needs of the individual or the fitness program. In our work, we measured the changes in the cytokines blood concentration before and 30 min after exercise based on the routine hematological protocols used in racehorses' fitness monitoring. We are aware that additional blood sampling time points might also be interesting to measure. However, the examination protocol needs to be implemented in everyday clinical practice. Thus,

in our preliminary study, we decided to check the values of the pre- and post-exercise measurements of these cytokines in racehorses' health status evaluation.

The main functions of IGF1 during exercise are the stimulation of muscle growth by muscle protein synthesis promotion or the activation of satellite cells [16,17]. In addition, IGF1 shares similarities with insulin and exerts insulin-like effects on glucose and amino acid uptake and metabolism in muscle cells. IGF1 promotes tissue healing and repair processes, including cell proliferation, collagen synthesis, and angiogenesis, as well as plays a crucial role in the regeneration and remodeling of musculoskeletal tissues, such as bone, cartilage, tendons, and ligaments [18]. In humans, as well as in horses, reduced level of IGF1 or impaired IGF1 signaling have been associated with tendon/ligament pathologies and compromised healing [19,20]. Also, some studies have examined the role of IGF1 in bone development, mineralization, and the repair of fractures [21]. Potential implications of understanding IGF1's involvement in exercise for optimizing training strategies and promoting overall health and well-being in humans and animals exist [22], as well as the potential clinical applications of IGF1 in exercise-related therapies, such as muscle wasting conditions or injury rehabilitation. In our study, we confirmed that the basal level of IGF1 was increased in a more advanced group of horses. In one study, the IGF1 expression was increased in catastrophically injured horses [6]. Another study suggested that IGF1 mRNA expression may act as a negative acute phase marker, which is in line with our results [12]. Thus, further investigation into the mechanisms underlying IGF1's effects on exercise and optimizing exercise protocols to maximize IGF1 responses is needed not only in horses but also in humans. However, we confirmed for the first time that pre-exercise measurements of its concentration may be a useful additional parameter for evaluating horses' performance.

Matrix metalloproteinase 2 (MMP2), also known as gelatinase A, is an enzyme that plays a crucial role in tissue remodeling and repair. While MMP2 is primarily associated with its involvement in pathological conditions such as cancer metastasis and cardiovascular diseases [23,24], recent research has also suggested its role in exercise [25,26]. During exercise, MMP2 is believed to be involved in the adaptation and remodeling of skeletal muscle in humans [27]. The main functions of MMP2 in exercise are extracellular matrix remodeling, angiogenesis, satellite cell activation, or collagen turnover [26]. It is important to note that MMP2 is generally considered beneficial in exercise-induced tissue remodeling [28], whereas excessive or dysregulated MMP2 activity can lead to tissue damage and impair recovery, which is especially visible during aging [29]. The balance between MMP2 activation and inhibition is crucial for maintaining tissue integrity during exercise. In a previous study, the MMP2 expression in injured horses was significantly elevated [5]. The authors suggested its possible chronic role in bone fractures formation, considering that these injuries are often associated with areas of pre-existing pathology. In our study, we did not observe any changes in the MMP2 serum concentration. However, we examined healthy horses; thus, the increased activity of MMP2 may only occur in animals with cartilage homeostasis distribution as well as those with overtraining syndrome. In this context, an evaluation of the MMP2 changes in overtrained horses may be useful for the prevention of fatigue injuries.

IL-1ra is a naturally occurring protein that acts as a competitive inhibitor of interleukin-1 (IL-1), a pro-inflammatory cytokine. IL-1ra plays an important role in modulating the inflammatory response in various physiological processes, including exercise. By inhibiting IL-1 signaling, IL-1ra can attenuate the production of pro-inflammatory cytokines and limit the extent of inflammation associated with exercise-induced muscle damage. Individual variability in the IL-1ra response to exercise must be acknowledged, and factors such as training status, age, heredity, and underlying health conditions can all influence IL-1ra levels and their impact on the inflammatory response. However, the available research on the IL-1ra response to exercise in horses is limited [5,9,30], and additional studies are needed to provide more specific and detailed information. In recent study, it was confirmed that IL-1ra increases after a 120 km endurance ride in Arabian horses, as well as in well-trained

Thoroughbred racehorses [9,30]. The obtained results may propose anti-inflammatory IL-1ra properties, as proteins intend to limit the extent of post-exercise inflammation [31]. Moreover, another study [5] showed a significantly lower expression of IL1RN (the gene responsible for encoding the protein IL-1ra) in horses with considerable injuries compared to non-injured animals. Thus, decreased IL1RN expression may indicate the occurrence of a pro-inflammatory physiology reaction, along with potentially decreased levels of IL-1ra, as an anti-inflammatory protein. The lower expression of IL1RN was especially visible in horses with proximal sesamoid bone (PSB) fractures, which may suggest the potential usage of this gene for the identification of horses specifically exposed to a PSB fracture. Nevertheless, as we underlined in the Introduction, the evaluation of mRNA expression seems to be a less effective compared to the examination of protein level. In our study, there was no difference in the IL-1ra serum concentration between horses with a different training status (inexperienced vs. experienced group), pre- and post-exercise samples, fold change, as well as between types of physical effort (race vs. training). However, only healthy horses were examined; therefore, eventual decreases in the IL-1ra serum concentration related to injuries could not be detected. Moreover, none of the studied animals participated in long-distance races (120 km), as opposed to those examined by Plisak et al. in 2022 [9]. Additionally, all horses included in this study were young adults (2–5 years old), whereas in Plisak et al.'s study, the endurance horses they studied were older (9–11 years). It was documented that IL-1ra levels increase with age, and this is often associated with a chronic low-grade inflammatory state that can occur with aging, sometimes referred to as "inflammaging" [32,33]. IL-1ra is considered an anti-inflammatory cytokine, and its elevated levels can be a response to counteract the pro-inflammatory effects of other cytokines. Thus, there is a potential usage of IL-1ra in preventing injury in endurance horses, but this is not necessary in racehorses.

Another novel anti-inflammatory marker evaluated by us was IL-13. Elevated levels of IL-13 serum concentration have been reported, especially following aerobic exercise, in humans [8]. The authors obtained results on the effect of exercise-induced inflammation on the promotion of tissue repair, remodeling, and improvement in endurance capacity. In a very recent study, this anti-inflammatory cytokine as also proposed as one of the markers for equine exercise monitoring [9]. A comparison of IL-13 changes in serum concentration during aerobic (endurance) and anaerobic (race) exercise in horses at different fitness levels in comparison to well-known anti-inflammatory cytokine interleukin 10 was conducted in the previously mentioned study. The results showed an increased level of IL-13 serum concentration after 100 km (endurance horses) and in a more advanced group after anaerobic (race) exercise. Additionally, increased levels of the IL-13 basal serum concentration in untrained racehorses decreased after exercise, whereas an increase in IL-13 after exercise occurred in experienced racehorses [9]. In the present study, the only significant changes were observed in the basal IL-13 serum concentration, which was increased in the more advanced group. Likewise, no changes between training and race effort between the beginner and more advanced groups were detected. These inconsistencies in the obtained results compared with our previous study may relate to the breeds of the different horses. In the previously mentioned study, only Thoroughbreds were included in the racehorses group, whereas in this study, Arabians were mostly exanimated. It is worth mentioned that Arabian horses differ to Thoroughbreds at the anatomical and physiological levels [7,34,35]. However, during this study, we did not notice any difference between those two breeds; thus, we evaluated them together. The exact cause of the differences in the obtained results needs further examination. It should be noted that the effects of varying exercise intensities, durations, and other environmental factors influencing immune responses could also impact the results.

The limitations of this study are the relatively low sample size, especially of the advanced group of horses (n = 6), as well as the low number of Thoroughbreds (n = 8) in the study. It is well known that the differences between Arabians and Thoroughbreds exist, such as breed origins, physical characteristics (size, body conformation, gait characteristic,

and genetic purity), or metabolic efficiency [36]. However, in our opinion, they are more likely linked with breeding lines which are used for show. A recent multinational study concluded that most racing Arabians range from a few percent Thoroughbred to over 60% [37]. In our study, we did not include showline Arabians. Thus, in our opinion, there are slight differences between these two breeds. On the other hand, the diversity of the used horse breeds has strengthened the value of this study because, nowadays, Arabian racehorses are becoming more popular every year.

Another limitation is the short post-exercise interval of the blood sampling. However, the samples were obtained during standard veterinary procedures, and additional sampling was not possible. On the other hand, in our previous study, changes in the measured cytokines were obvious after 30 mins of exercise [9]. We are aware that in the later time points, the changes may be more expressed. However, because this is a preliminary study, we decided to adapt it to the standard fitness measuring protocol in order to choose the proteins used for further examination in later studies.

5. Conclusions

The importance of understanding the factors that contribute to overtraining and musculoskeletal injuries in horses should be highlighted. In the present study, we confirmed that basal serum concentrations of IL-13 and IGF1 were increased in more advanced horses. These findings may indicate that basal cytokines values may be useful in predicting horses' readiness to race. This is convincing, especially when the examination protocol needs to be implemented in everyday clinical practice, not for research only. However, comparing all studies investigating various blood biomarkers' responses to exercise seems to be difficult due to the differences in the experimental design and examined animals. Thus, more studies are required to determine the exact effect of proposed blood biomarkers, since there are still numerous differences among the published data.

Author Contributions: Conceptualization, O.W.-P.; methodology, O.W.-P.; formal analysis, J.G., I.D. and O.W.-P.; investigation, J.G., I.D. and O.W.-P.; data curation, J.G. and I.D., writing—original draft preparation, P.K. and O.W.-P.; writing—review and editing, O.W.-P., J.G., I.D. and P.K.; visualization, I.D.; supervision, O.W.-P.; project administration, O.W.-P.; funding acquisition, O.W.-P.; resources O.W.-P. and M.P. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Samples collected from horses were a part of standard veterinary diagnostic procedures according to Polish legal regulations (art 1.2 (5) Ust. z dnia 15 stycznia 2015 r. o ochronie zwierzat wykorzystywanych do celów naukowych lub edukacyjnych, Dz.U.2018.0.1207 (Resolution on the animal's protection used for scientific and educational purposes); the European directive EU/2010/63 approval of the Local Commission for Ethics in Animal Experiments was not required.

Data Availability Statement: The data presented in this study are available on reasonable request from the corresponding author as well as on the website https://doi.org/10.18150/HZFODF, RePOD, V1.1.

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Conflicts of Interest: The authors declare no conflict of interest.

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