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A Comparison of Elevated Blood Parameter Values in a Population of Thoroughbred Racehorses

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ABSTRACT

In racing Thoroughbred horses, blood cell counts and key biochemistry parameters are used to monitor horse health during training. The most common measure is total white blood cell (WBC) count, usually coupled with estimates of the relative abundance of the five main types of WBC. However, WBC can go down and up when challenged, making interpretation difficult. In contrast, a large majority of health issues that impact training should trigger an inflammatory response. In this study, we test the potential for two inflammatory biomarkers, fibrinogen and serum amyloid A (SAA), to provide more reliable indicators of health issues across a large sample of horses in training. We find that although WBC and other cell counts are generally correlated with each other and other biochemistry parameters across their full range of values, fibrinogen and SAA exhibit the greatest concordance among the top 15% of values. Moreover, horses with the top WBC values do not overlap significantly with those having the top fibrinogen and SAA values. Because most horses are healthy, these patterns suggest that natural variation in cell counts and biochemistry largely occlude values that might indicate health issues. In contrast, the subset of unusual horses with elevated levels of both fibrinogen and SAA are strongly suggestive of the expected handful of animals with minor, undetected issues. We conclude that fibrinogen and SAA have excellent potential as biomarkers and are likely to be more informative about conditions relevant to horses in training compared with the widely used WBC.

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1. Introduction

Racing Thoroughbred horses have been selectively bred to produce optimal performances of speed and endurance on the racetrack. To achieve athletic excellence, the horse must undergo a rigorous exercise program. Just as human athletes strive to find the right balance between training hard enough to maximize performance but not so hard that stress induces either injury or a compromised immune system, so too with the horse trainers [1]. Because clinical symptoms in horses may only appear when overstressing has already occurred, methods to determine imminent problems at subclinical stages are at a premium.

Current methods of detecting when health is becoming compromised focus on blood biomarkers. Of three current measures, red blood cell (RBC) counts, white blood cell (WBC) counts, and blood biochemistry, the most commonly used is total WBC count, usually coupled with estimates of the relative abundance of the five main types of WBC—the neutrophils (Neut), lymphocytes (Lymph), monocytes (Mono), eosinophils (Eosin), and basophils (Baso). White blood cell counts can change rapidly in response to adverse health, but the changes tend to be transient and to differ depending on the stimuli. For example, the total WBC count may decrease to below normal in response to acute inflammation or virus attack, but may increase in response

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to prolonged inflammation or bacterial infection [2]. Similarly, Neut, which normally make up 60% of the total WBCs, may decrease quickly in response to acute stress but increase quickly when fighting acute infection [3]. Nonetheless, Neut and Lymph counts can be used to diagnose airway inflammation disease and recurrent airway obstruction [4] using bronchoalveolar lavage.

Although the various WBC counts have the potential to indicate a range of common conditions, there are a number of important issues. First, and most importantly, changes in WBC numbers can occur for reasons other than disease or injury, such as being agitated at the time of blood collection. Second, base levels are rather variable, with younger Thoroughbreds in particular differing greatly in their WBC counts from 1 week to another without any evidence of infection or inflammation [5]. Third, the fact that cell number can go down and up may cloud the interpretation of tests where multiple opposing stimuli are present. For these reasons, trainers often treat WBCs with skepticism as being too difficult to understand and too variable to provide a reliable indicator of a horse's overall health profile.

A more reliable tool should aim to reflect specifically the changes in blood biochemistry that occur at the onset of stress. When an animal suffers tissue injury, acute phase proteins are produced in the liver and released into the bloodstream, and the result is localized inflammation. Similar responses are noted for a wide range of conditions including trauma, arthritis, surgery or bacterial, viral, and parasitic infection [6–8], indicating that the acute phase response is generic and may be mounted to any form of tissue damage. Acute phase proteins thus appear a logical target for an improved test for stress-related injury during training. Two promising candidate proteins are fibrinogen, which has been the most commonly measured acute phase protein for some time, and serum amyloid A (SAA), which is becoming increasingly popular as a diagnostic of acute infection.

Fibrinogen is a plasma glycoprotein synthesized by the liver and is converted by thrombin into fibrin during blood coagulation. Fibrinogen is normally present between 2–4 mg/mL, but this rises after inflammation regardless of the cause. Indeed, fibrinogen may be the sole indicator of inflammation [9–11]. Elevated levels of fibrinogen may indicate chronic inflammation or reflect the progression of an infection [12]. Novel inflammation causes the level of fibrinogen to increase above normal within 24–48 hours and in proportion to the degree of inflammation and remains elevated for up to 10 days [13]. This relatively rapid response means that fibrinogen elevation may occur before clinical symptoms of illness [14,15].

Serum amyloid A is a second acute phase protein that is also produced in the liver. Normal levels in healthy horses are very low, but increase rapidly to peak 24–48 hours after infection [16]. Circulating SAA concentrations may increase up to 100-fold in response to an infection [13], but it disappears rapidly after the infection has abated [17], making it an excellent "real time" diagnostic tool for tracking progression and recovery. Previous studies have shown that elevated SAA may also be used for detecting the presence of inflammatory disease of the airways [6], gut, [18] and musculoskeletal system [7,19]. As with fibrinogen, the severity of the inflammation is reflected in the degree of elevation of SAA.

The purpose of the present study is to investigate the relationship between classic WBC counts and the two indicators of an inflammatory response, fibrinogen and SAA, across a large sample of Thoroughbred horses in training. We find evidence that WBC, fibrinogen, and SAA capture different aspects of a horse's physiology. White blood cell counts fluctuate across a rather narrow range and correlate well with parallel changes in many elements of blood chemistry, suggesting that they track normal homeostatic fluctuation. In contrast, fibrinogen and SAA tend to vary little except in a small subset of horses where both markers tend to show markedly elevated levels.

2. Materials and methods

A population of Thoroughbred horses bred for flat racing were screened at two random dates, once at the beginning of the racing season (May 01 and 02, 2012; $n = 105$) and once at the end of the racing season (September 02 and 03, 2012; $n = 118$). The horses were a random mixture of males and females, a mixture of grades, ranging in age from 2- to 5-year-old and had raced a maximum of five times each. All horses are managed in the same way with individual boxes, photoperiod of 4:30 AM to 9 PM, a natural indoor temperature (18°C–20°C), and the same feeding and training schedules. The horses underwent one workout of approximately 20–30 minutes per day between the hours of 6 AM and 10 AM. The horses were allowed to rest for a period of 4–7 hours after exercise before blood draw. Detailed veterinary analysis of each horse immediately after sampling would be desirable, but was beyond the scope of the present study. The horses names, existing injuries, illnesses, and medications were not recorded; however, it was noted by the veterinarian that all horses were fit for work. A large degree of overlap between the two sets of horses tested is expected. The complete blood count consists of the red cell series (RBC count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and platelets) and the white cell series (total WBCs, Neut, Lymph, Mono, Eosin, and Baso). The red series and the WBCs were assayed using a calibrated Advia 2120 (Abbott) analyzer.

In addition to cell counts, we also monitored a range of blood chemistry components: fibrinogen, SAA, creatine kinase, aspartate amino transferase, urea, creatine, total protein (TotP), glutamate dehydrogenase (GLDH), γ-glutamyl transaminase, alkaline phosphatase (ALP), lactose dehydrogenase, globulin (Glob), and albumin. The fibrinogen was measured using a calibrated ACL Elite analyzer from Instrumentation Laboratory. Serum amyloid A was measured using a calibrated Konelab 20 instrument from Thermo Scientific with the "Eiken" SAA test reagents supplied by Mast Diagnostic Ltd. The Eiken assay is a human immunoturbido metric method, which has been previously validated in horses [20]. According to the manufacturer, the range of the test is 5–500 μg/mL with a coefficient of variation of <10% and an accuracy of 85%–115% when a known concentration is measured. The measurement of 57

samples reported a correlation coefficient (r) as $r = 0.981$ and the regression line as $y = 0.971x + 2$ [21].

All tests were performed by the suitably qualified in-house laboratory technician. To minimize the impact of circadian fluctuations and to allow for horses to return to the resting state, blood was drawn between 2 PM and 3 PM according to in-house procedures and veterinary recommendation by the in-house vet. The blood was drawn into blood tubes appropriate for the parameters to be tested. The results for each of the parameters under analysis in this study for each of the 223 horses were compiled and analyzed using Microsoft excel.

3. Results

The three primary measures obtainable from blood, which we were most interested in, were the classical total WBC count and two proteins associated with the inflammatory response, fibrinogen and SAA. We began by asking whether, across the entire range of observed values, there was a general tendency for high and low values in one measure to be associated with high and low values in another. Because several of the trait value distributions were strongly not normal, we used nonparametric rank correlation tests rather than a standard Pearson correlation.

Rank correlations between our three primary measures and all other traits are presented in Table 1. Among the three primary measures, the two indicators of inflammation correlate positively and highly significantly with each other, but there is no association between either of these and WBC. As expected, WBC counts are positively correlated with many of the other subclasses of blood cell counts, particularly Neut, Lymph, and RBCs. Among the blood chemistry measures, WBC is associated with GLDH and ALP, whereas both the inflammation proteins correlate with TotP and Glob, but also exhibit weak correlations with several others. Red blood cells are interesting, because they correlate positively with WBC and SAA but negatively with fibrinogen.

From the point of view of diagnosing imminent health issues, weak correlations between two or more measures across all horses may or may not be biologically relevant. For example, mild dehydration might result in transiently higher protein concentrations across many and/or most molecules, and this could drive correlations even across a sample of equally healthy animals. More clinically relevant, therefore is the tendency for measures to show concordance when levels have risen outside what might be considered the “normal” range of values. We first explored published tables giving the “normal ranges” for different classes of horse (e.g. “Thoroughbreds” or “2-year-olds in training”), but several traits in these systems were in contradiction with one another and routinely yielded values outside the expected ranges depending on which reference method was applied. Consequently, we turned to a more unbiased approach. We arbitrarily assumed that the highest 15% of observed values for each parameter were “elevated” and used simple chi-squared tests to ask whether these elevated values at our three focal variables tended to be associated with elevated values in each of the other traits. Fifteen percent was chosen as a balance between a lower fraction that would have too little statistical power and a higher fraction that might be deemed unrealistic. This method, therefore, bypasses the need to predefine “normal” and “abnormal”.

Table 1
Correlation between values in diverse blood assays in 224 Thoroughbred racehorses

Parameter	Fibrinogen	SAA	WBC
SAA	$1.1 \times 10^{-09**}$		
WBC	.95	.21	
Neut	.92	.41	$3.8 \times 10^{-28**}$
Lymph	.005*	.01*	$3.0 \times 10^{-08**}$
Mono	.08	.09	$1.3 \times 10^{-04**}$
Eosin	.76	.49	.28
Plt	.03*	.39	.01*
Baso	.02*	.03*	.41
RBC	.02*	$2.69 \times 10^{-03*}$	$7.8 \times 10^{-07**}$
Hgb	.45	.03*	$3.1 \times 10^{-05**}$
Hct	.46	.03*	$1.1 \times 10^{-04**}$
TotP	$3.9 \times 10^{-05**}$	.02*	.10
Creat	.31	.17	.38
Urea	.02*	.45	.26
GGT	.09	.82	.27
AST	.33	.02*	.03*
CK	.009*	.03*	.13
LDH	.04*	.04*	.09
GLDH	.06	.42	$7.5 \times 10^{-05**}$
ALP	.10	.09	$1.1 \times 10^{-12**}$
ALB	.50	.18	.41
Glob	$2.2 \times 10^{-07**}$	$2.5 \times 10^{-03*}$	.18

ALB, albumin; ALP, alkaline phosphatase; AST, aspartate amino transferase; Baso, basophils; CK, creatine kinase; Creat, creatine; Eosin, eosinophils; GGT, γ-glutamyl transaminase; GLDH, glutamate dehydrogenase; Glob, globulin; Hct, hematocrit; Hgb, hemoglobin; LDH, lactose dehydrogenase; Lymph, lymphocytes; Mono, monocytes; Neut, neutrophils; Plt, platelets; RBC, red blood cell; SAA, serum amyloid A; TotP, total protein; WBC, white blood cell.
Two proteins associated with the inflammatory response, SAA and fibrinogen, and total WBC count are compared against each other and against 20 other cell count per protein assay. In each case, a nonparametric Spearman rank correlation is performed. Values presented are the resulting P values.
*Values significant at $P < .05$ are indicated.
**Values significant experiment-wide are indicated.

The chi-squared tests for concordance of high values are summarized in Table 2. With (overly) stringent full Bonferroni correction for conducting 63 tests, four tests are significant experiment-wide: Fibrinogen versus SAA ($\chi^2 = 43.7$; 1 degree of freedom [df]; $P = 1.4 \times 10^{-11}$), WBC versus Neut ($\chi^2 = 27.9$; 1 df; $P = 1.3 \times 10^{-7}$), WBC versus Lymph ($\chi^2 = 13$; 1 df; $P = 3.2 \times 10^{-4}$), and WBC versus ALP ($\chi^2 = 11.4$; 1 df; $P = 7.5 \times 10^{-4}$). In addition, a number of other combinations yield significance at $P = .05$ uncorrected, noticeably TotP Neut, and Glob, which all show associations with all three of our primary measures. It is reassuring that the strongest association, using both statistical models, by some way is the one between fibrinogen and SAA, the two measures of the inflammatory response. In all cases, the associations are positive, in which the highest values for one trait occur disproportionately frequently with high values at another trait.

4. Discussion

We explored the relationship between a number of standard blood parameters in a sample of Thoroughbred

Table 2
Concordance of occurrence of extreme values among diverse blood assays

Parameter	Fibrinogen	SAA	WBC
SAA	45.7**		
WBC	1.1	4.2*	
Neut	6.1*	7.2*	27.9**
Lymph	2.9	1.8	13.0**
Mono	2.1	1.4	6.2*
Eosin	0	0	0
Plt	0	0	0.5
Baso	4.4*	1.3	0
RBC	2.9	0.7	0.9
Hgb	3.4	1.0	0.5
Hct	1.7	1.0	0
TotP	3.9*	5.6*	8.7*
Creat	0.4	0.9	0.8
Urea	5.0*	0	0
GGT	0.4	0.1	0.1
AST	0.5	2.5	0.2
CK	0	0.1	0
LDH	2.3	2.1	0.5
GLDH	.2	6.5*	1.5
ALP	2.3	1.9	11.4**
ALB	1.1	0.9	1.2
Glob	3.9*	9.4*	8.7*

ALB, albumin; ALP, alkaline phosphatase; AST, aspartate amino transferase; Baso, basophils; CK, creatine kinase; Creat, creatine; Eosin, eosinophils; GGT, γ-glutamyl transaminase; GLDH, glutamate dehydrogenase; Glob, globulin; Hct, hematocrit; Hgb, hemoglobin; LDH, lactose dehydrogenase; Lymph, lymphocytes; Mono, monocytes; Neut, neutrophils; Plt, platelets; RBC, red blood cell; SAA, serum amyloid A; TotP, total protein; WBC, white blood cell.
Two proteins associated with the inflammatory response, SAA and fibrinogen, and total WBC count are compared against each other and against 20 other cell count per protein assay. In each case, a simple 2×2 test of homogeneity is conducted to test for an association between the top 15% of values observed. Values presented are Chi-squared statistics interpreted with 1 degree of freedom.
*Values significant at $P < .05$ are indicated.
**Values significant experiment-wide are indicated.

racehorses in training. Our data reveal that although the most commonly used indicator of health, total WBC count, correlates broadly with both individual cell subtype counts and several elements of blood chemistry, there is relatively poor agreement between horses with the highest WBC counts and the highest values in other measures such as the inflammatory markers, SAA and fibrinogen. In contrast, two components of the inflammatory response, SAA and fibrinogen, correlate relatively weakly with WBC and blood chemistry, but show excellent agreement with one another when it comes to high values. Furthermore, by application of two separate statistical models of analysis, similar trends can be observed demonstrating that this study group was indeed a random sample population of Thoroughbred racehorses and may not have been overly influenced by particularly “extreme” individuals.

Both blood chemistry and WBC counts are used routinely as indicators of health; however, readings in healthy horses are far from constant and vary with levels of hydration and other factors. For this reason, measurements are generally conducted in as standardized a way as possible, at the same time of day and the same time relative to feeding and exercise. Nonetheless, variation still seems likely because of factors such as individual-specific patterns in urination, environmental temperature, and anxiety, and this appears to be reflected in the way most of the WBC counts and blood chemistry measures exhibit some degree of cross-correlation.

To understand which part of the range of observed values of a given trait are associated with ill health as opposed to natural daily and hourly variation in homeostasis would involve tracking the fate of horses that were trained at a constant level until clinical symptoms developed. However, such an experiment is largely precluded by the need to act preemptively so as to maximize horse welfare. Instead, therefore, we focused entirely on correlations between the various blood analytes in general (Table 1), comparing these with the level of concordance seen between high value readings for the same measurements (Table 2). In this way, we can see the extent to which different measurements covary across their entire range, a pattern that would suggest correlation with some other factor such as diurnal variation in hydration, as opposed to a specific tendency for high values at one measure to be associated with high values at another, a pattern that tends to identify an unusual subset of horses. We presume that such subsets represent horses with, in this case, an ongoing inflammatory response.

Our argument is that, from experience, a small but unknown subset of our number of horses in training are likely to have incipient health issues. If these horses can be detected, they should be contributing unusually high trait values. Moreover, if two or more traits are useful as indicators, these should show good agreement in their highest values. When we interrogate our data in this way, we find a reversal with WBC showing weaker correlations among the highest 15% of values compared with fibrinogen and SAA. By implication, fibrinogen and SAA show agreement in identifying a subset of horses with unusual readings, most parsimoniously explained by these horses currently suffering some level of injury or illness involving the inflammatory response. The apparent lack of specificity of WBC counts likely reflects the large diversity of factors that can affect them, many of which are not directly related to health.

Our results raise questions both about what WBCs are detecting and what they are expected to detect as a preperformance assay. Cell counts undoubtedly fluctuate in a biologically meaningful way, but there are two complications. First, the correlation between WBC and many of the blood chemistry measures suggests that most of the variation in our sample is because of normal variation in blood concentration rather than specific responses to a particular challenge. Second, the range of stimuli capable of impacting WBC is wide, diverse, and some may even depress cell counts. Consequently, a single WBC is unlikely to tell us much about incipient problems. Better would be a monitoring program based on repeated measures so that sudden changes could be better identified, but even here the meaning of such changes may be difficult.

In comparison with WBC, fibrinogen and SAA appear to have considerably better discriminatory power, both largely agreeing with each other about a subset of horses with clearly elevated readings. The implication is that these horses may have an otherwise undetected health problem. From a diagnostic perspective, this brings both positive and

negative aspects. The negative aspect is that SAA and fibrinogen will not identify horses suffering from problems that are not currently causing an inflammatory response. The positive side is that these two blood proteins, in contrast to WBC, appear to identify a relatively specific state; that of horses exhibiting an inflammatory response.

5. Conclusions

We conclude that fibrinogen and SAA have excellent potential as biomarkers and are likely to be more informative about conditions relevant to horses in training compared with the widely used WBC.

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