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DIAGNOSTIC AND PREDICTIVE SIGNIFICANCE OF ACUTE PHASE RESPONSE AND NEOPTERIN LEVELS IN LAME RACING DROMEDARY CAMELS (*Camelus dromedarius*)

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ABSTRACT

The lameness of racing camels is a major health concern and a financial burden for many camel owners. This study was aimed to investigate different acute phase proteins, cytokines (CYTs), and neopterin (NPT) in the blood of racing lamed camels to highlight their role in disease diagnosis, pathogenesis and monitor treatment response. A 35 out of 315 racing camels exhibited clinical lameness. The mean serum levels of serum amyloid A (SAA) and CYTs (TNF-α, IL-1α, IL-1β, and IL-6) in lame dromedary camels with different detected causes (punctured foot and traumatic injury) were remarkably over than those detected in control healthy dromedary camels. It was found that lame dromedary camels had non-significant changes in serum levels of haptoglobin (HP) and NPT when compared with control healthy camels. A dramatic decline was detected in serum levels of SAA and CYTs of lame camels after 10 days of treatment whereas, the levels of HP and NPT remain at the same levels without any significant changes. The ROC curves were created for the tested biomarkers. The AUCs were calculated to evaluate each variable's accuracy to differentiate diseased from healthy camels. Based on the ROC curves and AUCs, both the SAA and CYTs provide similar and highly accurate diagnostic accuracy (AUC > 0.8) and monitoring of lameness treatment response.

Key words: Camel, cytokines, haptoglobin, lameness, neopterin, serum amyloid A

Lameness in camels can be caused by a variety of factors and extensively diverse causes including direct trauma, nutrition, fractures, punctured feet, abnormal limb conformation, and infection (Gahlot, 2007; Al-Juboori, 2013). Gahlot (2007) diagnosed lameness in camels, clinically manifested as partial or non-weight bearing by one or more limbs, swelling over joints, pain on palpation, toe-out postures, shivering of hind quarters while sitting, semi-flexed hocks in sitting postures, and an asymmetrical pelvis. Camels with lameness suffer substantial economic losses in the form of decreased milk production, decreased reproductive performance, and growth retardation, culling from competition or farms, decreased physiological vitality of the camels and additional treatment and care costs (Al-Juboori, 2010). Compared to cattle and horses, camels have a different pattern of lameness attributed to its peculiar anatomy, biomechanics, geo-climatic adaptation, and use (Gahlot, 2000).

Acute-phase response (APR) is a seditious response of the host in response to tissue damage. APR acts through pro-inflammatory cytokines (CYTs) such as interleukin-6 (IL-6) and acute phase proteins (APPs), confining microbial growth and maintaining homeostasis (Murata *et al,* 2004). During APR, acute phase proteins help the humoral and cell-mediated immunity elements from causing gratuitous damage to the host cells (Rossbacher *et al,* 1999). Because of various diseases, higher levels of bovine and ovine APPs (serum amyloid A; SAA and haptoglobin; HP) have been detected in the blood of diseased animals. These diseases include lower respiratory tract disease (El-Deeb and Elmoslemany, 2016; El-Deeb *et al,* 2020), mastitis (El-Deeb, 2013; El-Deeb *et al,* 2021) and hoof disorders (Ilievska *et al,* 2019; Kontturi *et al,* 2019). Moreover, APR was previously detected in camels with urinary tract infection (El-Deeb and Buczinski, 2015), *Trypanosoma evansi* infection (El-Deeb and Elmoslemany, 2015) and camels infected with *Coxiella burnetii* (El-Deeb *et al,* 2019). There was also evidence of higher cortisol levels, and a higher cortisol to dehydroepiandrosterone ratio in lame cows, which

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has been linked to inflammation (Almeida *et al,* 2008). A common method of testing the degree of inflammatory processes that may result from physical trauma, cardiovascular disorders, cancer, bacterial, parasitic, and viral infections is to measure neopterin concentrations in the urine and blood (Melichar *et al,* 2017).

The study of APR and neopterin (NPT) levels in camels against lameness is poorly understood. Accordingly, this study was aimed to investigate different APPs and NPT in blood of racing lame dromedary camels to highlight their role in disease diagnosis, pathogenesis, and to monitor treatment response.

Materials and Methods

Animal selection and sampling protocol:

A total of 315 racing dromedary camels, aged 5.5-8.1 years, were investigated between January and September 2019 in one herd in the eastern region, Saudi Arabia. Thirty-five of these camels exhibited recent acute clinical lameness. Punctured foot and traumatic hoof injuries were diagnosed as lesions accountable for clinical lameness in these racing camels. Bovine hoof tester and walk on sandy, pebbled, or hard tracks (Gahlot, 2000; 2007) were used to diagnose the camel foot lameness. Moreover, 20 clinically healthy racing dromedary camels with no lameness signs were included in control group. Whole blood samples were collected from the jugular vein of clinically lame dromedary camels (N = 35) and control healthy group (N= 20). Blood samples were centrifuged, serum separated and stored at -20°C for further biochemical analysis. Lame dromedary camels were subjected to treatment protocol including the use of non-steroidal anti-inflammatory drugs (NSAIDs) (2.2mg flunixin per kg body weight, IV injection), cleaning and disinfection of lesions for 5 consecutive days with complete rest.

Acute-phase proteins (APPs): Test kits (Tridelta Development Ltd., Kildare, Ireland) were used to measure haptoglobin (HP) and serum amyloid A (SAA) in serum samples.

Proinflammatory cytokines (CYTs): In order to estimate CYTs concentrations (TNF-α, IL-1α, IL-1β, and IL-6) in serum, commercial ELISAs (MyBioSource, San Diego, USA) were used according to the manufacturer's recommendations.

Neopterin (NPT): Commercial ELISA kit for measuring NPT concentrations in camel serum (Bovine NPT (Neopterin) ELISA Kit, Fine Test, Wuhan Fine Biotech, Wuhan, China) were used.

Data Analysis:

Comparisons in mean were performed by Kruskal–Wallis ANOVA on Ranks followed by Dunn's multiple comparisons. The different means were significant at $P<0.05$. Statistical analysis was performed using JMP software version 11.0.0 (SAS Institute, Cary, NC, USA). Graphpad Prism v5 software (Graphpad Software, Inc., San Diego, CA) was used to draw the figures. The correlation between parameters was evaluated using Spearman's rank correlation test. Each assay's diagnostic accuracy was evaluated by creating the ROC (receiver operator characteristics) curve and determining the area under the curve (AUC). An AUC of 0.7 to 0.9 was considered moderately accurate, an AUC of >0.9 highly accurate, and an AUC of 1 perfect (Gardner and Greiner, 2006).

Results and Discussion

The mean serum levels of SAA and CYTs(TNF-α, IL-1α, IL-1β, and IL-6) in lame dromedary camels with different detected causes (punctured foot, traumatic injury) were remarkably ($P < 0.0001$) over than those detected in control healthy dromedary camels (Fig 1). However, it was found that lame dromedary camels had non-significant changes in serum levels of HP ($P < 0.37$) and NPT($P < 0.75$) when compared with control healthy camels (Fig 1). The serum levels of APPs, NPT, and CYTs markers in lame dromedary camels' pre-and post-treatment was measured in this study. A dramatic decline ($P < 0.0001$) was detected in serum levels of SAA and CYTs of lame camels after 10 days of treatment, whereas the levels of HP ($P < 0.72$) and NPT ($P < 0.82$) remain at the same without any significant changes (Fig 2). Spearman's correlation was estimated for the study biomarkers in clinically lame camels and healthy ones and in lame camels before and after 10-days of treatment (Table 1). A positive significant correlation exists between SAA and all tested CYTs (TNF-α, IL-1α, IL-1β, and IL-6).

The diagnostic test characteristics of APPs and CYTs parameters in camel with lameness were presented in table 2 while the ROC curves were created as presented in Fig 3. The AUCs were assessed to evaluate the accuracy of each variable to distinguish diseased and healthy dromedary camels. Based on the ROC curves and AUCs; SAA ($AUC \geq 0.957$) and CYTs (AUCs ranged from 0.872 for IL-1α to0.927 for IL-1β) were highly diagnostic and predictive for treatment response in lame camels. Conversely, HP and NPT showed a poor diagnostic

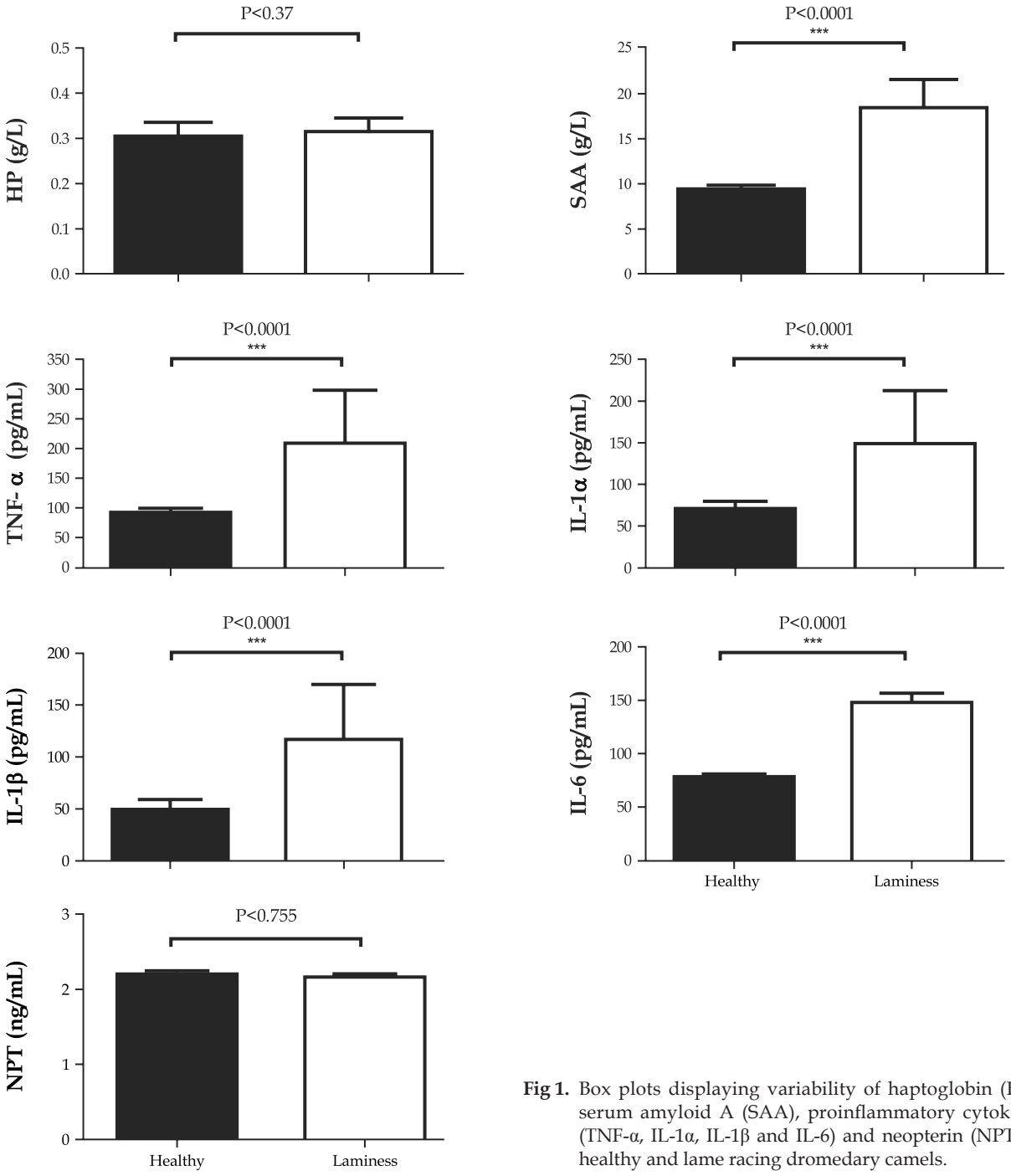

Fig 1. Box plots displaying variability of haptoglobin (HP), serum amyloid A (SAA), proinflammatory cytokines (TNF-α, IL-1α, IL-1β and IL-6) and neopterin (NPT) in healthy and lame racing dromedary camels.

ability to distinguish lame dromedary camels from control healthy ones and to evaluate the treatment response.

APR is a seditious response of the host in response to tissue damage. In this study, The SAA of the lame dromedary camels was the only APPs measured at higher levels than that of the control camels. Our findings are in harmony with other research studies that have also reported elevated SAA values for lame cows as well (Kujala *et al,* 2010; Tóthová *et al,* 2011). The levels of SAA were returned to normal levels 10 days after treatment with non-steroidal anti-inflammatory drugs and other lameness treatment protocols. It was suggested that mere pain may lead to an elevation of SAA concentration in the circulation (Tóthová *et al,* 2011), and this was supported by research work on human SAA (Zhang, 2007). In light of these results, we propose that lameness in camels can cause systemic APR, evident by the higher SAA levels. It was previously reported

that blood SAA concentration is related to the degree of tissue damage (Murata *et al,* 2004).

Monitoring, the healing advancement of lameness in dromedary camels is laborious. However, in present study, the alterations in tested biomarkers were estimated after 10 days of treatment. A marked decline in SAA was reported in lame racing camels after treatment protocol toward normal control values. Consequently, SAA could be used to monitor the healing process, the treatment of lame dromedary camels were evaluated and adjusted in a better way.

Unexpectedly, in this study, HP showed non-significant changes in the serum levels of lame dromedary camels when compared with control ones. The levels of HP in control healthy camels are in harmony with those reported by Nazifi *et al* (2006) and El-Deeb *et al* (2019). In contrast to findings in the present study in racing lame camels, HP has been recognised as a valuable biomarker of ruminant diseases where it offers further data to the classical haematological studies (Skinner *et al,* 1991). HP has been evenly recognised in calves' serum and broncho-alveolar lavage with experimental and clinical pasteurellosis (Katch *et al,* 1999; El-Deeb *et al,* 2020). In the same concern and in contrast to our results, high HP levels were detected at higher serum levels in 60 dairy cows with clinical lameness due to septic pododermatitis, pododermatitis circumscripta, interdigital necrobacillosis, and papillomatous digital dermatitis lesions (Smith *et al,* 2010). The authors concluded that lameness because of claw infections could be allied with a significant systemic APR and higher serum HP levels in cattle. Moreover, the authors mentioned that HP levels seemed effective for all claw disorders except for pododermatitis

Table 1. Correlation matrix among different acute phase proteins (HP and SAA), cytokines and neopterin in healthy and lame dromedary racing camels (20 control and 35 lame camels).

Parameter[a]	HP	SAA	TNF-α	IL-1α	IL-1β	IL-6	NP
HP	1.000						
SAA	0.197	1.000					
TNF-α	0.179	0.375	1.000				
IL-1α	0.008	0.411	0.440	1.000			
IL-1β	0.069	0.572	0.586	0.458	1.000		
IL-6	0.147	0.397	0.667	0.461	0.523	1.000	
NP	0.159	-0.042	-0.045	0.173	-0.063	-0.012	1.000

[a]HP, haptoglobin; SAA, serum amyloid A; TNF-α, tumour necrosis factor alpha; IL-1α; interleukin 1-alpha; IL-1β, interleukin 1-beta; IL-6, interleukin 6; NPT, neopterin.

Table 2. Diagnostic test characteristics of acute phase proteins, cytokines and neopterin in racing dromedary camels with clinical lameness.

Parameters [a]	Threshold	Diagnostic characteristics (%)		
		Se (95% CI)	Sp (95% CI)	AUC
HP	≥ 0.35	0.20 (0.08% to 0.36%)	0.80 (0.56% to 0.94%)	0.569
SAA	≥ 12.75	0.91(0.76% to 0.98%)	1.00 (0.83% to 1.00%)	0.957
TNF-α	≥ 114.80	0.80 (0.63% to 0.91%)	0.85 (0.62% to 0.96%)	0.907
IL-1α	≥ 108.30	0.77 (0.59% to 0.89%)	1.00 (0.83% to 1.0%)	0.872
IL-1β	≥ 88.74	0.74 (0.56% to 0.87%)	0.95 (0.75% to 0.99%)	0.927
IL-6	≥ 115.8	0.82 (0.66% to 0.93%)	0.90 (0.68% to 0.98%)	0.912
NP	≤ 2.70	0.08 (0.01% to 0.23%)	0.95 (0.75% to 0.99%)	0.515

[a] HP, haptoglobin; SAA, serum amyloid A; TNF-α, tumour necrosis factor alpha; IL-1α; interleukin 1-alpha; IL-1β, interleukin 1-beta; IL-6, interleukin 6; NPT, neopterin.

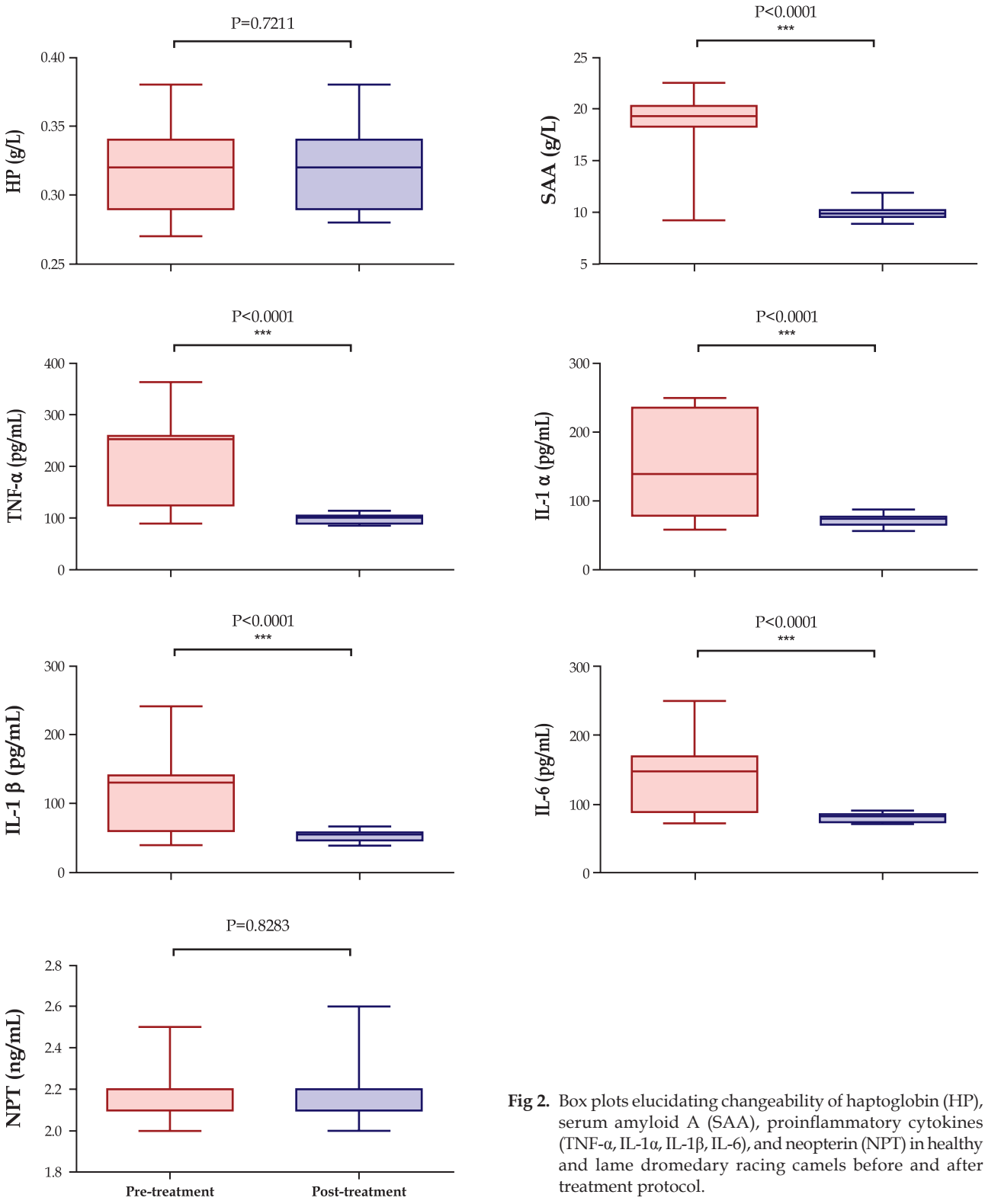

Fig 2. Box plots elucidating changeability of haptoglobin (HP), serum amyloid A (SAA), proinflammatory cytokines (TNF-α, IL-1α, IL-1β, IL-6), and neopterin (NPT) in healthy and lame dromedary racing camels before and after treatment protocol.

circumscripta. In a similar study, significantly higher fibrinogen, HP, and SAA levels in dairy cows suffering from limb diseases as compared to control cows were also reported (Jawor *et al,* 2008). Other studies have also shown SAA and HP as the major positive APPs, which can increase several folds after tissue injury in cattle (Murata *et al,* 2004).

The difference between the present findings and previous research work regarding HP levels might be attributed to the time of sampling and

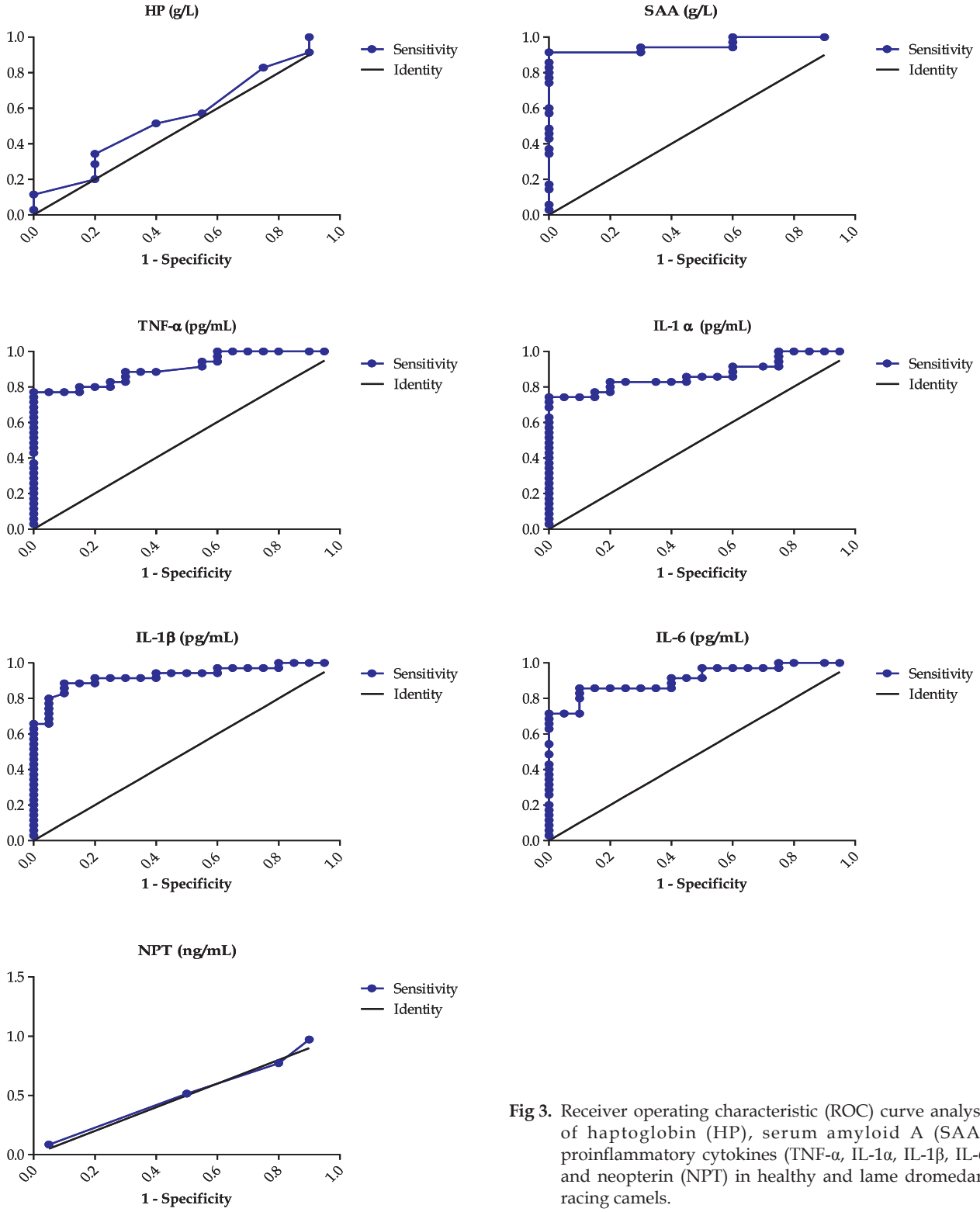

Fig 3. Receiver operating characteristic (ROC) curve analysis of haptoglobin (HP), serum amyloid A (SAA), proinflammatory cytokines (TNF-α, IL-1α, IL-1β, IL-6) and neopterin (NPT) in healthy and lame dromedary racing camels.

the non-infectious nature of lameness in racing camels under investigation (Ossent and Lisher, 1998). Moreover, the APR in camels toward lameness might be different from that of cattle. Consequently, further investigations of APR in lame camels at different time interval are recommended. Comparable to our assumption, Jacobsen *et al* (2004) and Petersen *et al*. (2004) proposed that synthesis of HP and SAA are controlled by dissimilar means. It appears that HP is not a very sensitive biomarker for this type of

lameness in racing dromedary camels. HP is related mainly to bacterial infection and not to non-infectious problems (Skinner *et al,* 1991).

Inflammatory responses in the peripheral and central nervous systems play key role in the development and persistence of many pathological pain states (Watkins *et al,* 2003). Certain inflammatory cytokines in the spinal cord, dorsal root ganglion (DRG), injured nerves or skin are known to be associated with pain behaviours and with the generation of abnormal spontaneous activity from injured nerve fibres or compressed/inflamed DRG neurons. Inflammatory reactions are up-regulated by proinflammatory cytokines, which are produced predominantly by activated macrophages. Numerous studies have demonstrated that CYTs such as IL-1β, IL-6, and TNF-α contribute to pathological pain (Watkins *et al,* 2003).

As evident in the results, the level of TNF-α, IL-1α, IL-1β, and IL-6 increased in the lame group of dromedary racing camels when compared to control camels. The reason behind this might be that CYTs appear to peak in blood in response to an inflammatory process (El-Deeb and Buczinski, 2015; El-Deeb *et al,* 2019; El-Deeb *et al,* 2020). However, following NSAIDs and other treatment protocols, a significant decline in TNF-α, IL-1α, IL-1β, and IL-6 indicates the improvement in lameness in dromedary camels after 10 days of treatment. The NSAIDs perform their action by hindering the production and release of prostaglandins and consequently resulting in decreased pain sensation and inflammatory process (Weissmann *et al,* 1987). Jawor *et al* (2008) assessed the levels of APPs at selected time points throughout the treatment protocol of cows with limb disorders with an aid to guide the treatment success and as a primary predictive biomarker of probable complications. The authors reported that sole ulcer, white line disease, and arthritis, were the most frequently diagnosed problems in cattle. Higher levels HP, SAA, and fibrinogen were reported at the beginning of the treatment protocols as also detected in lame camels in this study. In cattle, in which the treatment protocol went without clinical complications, a high gradual fall in APPs levels was detected. In this study, the fall in SAA and CYTs after treatment ascertained that the selected treatment protocol was appropriate and that it contributed towards decreasing the inflammatory process in lame racing dromedary camels. Based on these findings, lame camels which had higher APPs (SAA) or CYTs values post treatment had not been completely cured, and consequently, treatment process should be sustained.

The NPT marker is part of cell-mediated immunity system derived from monocyte/macrophages during the inflammatory conditions. In urine, serum, or plasma, NPT can be easily quantified (Rokos *et al,* 1985). It is detected before specific antibodies are formed, and often detected before disease states are fully manifested (Werner *et al,* 1990). Based on its ease of quantification and biochemical aspects in relation to the activation of the cellular immune system, NPT has gained increased use in human medicine as a sensitive and specific biomarker of disease activity in clinical situations including neoplasia (Weiss *et al,* 1993), autoimmune disease, viral, bacterial and parasitic infections (Facer, 1995), Crohn's disease (Judmaier *et al,* 1993), and HIV-1 (Fahey *et al,* 1990). In this study, the NPT levels did not altered in lame racing camels when compared to control healthy camels is also comparable to those detected in llamas (Stang and Koller, 1998). The normal levels of NPT in lame camels may be attributed to these injuries may not be allied with any detectable Th1-immune reaction. The AUCs were assessed for APPs, CYTs, and NPT to evaluate the diagnostic accuracy of each variable to distinguish lame and healthy camels. Based on the ROC curves and AUCs, SAA and CYTs provide highly accurate diagnostic accuracy and monitoring of treatment response for lame racing dromedary camels which are in agreement with the diagnostic accuracy guidelines (Gardner and Greiner, 2006).

According to this study, lame dromedary racing camels were linked to significant alterations in SAA and CYTs (TNF-α, IL-1α, IL-1β, and IL-6) indicators with non-significant changes in HP and NPT levels. Likewise, this study found that lame racing camels have higher levels of SAA and CYTs (TNF-α, IL-1α, IL-1β, and IL-6) biomarkers than healthy camels. It is concluded that in addition to clinical examination of lame camels, SAA and CYTs levels could be a useful diagnostic and predictive tool for lameness in racing dromedary camels.

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