Aus der
Klinik für Pferde, Allgemeine Chirurgie und Radiologie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Evaluation of the diagnostic value of asymmetric dimethylarginine for use as cardiac biomarker in horses and its assessment in endurance horses competing at 160 km

Inaugural-Dissertation
zur Erlangung des Grades eines
Doctor of Philosophy (PhD)
in Biomedical Sciences
an der
Freien Universität Berlin

vorgelegt von
Dr. Antonia Ertelt
Tierärztin aus Luckenwalde

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List of abbreviations

ADMA	asymmetric dimethylarginine
ANP	atrial natriuretic peptide
BNP	brain natriuretic peptide
cTNI	cardiac troponin I
ELISA	enzyme-linked immunosorbent assay
LC-MS/MS	liquid chromatography triple quadrupole mass spectrometry
NO	nitric oxid
NT-pro ANP	NT pro atrial natriuretic peptide
SDMA	symmetric dimethylarginine

1. Introduction

In veterinary medicine, blood parameters reflecting cardiac dysfunction in equines are lacking. In the past, different muscle enzymes (creatine kinase, aspartate aminotransferase), structural proteins (cardiac troponin I and T) and hormones (atrial natriuretic peptide) were used to assess cardiac disease in horses. However, they have not proven useful in diagnosing heart diseases in this species. This might be explained by the fact that most cardiac diseases in horses do not cause significant cardiomyocyte damage or necrosis. To evaluate heart disease that does not induce cardiomyocyte damage, a functional biomarker that increases as atrial and ventricular remodelling occurs is needed (Jesty 2012).

In human medicine, the amino acid derivative asymmetric dimethylarginine (ADMA) has been assessed and found useful to detect heart disease early and reliably (Kubes et al. 1991, Böger et al. 1995, Tsao et al. 1996, Wolf et al. 1997, Böger et al. 2000, Jiang et al. 2006, Ardigo et al. 2007, Bai et al. 2013,). Furthermore, ADMA has also predictive properties and can be used as a risk marker for future cardiovascular events and total mortality (Schlesinger et al. 2016).

However, human cardiac diseases are etiologically different from equine diseases. The most widespread cardiac disease in human medicine is ischemic heart disease, also referred to as coronary artery disease and atherosclerotic cardiovascular disease. In equine medicine, structural heart disease due to valvular insufficiency is common (Reef et al. 2014). Nevertheless, in both humans and horses, heart disease can result in progressive chamber remodeling and dysfunction as well as in the development of pulmonary hypertension and congestive heart failure.

Another commonality between humans and equines in the field of heart diseases is atrial fibrillation. Atrial fibrillation is prevalent in elderly humans, and it is the most common arrhythmia in horses (Reef et al. 2014). A study has shown that ADMA increases in humans with permanent atrial fibrillation (Horowitz et al. 2018). Atrial fibrillation has a huge impact on performance in racehorses and cardiac arrhythmias appear to be one reason for sudden death in sport horses and human athletes.

Animal welfare is becoming more important in equine sports, especially in endurance racing. The physical load for the cardiovascular system in a 160 km race is tremendous and fatal incidences can occur (Holbrook et al. 2006, Hewing et al. 2015, Flethøj et al. 2016). The cardiac biomarker troponin I as well as functional indices in echocardiography have been assessed in endurance horses racing 160 km and showed that cardiac stress occurred (Foreman et al. 1998, Flethøj et al.2016).

In an endurance race, horses must pass a series of veterinary inspections and examinations in the interest of the health, safety, and welfare of the horse. Only competitors, whose horses pass all the checkpoints, can proceed in the race. However, it is not always straightforward to decide, which horse is fit to continue and which is not. A panel of different blood parameters taken with a rapid test may help to make the right decision by the responsible vet and this can help to prevent injuries or even deaths.

The aim of this doctoral thesis was to evaluate, if measuring serum ADMA concentrations via ELISA allows the detection of cardiac disease in horses. We hypothesized that ADMA serum concentration might be higher in horses with cardiac disease compared to healthy horses. In this context, reference values in horses were established. Furthermore, the objective of this doctoral thesis was to assess changes in ADMA concentrations over a 160 km endurance race, and to evaluate any differences between horses that failed to complete the race and horses that were successful. We hypothesized that ADMA would be higher before racing or show higher increases after racing in horses that failed to complete the race.

2. Review of literature

2.1. Asymmetric dimethylarginine in human medicine

Asymmetric dimethylarginine (ADMA) is an analogue of the amino acid L-arginine. It is produced as a by-product of the proteolysis of post-translational methylated proteins and released into plasma in exchange for arginine (Böder et al. 1998, Morris et al. 2007, Tain et al. 2017). Approximately 15 % of circulating ADMA is excreted through the renal system and over 80 % is eliminated through enzymatic degradation by the enzyme dimethylarginine dimethylaminohydrolase. (Kielstein et al 2004, 2006, Schwedhelm et al. 2011).

ADMA acts as a competitive inhibitor of nitric oxide synthase, which catalyses the synthesis of nitric oxide from the amino acid L-arginine (Vallance et al. 1992, Böger et al. 1995, De Gennaro et al 2009, Dias et al. 2011, von Leitner et al. 2011). There are at least three NO synthases: endothelial nitric oxide synthase, neuronal nitric oxide synthase and inducible nitric oxide synthase. Endothelial nitric oxide synthase is most strongly expressed in vascular endothelial cells. The vascular endothelium is crucial in the regulation of vascular structure and function due to the formation of endothelium-derived NO (Argido et al. 2007, Dias et al. 2011, Tousoulis et al. 2012). The effects of ADMA on NO synthesis and NO-mediated pathophysiological processes have been assessed in numerous human cardiovascular diseases (Böger et al. 2003,2004, Schlesiger et al. 2016). Because ADMA competes with L-arginine for nitric oxide synthase, the bioavailability of NO depends on the balance between the two (Argido et al. 2007, Bode-Boger et al. 2007, Dias et al. 2011, Tousoulis et al. 2012). Thus, the L-arginine/ADMA ratio is gaining more interest in the field of research as a potential marker of cardiovascular diseases in human medicine (Bode-Boger et al. 2007).

Elevated ADMA levels in plasma have been found in patients with chronic heart failure (Liu et al. 2016, Schlesiger et al. 2016). In chronic heart failure, coronary or systemic vasodilatation are attenuated, in part due to decreased vascular NO bioavailability (Chen et al. 2002). NO helps to preserve cardiac function in the setting of heart failure and plays an important role in maintaining cardiomyocyte function (Schwerrer-Crosbie et al. 2001, Jones et al. 2003). The protective effects of nitric oxide synthase are largely attributed to cyclic guanosine monophosphate production and activation of protein kinase G. NO activates soluble guanylate cyclase and therefore promotes cyclic guanosine monophosphate production. Subsequently, the cyclic guanosine monophosphate dependent protein kinase G is activated. Both substrates target several proteins involved in cardiac contractility, hypertrophy, and remodeling. Protein kinase G phosphorylates several essential intracellular targets that cause smooth muscle relaxation and increased blood flow (Ignarro 2002, Feil et al. 2003). ADMA can downregulate

nitric oxide synthase activity. When ADMA increases, NO decreases and leads to constriction of coronary vessels and to a reduction in coronary perfusion (Liu et al. 2016, Zuchi et al. 2020).

In addition to its importance in cardiovascular disease, ADMA also increases in patients with permanent atrial fibrillation (Horowitz et al. 2018). The precise mechanism(s) whereby ADMA concentrations modulate outcomes in patients with chronic atrial fibrillation remains somewhat putative. However, there is evidence that myeloperoxidase may inactivate the enzyme dimethylarginine dimethylaminohydrolase, which in turn degrades ADMA (von Leitner et al. 2011). Myeloperoxidase is an enzyme of leukocyte origin that has been implicated in the pathogenesis of atrial fibrillation.

So far, ADMA has been assessed not only in humans suffering from cardiovascular disease or arrhythmias, but also in healthy athletes. The studies in athletes showed reduced levels of circulatory L-arginine and a generally reduced level of amino acid-bound nitrogen after completion of prolonged exercise (Haralambie and Berg 1976, Cuisinier et al. 2001, Schrader et al 2020, Nyborg et al.2021). Furthermore, a study conducted by Noyburg et al. (2021) found increased levels of ADMA and a reduced L-arginine/ADMA ratio the day after the race. These changes indicate a state of reduced nitric oxide synthase activity and reduced endothelial function after the race and warrant further studies.

2.2. Cardiac biomarkers in horses

Cardiac biomarkers are indicators of processes, events, or conditions happening within the cardiovascular system. They can indicate physiological or pathophysiological processes that occur with cardiac damage or heart failure. As such, cardiac biomarkers can aid in the diagnosis and prognosis of heart diseases within individuals. Biomarkers can be subdivided into leakage enzymes that are released from damaged cells and functional markers, which increase or decrease in response to a biological process or disease (Jesty 2012).

The most studied cardiac biomarkers in equine medicine include cardiac troponin I and atrial natriuretic peptide.

Cardiac troponin I reflects leakage from cardiac myocytes due to increased oxidative stress, increased wall stress, ischemia, altered calcium handling, increase in inflammatory cytokines and altered neuro-hormonal activity (Holbrook et al. 2006, Trachsel et al. 2013, Park et al. 2017, Baker et al. 2019, Rossi et al. 2019). Reasons for an increase of cTNI are diverse and can be due to cardiac diseases, especially myocarditis, or secondary to other pathologies like sepsis, toxicities, endotoxemia, haemorrhage or myopathies (Holbrook et al. 2006, Trachsel et al. 2013, Park et al. 2017, Baker et al. 2019, Rossi et al. 2019). Despite the high sensitivity

and specificity of cTNI for cardiomyocyte damage, it has not proven very useful in diagnosing heart failure or heart diseases in horses. This is likely because most cardiac diseases in horses do not cause significant cardiomyocyte damage or necrosis.

A functional cardiac biomarker is ANP. ANP is a circulating cardiac hormone that takes part in the neuroendocrine control of fluid regulation and is secreted in response to increased stretching, volume, or pressure overload of the cardiac atrium (Trachsel et al. 2013). ANP counteracts the effects of the renin–angiotensin–aldosterone system and regulates blood pressure and fluid balance by a series of renal, vascular, and cardiac mechanisms (McGrath et al. 2005). Myocardial stretch, volume or pressure overload can occur in many chronic heart diseases prior to the event of obvious heart failure (Jesty 2012). The increased ANP secretion is of particular interest in equine cardiology, since horses frequently suffer from mitral regurgitation or atrial fibrillation, which both affect atrial size and mechanical function. Trachsel et al. (2015) found an increase in ANP in horses with heart disease. The increase was associated with altered left-sided chamber dimensions and/or function. However, the diagnostic value of ANP was compromised by poor sensitivity in this study. Other studies have also examined the association between left-sided chamber dimensions and ANP concentration and showed inconsistent results concerning the increase in ANP concentration with left atrial dilatation in horses (McKeever et al. 1992, van der Vekens et al. 2016).

In cardiac myocytes, ANP is stored as the prohormone proANP and is cleaved into the biologically active C-terminal fragment and the biologically inactive N-terminal fragment. The biologically inactive N-terminal fragment NT-ProANP is more stable. Trachsel and colleges (2012) found a correlation of NT-proANP concentration and left atrial size in horses with heart disease. However, another study found no relationship with NT-ProANP and left atrial dilatation (van der Vekens et al. 2016).

Furthermore, ANP has been reported to be increased with exercise (McKeever et al. 1991, 1992). Trachsel and co-workers (2013) showed that ANP increased in the exercising horse in relation to left atrial pressures. Horses with mitral regurgitation had even higher left atrial pressures and higher ANP at rest and after exercise compared to healthy horses.

2.3. Cardiac biomarkers in the exercising horse

Besides orthopaedic and respiratory problems, cardiovascular disease can be a reason for poor performance in horses. Two-dimensional echocardiography is a well-established examination tool in the resting horse. However, its applicability is limited in assessing myocardial function during exercise. For this reason, it would be an advantage to have a

biomarker that can reliably report about heart function during physical activity. A few studies have assessed the cardiac biomarker troponin I and have shown that increases in exercising horses occur (Holbrook et al. 2006, Nostell and Haggstrom 2008, Flethøj et al. 2016, Pourmohammad at al. 2020). In Standardbred racehorses, an increase in cTNI post-exercise was detected with peak concentrations occurring 2-6 hours post-exercise (Rossi et al. 2019). Cardiac troponin I was also assessed in endurance horses and increased significantly after 160 km and 80 km races (Holbrook et al. 2006, Flethøj et al. 2016). Holbrook and co-workers (2006) revealed that in endurance horses failing to finish competition, the degree of cTNI increase was not greater than the increase over baseline seen in the horses that successfully completed competition. The increase after exercise is generally mild and can occur in horses performing normally as well as those with poor performance (Holbrooke et al. 2006, Nostell and Haggstrom 2008). The ability of cardiac troponins to indicate subtle cardiac disease is hampered by the lack of a gold standard for assessing cardiac damage in horses, and because of the incomplete understanding of the effect of strenuous exercise on the concentration of cTNI in healthy horses (Jesty 2012). It is well accepted that regular moderate intensity exercise is beneficial for health. It remains unclear whether this still applies to endurance horses participating in strenuous prolonged exercise. Although some theories have been proposed to explain the mechanism underlying cTNI release following exercise, none has been confirmed. Without understanding the mechanism of cTNI increase after prolonged exercise, clarifying the clinical significance is difficult. Currently cTnI elevation following exercise is considered benign and the most well recognised mechanism is that of increased membrane permeability of cardiomyocytes, whereby unbound cTNI found in the cytosol diffuses across a concentration gradient from the intra- to the extra-cellular compartment (Shave et al. 2012, Baker et al. 2019, Rossi et al. 2019).

In addition to cTNI, the functional cardiac marker ANP was examined in horses after exercise and was found to be increased in horses during exercise due to physiological response to increased heart rate, increased atrial pressure, and/or changes in blood volume (Trachsel et al. 2013). The increase in ANP concentration only lasts as long as the pressure in the atrium is increased and decreases as soon as the pressure returns to normal. However, Pourmohammad and colleges (2020) revealed a non-significant increase of ANP concentrations 18 hours after exercise. Although the actual reason for increase of ANP 18 hours after exercise remains unknown, the researchers discussed that other factors could contribute to the response of ANP release, such as expression of ANP by remodelled left ventricular myocytes, activation of the renin-angiotensin system and sympathetic stimulation.

3. Summary of literature

In equine medicine, just cTNI and ANP have been related to heart disease (Jesty 2012). Additional functional biomarkers that increase with various heart diseases and during heart failure are missing.

Researchers agree that cardiac stress occurs in horses after prolonged physical exercise. In human medicine, studies have shown impaired cardiac function in athletes after prolonged physical exercise. In 1987, the concept of exercise-induced cardiac fatigue was used for the first time (Douglas et al. 1987). This phenomenon is defined as a transient decrease in systolic and diastolic ventricular function and is sometimes associated with an increase in cTNI (Douglas et al. 1987, Shave et al. 2010). So far, only two cardiac biomarkers, cTNI and ANP, have been investigated in exercising horses (Holbrook et al. 2006, Trachsel et al. 2013, Flethøj et al. 2016, Pourmohammad et al. 2020).

In contrast and despite promising potential, research on the topic of ADMA in cardiac disease as well as in horses performing prolonged physical exercise is currently lacking. This is unfortunate since due to the species-independent chemical structure, ELISA techniques developed for humans have emerged as useful and cost-efficient screening tool in veterinary medicine (Hall et al. 2014, Naby et al. 2015).

4. Paper I

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Author contribution

Ertelt A: conception of the work, clinical examinations and analysis, interpretation of data, writing most of the paper

Stumpff F: assisting in data interpretation, writing parts of the paper concerning physiological associations in discussion, proof reading

Merle R: statistical analysis, interpretation of statistical data, writing statistical analysis in material and methods, proof reading

Kuban S: helping with clinical examinations and offline analysis of echocardiography, proof reading

Bollinger L: helping with sample collection, proof reading

Gehlen H: conception of the work, fund raising, proof reading, approval of the version to be published

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Asymmetric dimethylarginine—A potential cardiac biomarker in horses☆

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Reference values

Abstract *Introduction/objectives:* Asymmetric dimethylarginine (ADMA) is a cardiac biomarker in humans, symmetric dimethylarginine (SDMA) a renal biomarker in humans, cats, and dogs. The purpose of this prospective study was to investigate if measuring serum ADMA and SDMA concentrations via ELISA allows detection of cardiac disease in horses in a routine laboratory setting. In this context, reference values in horses were established.
Animals, materials, and methods: Seventy-eight horses with no known medical history were compared to 23 horses with confirmed structural cardiac disease with/or without arrhythmias. Horses underwent physical examination, electrocardiography, echocardiography and venous blood sampling and were staged based on the severity of cardiac disease from 0 to II. Asymmetric dimethylarginine and SDMA were measured via ELISA and crosschecked using liquid chromatograph triple quadrupole mass spectrometry. Reference intervals with 90th percent confidence intervals were evaluated and standard software was used to test for significant differences in ADMA, SDMA, and the L-arginine/ADMA ratio between groups.
Results: The reference ranges were 1.7—3.8 μmol/L and 0.3—0.8 μmol/L for ADMA

☆ A unique aspect of the Journal of Veterinary Cardiology is the emphasis of additional web-based materials permitting the detailing of procedures and diagnostics. These materials can be viewed (by those readers with subscription access) by going to . The issue to be viewed is clicked and the available PDF and image downloading is available via the Summary Plus link. The supplementary material for a given article appears at the end of the page. To view the material is to go to and enter the doi number unique to this paper which is indicated at the end of the manuscript.

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and SDMA, respectively. Serum ADMA was higher in horses with heart disease compared to healthy horses ($p < 0.01$) and highest in horses with stage II heart disease ($p = 0.02$). The L-Arginine/ADMA ratio was significantly higher in healthy animals than those with cardiac disease ($p = 0.001$).
Conclusions: Reference values for serum ADMA and SDMA using ELISA methods are presented in horses. This study confirms the association between heart disease and increased serum ADMA concentration as well as a decreased L-Arginine/ADMA ratio in horses.

Abbreviations

ADMA	asymmetric dimethylarginine
LC-MS/MS	liquid chromatograph triple quadrupole mass spectrometry
NO	nitric oxide
SDMA	symmetric dimethylarginine

Introduction

Asymmetric dimethylarginine (ADMA) is a naturally occurring modified amino acid that inhibits the production of nitric oxide (NO), a key regulator of vascular tone [1—3]. Nitric oxide is produced from L-arginine as the natural substrate of NO synthase [2—4]. However, NO synthesis can be reduced by at least two of three different derivatives of L-arginine: symmetric dimethylarginine (SDMA), ADMA, and possibly also by monomethyl-L-arginine [4—6]. All three are generated via intracellular methylation of arginine via protein arginine methyltransferases and released into plasma in exchange for arginine and other cationic amino acids [4—7].

An increasing number of studies have linked elevated concentrations of circulating ADMA to cardiovascular disease [3,8—14]. Furthermore, a growing number of studies showed a strong link between the L-arginine/ADMA ratio and the severity of chronic heart failure in humans [15—17], raising the question whether this ratio might also be useful for identifying cardiovascular risk in equines.

After intracellular uptake by major organs such as the liver, brain, or kidney over 80% of circulating ADMA is eliminated through enzymatic degradation by the enzyme dimethylarginine dimethylaminohydrolase [18—20]. Symmetric dimethylarginine is primarily eliminated through renal filtration and correlates with both measured and estimated glomerular filtration rate. The importance of SDMA as an endogenous marker of renal function in humans, cats, and dogs is rising [19—24]. In dogs and cats, SDMA is used both for early identification and monitoring of decreased renal function in kidney disease [21,22].

In contrast and despite promising potential, research on the topic of SDMA and ADMA in cardiac or renal disease in horses is currently lacking. This is unfortunate since due to the species-independent chemical structure of SDMA, ELISA techniques developed for humans have emerged as useful and cost-efficient screening tools in veterinary sciences under practical clinical conditions [21,22].

The purpose of this prospective study was to investigate if measuring serum SDMA and ADMA concentrations via ELISA in a routine laboratory setting allows detection of cardiac disease in horses. We hypothesized that ADMA serum concentration might be higher in horses with cardiac disease compared to healthy horses. In this context, reference values in horses were established.

Animals, materials, and methods

The study was performed in compliance with guidelines from the Ethical Committee and European Union legislation. Permission to conduct the study was attained from national authorities (license number 0206/18). Horses were recruited either at national or international horse competitions or by a public call for study participation.

Horses without cardiac disease served as reference group. Inclusion criteria for the healthy group were no known history or clinical signs of cardiac disease or any other diseases. Cardiovascular

disease was excluded based on echocardiography and electrocardiography. Horses with a physiological atrioventricular block were included in the healthy group, provided that the block disappeared during physical activity. Horses with more than trivial (physiological) valve regurgitation on colou flow Doppler echocardiography or a pathological arrhythmia on electrocardiogram were excluded from the healthy group. Cardiovascular disease was further excluded based on venous blood sampling for cardiac biomarkers (cardiac troponin I, lactate dehydrogenase, α-hydoxybutyrate dehydrogenase). For exclusion of renal disease creatinine and urea were measured. For horses to be included within the reference group, cardiac troponin I, lactate dehydrogenase, α-hydoxybutyrate dehydrogenase, creatinine, and urea had to be within the reference ranges. Only horses with confirmed heart disease were included in the second group of horses with cardiac disease. Animals with the presence of any other disease were excluded from the study.

In addition to obtaining the medical history for each animal, all animals were subjected to a general physical examination, echocardiography, electrocardiography, and venous blood sampling for cardiac and renal biomarkers. Heart rate, respiratory rate, rectal temperature, pulse quality, mucous membranes, and heart sounds were assessed.

Echocardiography and electrocardiography

Transthoracic echocardiography was performed by means of a portable ultrasound unit[d] with a phased array transducer and simultaneous electrocardiogram recording. A single observer assessed cardiac structures, valvular competence, chamber dimensions and the diameters of the aorta, the pulmonary artery, and left ventricular systolic function by routine two-dimensional, motion mode, and color flow Doppler echocardiography[e].

Electrocardiographic recordings were obtained with a Holter recording system[f] with two channels and bipolar leads. Electrodes were secured with adhesive foam patches.

Grading of cardiac disease

Severity of cardiac disease was graded using a modification of the system proposed by Gehlen 2010 [25]. Horses in stage 0 were healthy and horses in stage I had mild to moderate valvular incompetence and cardiac dimensions within the reference range. Horses in stage II had increased cardiac dimensions due to severe valvular incompetence, in some cases with concomitant additional arrhythmia or increased cardiac troponin I and pericardial effusion. The valvular incompetence was graded according to the quantification methods defined by Gehlen et al. [25,26], Stadler et al. [27], Vahanian et al. [28] and Young et al. [29].

Laboratory analysis

Blood samples were obtained from the jugular vein via 18-gauge needles in all horses. Samples were collected in 6 mL EDTA tubes and 20 mL serum separator tubes. Serum was separated by centrifugation at a force of 1800×*g* for 10 min after clotting was completed. EDTA-plasma was also separated by centrifugation at a force of 1800×*g* for 10 min. Serum and EDTA-plasma were transferred into 2 mL cryotubes and frozen on dry ice until storage at −80°C. Serum and EDTA-plasma were sent on dry ice for analysis to different laboratories. Total storage time was 8 months at −80°C. Lactate dehydrogenase, α-hydoxybutyrate dehydrogenase, creatinine, and urea were measured with ELISA technology with conventional methods from serum samples[g]. Cardiac troponin I was determined in serum by an immunoassay system[h].

Symmetric dimethylarginine and asymmetric dimethylarginine were measured in serum samples in duplicate with commercial human ELISA

[d] Vivid I, GE Healthcare GmbH, Torgauer Str. 12−15, Berlin, Germany.

[e] EchoPAC, clinical workstation software, GE Healthcare GmbH, Torgauer Str. 12−15, Berlin, Germany.

[f] Televet-100 electrocardiogram device from Engel Engineering Service GmbH, Heusenstamm, Germany.

[g] Analyses of lactate dehydrogenase, α-hydoxybutyrate dehydrogenase, creatinine, and urea were run by Laboklin GmbH & CoKG with an analyzer from Bio Aim Scientific Inc., Steubenstraβe 4, 97,688 Bad Kissingen, Germany.

[h] Analysis of cardiac troponin I was run by Laboklin GmbH & CoKG with Immulite 2000XPi Siemens, Steubenstraβe 4, 97,688 Bad Kissingen, Germany.

technology[i]. The average of the duplicate determinations was used.

Ten values, six of the highest measured values from the group with cardiac disease and four values within the medium range measured in the control group, were crosschecked using liquid chromatograph triple quadrupole mass spectrometry (LC-MS/MS)[j].

For L-arginine analysis 400 μL EDTA plasma, 400 μL sample dilution buffer (including standard norleucine 100 nmol/mL), and 200 μL precipitation solution for the deproteinization were stored at 4°C for 20 min. The suspension was centrifuged at a force of 13,150×*g* for 5 min and the supernatant subsequently filtered with a membraspin filter at a force of 13,150×*g* for 5 min. L-arginine was analysed by the amino acid analyser Aracus[k]. Integration and calculation of the analysis were performed via aminoPeak software[k].

Statistics

Reference intervals were determined from 78 healthy horses for SDMA and ADMA. Data were evaluated according to Principles of Quality Assurance and Standards for Veterinary Clinical Pathology guidelines and calculated using the program Reference Value Advisor [30]. Except for SDMA, data were not normally distributed (Anderson-Darling test). The instructions of the International Federation of Clinical Chemistry—Clinical and Laboratory Standards Institute C28-A3 guidelines were followed in order to calculate nonparametric 90% reference intervals using bootstrap methods [31]. The 95% reference intervals were calculated by nonparametric methods, and then, the 90% confidence intervals about the lower and upper limits of the reference interval were calculated.

Statistical Package for the Social Sciences version 25 was used for all statistical analyses described in the following. Differences between horses with and without cardiac disease regarding ADMA, SDMA, and L-arginine/ADMA ratio were investigated using the t-test (for SDMA) and the Mann-Whitney U test (two groups, healthy (stage 0) and horses in stage I and II) or Kruskal-Wallis test (comparison of healthy horses (stage 0), horses stage I to II), as appropriate. p-values < 0.05 were regarded as statistically significant.

Lin's Concordance Coefficient was calculated to assess agreement between ten laboratory ADMA and SDMA values estimated with the ELISA[i] and the reference method LC-MS/MS[j]. Since some ADMA samples ran far out of the human reference range for the LC-MS/MS[j] method, samples were confirmed once again after dilution.

Results

The study population involved 78 healthy horses and 23 horses with confirmed cardiac disease.

The study population of healthy horses consisted of animals without known clinical disease, aged between 4 and 21 years (average: 11.9 years, median: 12 years). Animals weighed between 285 and 575 kg (average: 417 kg, median 410 kg).

All horses in the group exercised regularly and most horses were used for endurance sport (n = 66). Horses originated from diverse locations (Germany, Switzerland, Belgium, Italy, Austria, Sweden, Norway, Portugal, Croatia, the Netherlands, United Kingdom, Algeria, Australia, Chile, Uruguay, Columbia, Brazil, Ecuador, USA, Malaysia, Thailand, South Africa, and Bahrain). Breeds included Arabian Thoroughbred (n = 34), Arabian Partbred (n = 5), Shagya Arabian (n = 4), Anglo-Arabian (n = 7), German Warmblood (n = 3), Standardbred (n = 5), German Riding Pony (n = 4), Haflinger crossbreed (n = 1), Pinto (n = 1), Appaloosa (n = 1), Arabian Akhal-Teke crossbreed (n = 1), Karbarda (n = 1), and unknown (n = 11). There were 43 geldings, one stallion, and 34 mares.

Horses in the group with cardiac disease (n = 23) were between 2 and 29 years of age (average: 15.8 years, median: 15 years) and weighed between 240 and 750 kg (average 536 kg, median 580 kg). All horses came from Germany. Breeds consisted of German Warmblood (n = 14), Standardbred (n = 2), German Riding Pony (n = 2), Arabian Thoroughbred (n = 1) Appaloosa (n = 1), American Quarter Horse (n = 1), Pinto (n = 1), Trakehner (n = 1). The group included 13 geldings, three stallions, and seven mares. One horse that initially presented as healthy was excluded from the study, since it had a heart murmur 3/6 and more than trivial aortic valve regurgitation on color flow Doppler echocardiography.

[i] Analyses of ADMA and SDMA via Fast ELISA were run by DLD Gesellschaft für Diagnostika und medizinische Geräte mbH, Adlerhorst 15, 22,459 Hamburg, Germany.

[j] Analyses of ADMA and SDMA via LC - MS/MS were run by Medizinisches Labor Bremen GmbH, Haferwende 12, 28,357 Bremen, Germany.

[k] Analysis of L-arginine was run by MembraPure, Gesellschaft für Membrantechnik mbH, Wolfgang Küntscher Str. 14, 16,761 Hennigsdorf/Berlin, Germany.

Table 1 Reference range (90th percent confidence interval) in μmol/L for asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) in healthy animals. Horses with cardiac disease in stage I and II, with median and average ADMA (μmol/L) concentration. An asterisk (*) indicates data are normally distributed and a section sign (§) indicates data are not normally distributed. Values with different superscripts (a, b) are significantly different ($p < 0.005$). ADMA: asymmetric dimethylarginine; SDMA: symmetric dimethylarginine.

Stage	Reference range SDMA (μmol/L)	Reference range ADMA (μmol/L)	SDMA (μmol/L) * Mean ± SD	ADMA (μmol/L)§ Median (25 -percentile, 75 -percentile)	Number of horses
0	0.3—0.8	1.7—3.8	0.525 ± 0.114[a]	2.53 (2.16, 2.80)[a]	78
90% CI for lower limit	0.3—0.3	1.5—1.8			
90% CI for upper limit	0.7—0.9	3.5—3.8			
I			0.518 ± 0.092[a]	2.73 (2.50, 3.19)[a,b]	15
II			0.584 ± 0.055[a]	3.19 (3.00, 3.57)[b]	7

Reference values for ADMA and SDMA were determined in 78 healthy horses (Table 1). Lin's Concordance Coefficient revealed an excellent test agreement between the ELISA[i] and the reference method (LC-MS/MS[j]) with 0.91 for ADMA and 0.94 for SDMA.

Of the horses with cardiac disease, 14 were in stage I and 9 in stage II (Table 1). The values of ADMA and cardiac troponin I as well as the clinical symptoms (stage II) are given in Table A (available in Supplemental Material online).

Apart from one horse with pericardial effusion (horse stage II), cardiac troponin I was within the reference range (<0.03 ng/mL) (Table A, available in Supplemental Material online).

The values of (normally distributed) SDMA data did not differ significantly between healthy horses and horses with cardiac disease ($p = 0.6$), but the values of ADMA differed significantly, with horses suffering from cardiac disease having higher values than healthy animals ($p = 0.003$) (Fig. 1). Likewise, no significant differences could be detected between animals in stage 0 to II ($p = 0.2$) for SDMA, but ADMA showed significant differences between the groups ($p = 0.007$) with significantly higher values in stage II horses compared to healthy ones (adjusted $p = 0.02$) (Table 1, Fig. 2). In addition,

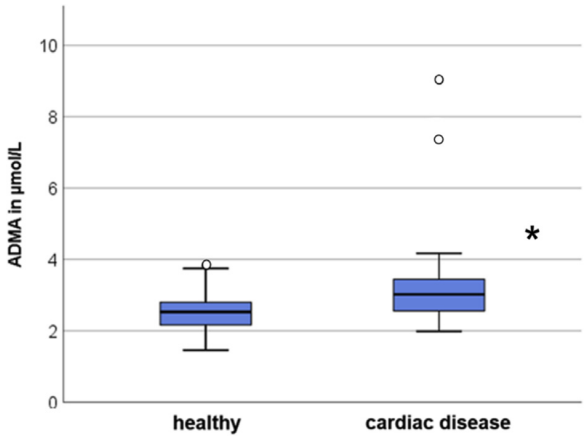

Fig. 1 Boxplot showing serum asymmetric dimethylarginine (ADMA) concentration in μmol/L in healthy horses and horses with cardiac disease. Asymmetric dimethylarginine (μmol/L) showed significantly higher values in horses with cardiac disease (stage II) compared to healthy animals. The circles (∘) represent outliers between 1.5 and 3.0 times the interquartile range (IQR). The asterisk (*) indicates $p = 0.003$. ADMA: asymmetric dimethylarginine.

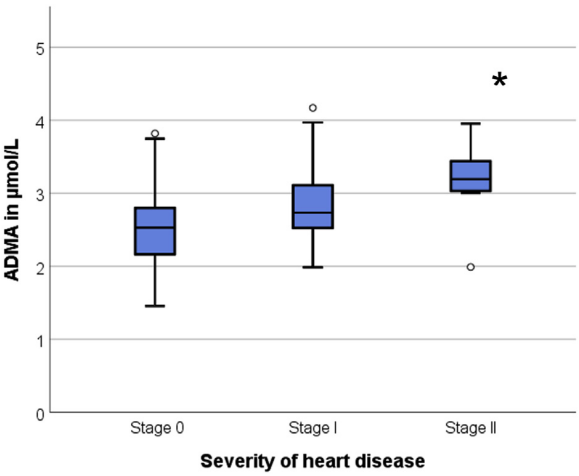

Fig. 2 Boxplot showing serum asymmetric dimethylarginine (ADMA) concentration in μmol/L in healthy horses (stage 0) and horses with cardiac disease in stage I and II. The circles (∘) represent outliers. The asterisk (*) indicates a significant difference between stage 0 and stage II horses, $p = 0.007$. ADMA: asymmetric dimethylarginine.

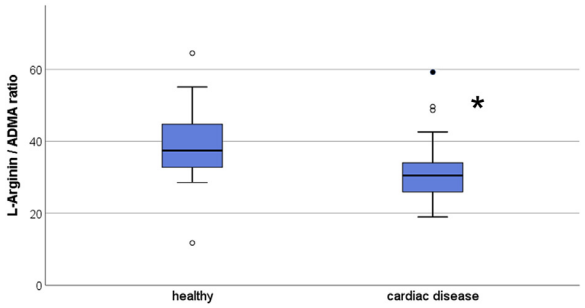

Fig. 3 Boxplot showing the distribution of the ratio between L-arginine (μmol/L) and asymmetric dimethylarginine (ADMA) (μmol/L) measured in healthy horses and in horses with cardiac disease, showing a clear shift of the distribution toward higher values in healthy animals. Differences between healthy horses and horses with cardiac disease were statistically significant ($p = 0.001$, Mann-Whitney U test). The circles (∘) represent outliers between 1.5 and 3.0 times the interquartile range (IQR), the filled circle (•) indicates outliers beyond 3.0 times IQR. The asterisk (*) indicates $p = 0.001$. ADMA: asymmetric dimethylarginine.

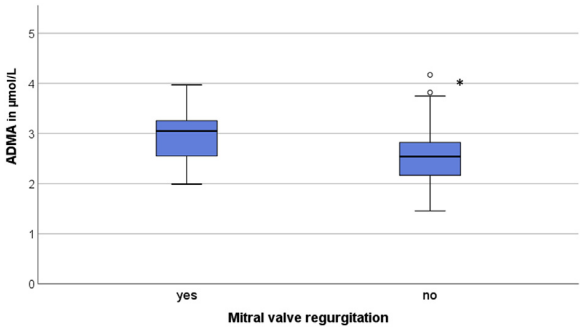

Fig. 4 Boxplot showing asymmetric dimethylarginine (ADMA) serum concentration in μmol/L in horses with mitral valve regurgitation (n = 14, 8 in stage I and 6 in stage II) and in healthy horses (without valve regurgitation) (n = 78). Horses with mitral valve regurgitation had significantly higher serum ADMA (μmol/L) concentrations than healthy horses. The circles (∘) represent outliers. The asterisk (*) indicates $p = 0.007$. ADMA: asymmetric dimethylarginine.

the ratio between L-arginine and ADMA was significantly higher in healthy horses than in horses with cardiac disease ($p = 0.001$) (Fig. 3).

Six horses suffered from atrial fibrillation, three with and three without increased left atrial diameter and area. Furthermore, one horse was diagnosed with premature atrial complexes.

Horses with arrhythmia had an increased ADMA serum concentration. Unfortunately, the data structure did not make it possible to apply a multivariable analysis, which would have given further information concerning other influence factors.

Horses with arrhythmia ($p = 0.01$), mitral valve regurgitation ($p = 0.007$) (Fig. 4), and regurgitation of more than two valves ($p = 0.02$) were associated with a significantly higher ADMA.

Discussion

Immunoassays have become the method of choice for measurement of diagnostic markers in biouids such as serum. In the present study, ADMA differed significantly between healthy horses and horses with cardiac disease. Animals with cardiac disease had higher values than healthy animals. In contrast to humans, the L-arginine/ADMA ratio in horses showed no advantage compared to ADMA alone. However, the finding that the L-arginine/ADMA ratio is significantly higher in healthy horses than in horses suffering from cardiac disease is an important finding. Since ADMA competes with L-arginine for NO synthase, the bioavailability of NO depends on the balance between the two. The L-arginine/ADMA ratio is an indicator of NO bioavailability and is therefore emerging as a potential marker of cardiovascular diseases in human medicine [32]. In what way the L-arginine/ADMA ratio affects horses and if it is a cause or the result of the associated cardiac disease cannot be clarified in the present study.

Despite our finding that ADMA differed significantly between healthy animals and animals with cardiac disease, the latter group rarely exceeded the upper reference range of 3.8 μmol/L (Table A, available in Supplemental Material online). This is most likely a result of the limited number of horses and the wide range of data included in the study, leading to a high reference range for the 90th percent confidence interval and calling for the necessity of follow-up studies with a greater number of animals.

In humans, ADMA measurement can be useful for assessment of endothelial dysfunction as a predictor of cardiovascular disease risk [3,6,8]. However, horses do not suffer from "typical" human cardiovascular diseases, such as hypertension, atherosclerosis, carotid artery intima-media thickness, angina pectoris, and heart attacks. However, atrial fibrillation is a disease seen in both humans and horses.

A study conducted by Horowitz and coworkers has shown that ADMA is elevated in human patients with permanent atrial fibrillation [33]. Notably, under conditions of NO synthase substrate (arginine) depletion, ADMA leads to an "uncoupling" of

NO synthase so that electron transfer is shifted from L-arginine to molecular oxygen, yielding the radical superoxide anion. The role of endogenous oxygen radicals in targeting DNA, proteins, lipids, and other components of the cell has long been known. Interestingly, studies on knockout mice that lacked an enzyme responsible for mitochondrial radical superoxide anion removal exhibited perinatal lethality due to cardiac dysfunction and congestive heart failure [34]. However, it appears likely that elevated levels of ADMA are the consequence and not the cause of cardiac disease in horses.

Horowitz and coworkers [33] mentioned that the precise mechanism(s) whereby ADMA concentrations modulate outcomes in patients with chronic atrial fibrillation remains somewhat putative. Dimethylarginine dimethylaminohydrolase, the enzyme that plays a primary role in ADMA metabolism is inhibited by oxidative stress [35] and there is evidence that myeloperoxidase, an enzyme of leukocyte origin that has been implicated in the pathogenesis of atrial fibrillation, may deactivate dimethylarginine dimethylaminohydrolase [36]. Determination of concentrations and activity of both dimethylarginine dimethylaminohydrolase and myeloperoxidase might have helped to further delineate the precise mechanism(s) [36].

The immunoassay used in the current study has been evaluated for SDMA in humans, cats, and dogs and for ADMA in humans [21,22,37—40]. The immunoassay showed adequate precision with intra-and inter assay coecients of variation lower than 15% [21,22,38,39]. Similar studies in horses have not been published so far. The reference values for equine SDMA determined in the present study ranged between 0.3 and 0.8 μmol/L. Reference values for SDMA in humans, cats, and dogs cited by DLD Diagnostika GmbH[j] are quite similar (humans and cats: 0.30—0.75 μmol/L, dogs: 0.30—0.65 μmol/L) [39]. The equine reference values for ADMA established in this study appear to be noticeably higher than those found in humans (0.40—0.75 μmol/L) [37,40]. This raised the question why values in horses are so much higher or whether possibly, these values were falsely high. For this reason, we crosschecked six outliers and four controls from the medium range using mass spectrometry. In total, ten ADMA and SDMA values were crosschecked using LC-MS/MS[j] and both methods agreed very well. Notably, the ADMA ELISA test[i] used in the present study has already been assessed in humans and compared to the LC-MS/MS[j] method [37,40], also showing good agreement. Since ADMA and SDMA are well-defined, small compounds with a structure that is identical in humans and equines, our finding that both methods correlate well in horses is hardly surprising.

Therefore, there appears to be other reasons for the higher ADMA levels in horses. Notably, the uptake of ADMA into the cytosol for degradation occurs via an exchanger from the anion/cation transporter family that requires intracellular presence of dibasic amino acids such as arginine, lysine, and ornithine as a substrate [41]. As a herbivore, horses may well have a different amino acid profile than carnivorous species [42—44], possibly leading to a different profile.

The major limitation of the study is the small sample size of 23 horses with cardiac disease that lowered statistical power and might have impacted the ability to detect differences between the stages of cardiac disease, especially in stage II. Furthermore, there is the possibility of a breed effect on ADMA and SDMA concentration. Therefore, the reference intervals might not be relevant to other breeds of horses. Although samples from the entire range were selected for comparison of the ELISA[i] and LC-MS/MS[j] methods, sampling was not random since six of the highest measured values were included. Strictly speaking, it is thus not possible to generalize the results, although they are in good agreement with previous studies [37,39,40]. Furthermore, storage time of the samples was 8 months and therefore values could have decreased over time. No data on storage time for ADMA or SDMA has been published to date. The laboratory that carried out the analysis[i] has real time data on storage time for at least 5 years from 32 quality control samples (human serum) that were frozen at −23°C. Over the past 5 years, these quality control samples were measured seven times and showed almost identical results. At −80°C ADMA and SDMA should be at least as stable.

Conclusions

In the present study, horses with cardiac disease had significantly higher serum ADMA concentration and a decreased L-arginine/ADMA ratio compared to healthy horses. Although the physiological reasons for higher serum ADMA concentration and a decreased L-arginine/ADMA ratio in horses with cardiac disease remain elusive, this study demonstrates that ADMA is a promising biomarker not just in humans, but also shows diagnostic potential in identifying cardiac disease in horses under routine conditions.

The manuscript is original. No part of the manuscript has been published before, nor is any part of it under consideration for publication in another journal. There are no conflicts of interest to disclose.

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Supplementary data

Supplementary data to this article can be found online at .

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5. Paper II

Ertelt A., Merle R., Stumpff F, Bollinger L, Liertz S, Weber C, Gehlen H.
Evaluation of different blood parameters from endurance horses competing at 160 km.
JEV 2021;104:103687.

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Author contribution

Ertelt A: conception of the work, clinical examinations and analysis, interpretation of data, writing most of the paper

Merle R: conception of the work, statistical analysis, interpretation of statistical data, writing statistical analysis in material and methods and parts in the result section, proof reading

Stumpff F: assisting in data interpretation, proof reading and writing parts of the paper in discussion

Bollinger L: helping with sample collection, proof reading

Liertz S: helping with clinical echocardiographic examination and sampling, proof reading

Weber C: conception of the work, sample analysis, proof reading

Gehlen H: conception of the work, fund raising, proof reading, approval of the version to be published

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6. Discussion

This is the first study assessing the reference interval of plasma ADMA in healthy horses. Furthermore, the study showed that ADMA can detect horses with cardiac disease. In the present study, ADMA was measured using a human enzyme-linked immunosorbent assay. Currently, high performance liquid chromatography, with fluorescence or mass spectrometric detection and enzyme-linked immunosorbent assay are the most frequently used analytical methods to determine ADMA concentrations. High performance liquid chromatography has high maintenance cost and is time-consuming. ELISA is an easy-to-use, fast method with the disadvantage of a slightly lower sensitivity. Regarding the comparability of these methods, Schulze and co-workers (2004) found nearly identical ADMA levels measured by liquid chromatography triple quadrupole mass spectrometry (LC-MS/MS) and enzyme-linked immunosorbent assay (ELISA). Fortunately, the amino acid analogue ADMA is highly conserved among species, allowing the use of commercially available human assays to measure plasma ADMA concentrations in horses.

In the present study, the equine reference range for ADMA (1.7-3.8 µmol/L) is noticeably higher than that found in humans (0.40 - 0.75 µmol/L) (Schulze et al. 2004, https://www.dld-diagnostika.de/product/84). One reason for this might be that the measured values were falsely high. For this reason, six outliers and four controls from the medium range were cross - checked using LC-MS/MS. As expected, both methods agreed very well. However, for comparison of ELISA and LC-MS/MS samples were not randomly selected. Therefore, it's not possible to generalize the results.

Another reason for the higher reference range can be the different amino acid utilisation in horses compared to humans. Horses are herbivores and may have a different amino acid profile than carnivorous species (Teerlink et al. 2009, DeBoer et al. 2019), possibly leading to a different profile. Unfortunately, specific details on amino acid utilisation in horses are not readily available.

A further point to consider is that the reference interval might not be relevant to other breeds of horses. Because the main breed used in the present study were well trained Arabian horses and crossbreeds. Therefore, there is the probability of a breed and training effect on the reference intervals.

An important finding in the present study is that ADMA differed significantly between healthy horses and horses with cardiac disease. Compared to healthy controls elevated ADMA concentrations were found in patients suffering from cardiac structural disease. However, only horses with severe heart disease exceeded the reference range. Same was evident with the

L-arginine/ADMA ratio. In human medicine, ADMA is a one of the strongest predictors of mortality in patients who suffer from chronic left heart failure. ADMA plays an important role in maintaining nitric oxide bioavailability and it is presumed that an abnormal NO production contributes to heart failure (Bode-Boger et al. 2007). Furthermore, ADMA has been shown capable of uncoupling electron transport between arginine and nitric oxide synthase resulting in production of reactive oxygen species (Lu et al. 2003, Sydow and Münzel 2003). To what extent ADMA affects horses with cardiac disease and if it is a predisposing factor or a consequence of heart disease, cannot be clarified in the present study. It appears more likely though that elevated levels of ADMA in horses are the consequence. The major limitation of the present study is the small sample size of 23 horses with structural cardiac disease. This fact lowered statistical power and might have had an impact on the ability to detect differences between the stages of cardiac disease. Furthermore, the main breed in the control group were well-trained Arabian breeds, while in the diseased group mainly unridden warmbloods were affected. We know from human athletes that ADMA increases after physical performance (Haralambie and Berg 1976, Cuisinier et al. 2001, Schrader et al 2020, Nyborg et al. 2021) and this might have resulted in a wide reference range where untrained horses with mild and moderate heart disease not exceeding the upper range.

Remarkably, the mean ADMA concentration in horses with severe heart disease is similar in horses finishing a 160 km endurance race. In the general linear model, the running distance was associated with a greater increase of ADMA. In human studies, reduced levels of circulatory L-arginine and amino acid-bound nitrogen after completion of prolonged exercise were found (Haralambie et al. 1976, Nyborg et al. 2021). NO is synthesized by the endothelial nitric oxide synthetase from the amino acid L-arginine and leads to vasodilation. The reduction of L-arginine is believed to be due to increased gluconeogenesis and enhanced NO production during prolonged exercise (Haralambie et al. 1976, Cuisinier at al. 2001). Thus, low levels of L-arginine, in part, explain the reduced endothelial function after prolonged exercise (Nyborg et al. 2021). We found an elevated concentration of ADMA and a reduced L-arginine/ADMA ratio after prolonged exercise in endurance horses in the present study. The longer the completed distance, the higher the ADMA increase. ADMA reduces the activity of endothelial nitric oxide synthetase through competitive inhibition at the binding site for L-arginine (Vallance et al. 1992, Böger et al. 1995, De Gennaro et al 2009, Dias et al. 2011, von Leitner et al. 2011). Therefore, the L-arginine/ADMA ratio is of importance for NO synthesis and endothelial function. Besides the reduced levels of the amino acid L-arginine, an increase of ADMA concentration occurs after prolonged exercise, further contributing to endothelial dysfunction and leading to constriction of certain parts of the vascular system. The activity of the enzyme dimethylarginine dimethylaminohydrolase is reduced during oxidative stress (De Gennaro et

al. 2009), which occur during prolonged exercise. Therefore, plasma ADMA concentrations increase due to reduced degradation. It is possible that the reduced perfusion of the intestine may help the horse compensate for dehydration and volume loss. In addition to that, coronary perfusion is also reduced, and the tissue-protective function of NO as a scavenger of cytotoxic oxygen radicals is decreased (Zuchi et al. 2020). Whether these mechanisms are in part responsible for cardiac impairment seen in horses after endurance races (Flethoj et al. 2016) needs further evaluation.

Unfortunately, a significant difference between horses that failed to complete the race and horses that were successful could not be detected. Our results did not support the hypothesis, that ADMA would be higher before racing or increase more after racing in horses that failed to complete the race.

However, the highest measured ADMA value after racing was 5.3 µmol/L (reference range 1.7-3.8 µmol/L) in a horse that failed to qualify due to metabolic reasons.

The second highest value with 5.2 µmol/L was seen in a 13-year-old gelding from Bahrain that finished the race. This horse also had one of the highest cTNI concentration (8.2 ng/mL, reference range < 0.03 ng/mL). Despite these increases in cardiac markers, the horse finished the endurance race and passed all the veterinary inspections. The cause for the severe increase in the biomarkers was not determined and it did not undergo any kind of treatment at the local event. The official next start of this horse was 3 years later in a 160 km CEI* and 120 km CEI2*. In both races the horse failed to qualify. For animal welfare reasons, integrating a laboratory assessment for certain biomarkers with rapid tests in the veterinary checks during the loops is recommended.

Therefore, a limitation of the present study is that no additional examination was undertaken that could have detected the underlying reason why the non-finishers failed to qualify or whether finishing horses had adverse health effects. Another limitation of the study is that only one sample was taken after the competition, which is most likely insufficient to obtain maximal or minimal values depending on the kinetics of the parameter. In future studies, serial measurements at various points during and after the race could be beneficial.

7. Summary

Evaluation of the diagnostic value of asymmetric dimethylarginine for use as cardiac biomarker in horses and its assessment in endurance horses competing at 160 km

Dr. Antonia Ertelt

Asymmetric dimethylarginine (ADMA) is a well-established cardiac biomarker in humans. The purpose of this prospective study was to investigate if measuring serum ADMA concentration via ELISA allows detection of cardiac disease in horses. In this context, ADMA reference values in horses were established. Furthermore, a change in ADMA serum concentration before and after a 160 km endurance race was assessed and the differences in ADMA concentration between horses that completed the race and horses that did not was evaluated.

For this propose 78 horses with no known medical history were compared to 23 horses with confirmed structural cardiac disease with or without arrhythmias. Horses with heart disease were staged based on the severity of cardiac disease from I to II. Furthermore, 52 healthy endurance horses competing in a 160 km race were assessed. The endurance horses were assigned to three groups: horses that successfully completed the race, horses that failed to qualify at the veterinary check for primarily metabolic reasons and horses that failed to qualify at the veterinary check for primarily gait related reasons. The latter two groups were combined to form a final group of “non-finishers” that were excluded for either “gait related” or “metabolic” disorders.

Horses underwent physical examination, electrocardiography, echocardiography, and venous blood sampling. In the endurances horses venous blood samples were taken before and after the endurance race. Asymmetric dimethylarginine was measured via ELISA, with a subset cross-checked using liquid chromatograph triple quadrupole mass spectrometry.

Reference intervals with 90th percent confidence intervals were established according to ASVCP-guidelines. Standard software was used to test for significant differences in ADMA, and the L-arginine/ADMA ratio between groups.

The reference range for ADMA in healthy horses was 1.7 - 3.8 µmol/L. Serum ADMA was higher in horses with heart disease compared to healthy horses ($p<0.01$) and highest in horses with stage II heart disease ($p=0.02$). However, only a few horses with stage II heart disease exceeded the reference range. The L-Arginine/ADMA ratio was significantly lower in healthy animals than in those with cardiac disease ($p=0.001$). ADMA ($p=0.002$) increased significantly after the endurance race in the finisher group. The longer the completed distance the higher

was the ADMA concentration. No differences between the groups “finisher”, “metabolic” or “gait related” could be found.

This study confirms the association between heart disease and increased serum ADMA concentration as well as a decreased L-Arginine/ADMA ratio in horses. Regardless of clinical assessment, there was a strong correlation between ADMA concentration and the completed distance in endurance horses. Our results did not support our hypothesis that ADMA would be higher before racing or increase more after racing in horses that failed to complete the race. Although the physiological reasons for higher serum ADMA concentration in horses with cardiac disease and horses completing a 160 km endurance race remain elusive, this study demonstrates that ADMA is a promising biomarker not just in humans, but also shows diagnostic potential in identifying cardiac disease respectively cardiac stress in horses under routine conditions.

8. Zusammenfassung

Evaluierung des diagnostischen Potentials von Asymmetrischem Dimethylarginin bei Pferden mit einer Herzerkrankung und dessen Beurteilung bei Distanzpferden nach einem 160km Distanzritt

Dr. Antonia Ertelt

Asymmetrisches Dimethylarginin (ADMA) ist ein etablierter und sensitiver kardialer Biomarker beim Menschen. In dieser prospektiven Studie sollte untersucht werden, ob die Messung der ADMA-Konzentration im Serum mittels ELISA den Nachweis einer Herzerkrankung bei Pferden ermöglicht. In diesem Zusammenhang wurden ADMA-Referenzwerte für Pferden ermittelt. Darüber hinaus sollte eine Veränderung der ADMA-Serumkonzentration vor und nach einem 160 km Distanzritt untersucht werden. In diesem Zusammenhang sollte geprüft werden, ob es Unterschiede in der ADMA-Konzentration zwischen Pferden gibt, die das Rennen beenden und die vom Rennen aufgrund einer gesundheitlichen Einschränkung ausgeschlossen werden müssen.

Für den ersten Teil der Studie wurden 78 herzgesunde Pferde mit 23 Pferden mit einer bestätigten strukturellen Herzerkrankung mit oder ohne Arrhythmie verglichen. Für den zweiten Teil der Studie wurden 52 gesunde Distanzpferde, die an einem 160 km Distanzrennen teilnahmen, untersucht. Die Distanzpferde wurden in drei Gruppen eingeteilt: Pferde, die das Rennen erfolgreich abgeschlossen haben, Pferde, die aufgrund eines metabolischen Problems im Veterinärcheck disqualifiziert wurden und Pferde, die beim Veterinärcheck aufgrund eines veränderten Gangbildes disqualifiziert wurden.

Es erfolgte eine Allgemeinuntersuchung, eine Elektrokardiographie und eine Echokardiographie der Pferde sowie eine venöse Blutentnahme. Bei den Distanzpferden wurde vor und nach dem 160 km Distanzritt venöse Blutproben entnommen. Aus den Blutproben wurde die ADMA-Konzentration mittels ELISA im Serum bestimmt. Basierend auf der Schwere der Herzerkrankung wurden die Pferde in Stadien von I bis II eingestuft. Bei einem Teil der Proben erfolgte eine Doppelmessung mittels Flüssigchromatographie-Triple-Quadrupol-Massenspektrometrie. Die Referenzintervalle für ADMA wurden entsprechend der ASVCP-Richtlinien eruiert. Die Standardsoftware SPSS wurde verwendet, um signifikante Unterschiede in der ADMA-Konzentration und der L-Arginin/ADMA-Ratio zwischen den Gruppen zu testen.

Der in der vorliegenden Studie ermittelte Referenzbereich für ADMA liegt zwischen 1,7 - 3,8 µmol/L. Des Weiteren ergab die Studie, dass bei Pferden mit Herzerkrankungen die ADMA Konzentration im Serum im Vergleich zu herzgesunden Pferden signifikant höher ($p<0,01$) ist

und dass der Anstieg bei Pferden mit schweren Herzerkrankungen (Stadium II) am höchsten (p=0,02) ist. Jedoch überschritten nur sehr wenige der schwer herzkranken Pferde den Referenzbereich. Die L-Arginin/ADMA-Ratio war bei gesunden Pferden signifikant geringer als bei herzkranken Pferden (p=0,001).

Bei Pferden, die den Distanzritt von 160 km erfolgreich beendeten, stieg die ADMA-Konzentration im Serum (p=0,002) signifikant an. Es ergaben sich keine signifikanten Unterschiede in der ADMA-Konzentration zwischen Pferden, die aus dem Rennen ausgeschieden sind oder dieses erfolgreich beendet haben. Die ADMA-Konzentration stieg proportional zu den zurückgelegten Kilometern an.

Die Untersuchungen ergaben, dass ADMA nicht nur beim Menschen ein vielversprechender kardialer Biomarker ist, sondern auch ein diagnostisches Potenzial zur Identifizierung von Herzerkrankungen oder kardialem Stress bei Pferden aufweist.

Die Hypothese, dass die ADMA-Konzentration vor oder nach dem Rennen bei Pferden höher ist, die disqualifiziert werden, konnte nicht gestützt werden. Weitere Studien zur Untersuchung der ADMA-Konzentration in den verschiedenen Vet-Gates wären von Vorteil und könnten weitere Hinweise geben, ob ein Ausscheiden aus dem Rennen gegebenenfalls vorherzusehen ist.

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13 Conflicts of interest

No conflicts of interest to declare.

14 Declaration of academic honesty

I hereby declare that I have written the present thesis independently. I assure that I have used only the sources and help indicated.

Dr. Antonia Ertelt

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