Analysis of neurofilament concentration in healthy adult horses and utility in the diagnosis of equine protozoal myeloencephalitis and equine motor neuron disease

Adriana M. Morales Gómez[a], Sophie Zhu[a], Scott Palmer[a], Emil Olsen[a], Sally L. Ness[a], Tom J Divers[a], Karyn Bischoff[a], Hussni O. Mohammed[a, *]

[a] College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA

*Corresponding author at: S1 070 Schurman Hall, Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA.
E-mail address: hom1@cornell.edu (H.O. Mohammed).

Running title: Neurofilaments as biomarker for neurological disorders

Abstract: Neurofilaments (NFs) are structural proteins of neurons that are released in significant quantities in the cerebrospinal fluid and blood as a result of neuronal degeneration or axonal damage. Therefore, NFs have potential as biomarkers for neurologic disorders. Neural degeneration increases with age and has the potential to confound the utility of NFs as biomarkers in the diagnosis of neurologic disorders. We investigated this relationship in horses with and without neurological diagnosis. While controlling for horse type (draft, pleasure, and racing), we evaluated the relationship between serum heavy-chain phosphorylated neurofilaments (pNF-H) and age, sex, and serum vitamin E concentrations. Serum pNF-H concentrations increased by 0.002 ng/mL for each year increase in age. There were significant differences in the serum pNF-H concentration among the type of activity performed by the horse. The highest serum pNF-H concentration was found in horses performing heavy work activity (racehorse) and with lower serum pNF-H concentration found among light (pleasure riding) and moderate (draft) activity. There was no significant association between the pNF-H concentration and sex or vitamin E concentration. Serum pNF-H concentration was elevated among horses afflicted with EMND and EPM when compared with control horses without evidence of neurologic disorders. Accordingly, serum pNF-H concentration can serve as a

useful biomarker to complement the existing diagnostic work-up of horses suspected of having EPM or EMND.

Key words: Biomarker; equine motor neuron disease; equine protozoal myeloencephalitis; neurofilaments.

1. Introduction

Current diagnostic methods used to diagnose equine neurologic disorders such as equine protozoal myeloencephalitis (EPM) and equine motor neuron disease (EMND) are based heavily on clinical examinations and invasive laboratory tests, i.e., tissue biopsies and cerebrospinal centesis, and definitive diagnosis can only be determined by autopsy (Dubey et al., 2001; Reed et al., 2013).

Neurofilaments (NFs) are structural proteins of neurons that are densely located in axons of the neurons (Boylan et al., 2009; Gresle et al., 2014) and mainly consist of 3 subclasses: light (NF-L), medium (NF-M), and heavy (NF-H) (Gresle et al. 2014). These proteins are released into the cerebrospinal fluid (CSF) and blood as a result of neuronal degeneration or axonal damage (Inoue et al., 2017; Petzold, 2005). Neurofilaments have been demonstrated to be stable in blood, serum, or CSF over time, and no effect has been detected in pNF-H concentrations when freezing CSF or serum (Gendron et al., 2017; Hamishehkar et al., 2016). The fact that NFs have a relatively long half-life makes the concentration of NFs in serum a possible biomarker for neurologic diseases or neuronal disintegration (Millecamps et al., 2007; Yuan et al., 2009).

Our studies and those of others have confirmed elevated concentrations of neurofilaments in animals affected with several pathologic conditions or trauma (Intan-Shameha et al., 2017; Nishida et al., 2014). Little is known about the changes in the concentrations of NFs during the normal aging process. Our suspicion is that the concentrations of the phosphorylated NFheavy (pNF-H) in the serum increases with age as a result of progressive neuronal loss and axonal disintegration. The literature on the relationship between age and neuronal degeneration is conflicting. Although one study in humans reported that the serum concentration of phosphorylated NF-light (pNF-L) increases with age (Burianová et al., 2015); studies on the relationship between the serum concentration of NFs and age are lacking in horses. A study in rats demonstrated non-significant changes in the number of neurons with age Vågberg et al., 2015). If the serum concentration of NFs increases significantly with age in the horse, this finding has the potential to confound the utility of NFs as a test for equine neurologic disorders. Furthermore, in a previous study we reported that horses afflicted with EPM had significantly increased concentrations of pNF-H compared to healthy horses. Since we did not account for the age of the animals in that report (Intan-Shameha et al. 2017), we conducted this study to determine if there was a relationship between pNF-H concentrations and age.

Another factor that has the potential to influence the concentrations of NFs in animals is treatment with vitamin E.

Vitamin E is commonly administered to animals and humans for disease prevention or treatment of neurodegenerative diseases, including EMND and motor neuron diseases in other species (Brown et al., 2017; Finno et al., 2017; Mohammed Dr. et al., 2012; Ng et al., 2017). The impact of serum concentrations of vitamin E on the concentration of NFs is not clear and has a potential to confound the utility of this biomarker as a diagnostic parameter for neurologic disorders.

In this study we aim to investigate the association between pNF-H and the age of the horse, while controlling for other possible confounding factors, (effect of serum levels of vitamin E, level of activity of the horse, and sex) in order to evaluate the potential use of these proteins as biomarkers for 2 common neurologic disorders of the horse: EMND and EPM.

2. Materials and methods

2.1. Study design, target and study population

This cross-sectional study included horses residing in New York State. The study population included healthy horses and horses with a confirmed neurologic diagnosis of EMND or EPM. The case horses were either admitted to the Cornell University Hospital for Animals (CUHA) or had blood samples submitted to the Animal Health Diagnostic Center (AHDC). Control horses included horses admitted to CUHA for evaluation and treatment of non-neurologic conditions or horses without reported neurologic clinical signs whose blood was submitted to the AHDC for determination of Vitamin E levels. Horses with or without neurologic disorders were categorized into 3 activity categories: light (pleasure or trail riding horses), moderate (working draft horses or low-level event horses) and heavy (Thoroughbred race horses).

2.2 Inclusion criteria

The inclusion criteria for enrollment of case horses with neurological diseases was a definitive diagnosis of EMND or EPM (based on necropsy findings that included histopathological examination of the brain and spinal cord and vitamin E values in their medical record. All horses without a neurologic disorder (lack of reporting of neurologic signs) were included as healthy horses.

2.3 Determination of serum pNF-H concentration

The pNF-H assay was conducted using the pNF-H sandwich enzyme-linked immunosorbent assay (ELISA) kit (ELISA, EMD Millipore, Billerica, MA). The protocol used chicken polyclonal antibodies generated against pNF-H, which were pre-coated onto a 96–well plate, later rabbit polyclonal antibodies and a goat anti-rabbit alkaline phosphatase conjugate were used to detect the captured pNF-H from the samples.

The pNF-H ELISA kit uses antibodies specific for pNF-H from mammalian species, additional ELISA protocol details are described previously (Anderson et al., 2008; Intan-Shameha et al., 2017). Frozen serum samples from each horse were thawed prior to assay. All samples were tested in duplicate and the assay was performed according to the manufacturer's protocol. The person who performed the assays was completely blinded to the clinical information. The mean absorbance of the pNF-H standard, measured as optical density (OD), was plotted on a logarithmic scale. As a result, a standard curve was created and was used to calculate the pNF-H concentration of each sample (range of detection was 0.0293 ng/mL to 15 ng/mL), since duplicates were used for every sample, an average value of each sample was calculated.

2.4 Determination of vitamin E concentration

Serum was harvested from the horse whole blood samples, and 1 mL of serum was added into a sterile polypropylene microtubes containing an antioxidant mixture, consisting of 100 mL of an ethanol mixture of propyl gallate and EDTA. The samples were then frozen at –75°C until testing. Serum concentrations of α-tocopherol were measured at the AHDC at Cornell University by means of high-performance liquid-liquid partition chromatography. Analytes of interest were detected by spectrophotometry (molecular fluorescence emission at 330 nm for 7.05 minutes) with a tandem arrangement of a variable-wavelength UV detector and a spectrofluorometric detector. The concentration of vitamin E was reported as µg/ml. Further details on method are delineated previously (Mohammed et al., 2007).

2.5 Data collection and analysis

Data on the age, sex and type of activity (draft, pleasure, or racing; which recoded into light, moderate, or heavy) of the horse was acquired from the CUHA horses' medical records or collected by personal interviews with the horse's owner/trainer.

The data was initially reported using frequency distribution and graphics, and the measure of central tendency (mean or median) and dispersion (standard deviation and range) were calculated. The bivariate relationship between each of the factors/variables (age, vitamin E concentrations and type of horse activity) and the concentration of pNF-H was assessed using regression analysis or analysis of variance for categorical variables. In the final analysis, factors that were significantly associated with the concentrations of pNF-H in the bivariate analysis were evaluated jointly using the general linear model to assess the significance of association of each factor while simultaneously controlling for other factors. The probability of neurologic diseases (EMND or EPM) was calculated from the logistic regression analysis equation and the dependent variable was whether the horse had neurologic disorder or not. Only horses with vitamin E values were included in this analysis. All statistical analyses were performed using the SPSS v.24 (IBM SPSS Statistical Software, White Plain, NY) and statistical significance differences were considered a type I error (*p-value*) of 0.05.

3. Results

3.1 pNF-H as a function of age, sex and activity

A total of 169 horses without clinical signs of neurologic disease met the inclusion criteria and were enrolled for this part of the study. **Table 1** shows the distribution of the pNF-H horse serum concentrations by the type of activity, sex and age of the horse. There was significant variation in the serum neurofilament concentrations among horses with different levels of work activity (light, moderate, and heavy).Those horses with heavy activity (Thoroughbred race horses), had significantly higher serum levels of neurofilaments compared to either horses with moderate work activity (working draft horses or low-level event horses) or light work activity (pleasure horses or trail horses) (**Table 1**). There was no significant difference in the serum concentration of neurofilaments among horses in our study population based upon the sex of the horse.

The average age of the healthy horses in our study was 11.65 years (SD = 6.8 years) (**Table 1**). There was a tendency for the concentrations of pNF-H to increase with the age of the horse (**Figure 1**). The initial correlation (bivariate) between age and the level of neurofilaments was evaluated using a regression analysis with different transformation (linear and second order) to ensure capturing any variability in age. Although there was significant positive linear relationship between the age of the horses in this study and the concentrations of the pNF-H; the concentrations of pNF-H increased by only 0.002 ng/ml for each year of increase in age.

3.2 pNF-H concentration and vitamin E concentration

Serum vitamin E concentrations were obtained from 93 healthy horses. The average serum vitamin E concentration in healthy horses was 2.56 µg/ml (**Table 1**). There was no significant correlation between the concentration of vitamin E and the concentration of pNF-H in our study (**Figure 2**) and in the bivariate analysis.

3.3 Multivariate Analysis

Table 2 shows the results of the multivariate analysis for the relationship between the age of the horses and the serum concentrations of pNF-H when controlled for the activity of the horses. The concentrations of neurofilaments increased by 0.002 ng/ml for each year of increase in age of the horse (regression coefficient). That means for each year increase in age of the horse the concentration of pNF-H increases by 0.002 What that means

There was a significant association between the type of activity of the horse and the concentrations of pNF-H. The adjusted mean pNF-H values for the reference category of the activity was 0 ng/ml, horses with heavy activity was 0.359 ng/ml which was significantly higher than that for moderate (0.225 ng/mL) or light (0.0246 ng/mL) activity horses (**Table 2**).

3.4 pNF-H and neurologic disorders

We investigated the association between serum pNF-H concentration and the likelihood of neurologic disorders using a logistic regression analysis. A total of 61 horses with confirmed diagnosis of EMND (23 horses) or EPM (38 horses) were identified. The probability of neurologic disorder given the concentrations of neurofilaments was calculated using the logistic regression analysis as follows: $P(Neurolog\ ic) = \frac{1}{1 + (\exp^{-(\alpha + \beta(pNFH))})}$. Where P (Neurologic) is the probability of neurologic disorders (EMND or EPM), α is the constant of the logistic regression, and β is the regression coefficient for the changes in the probability of pNF-H per unit change in the pNF-H concentrations. In the analysis the constant value was -3.786 and the regression coefficient was 2.977. **Figure 3** shows the relationship between the probability of neurologic disorder and the pNF-H serum values. The probability of a neurologic disorder reaches 0.9 as the concentrations of pNF-H reaches 2.0 ng/ml (**Figure 3**).

4. Discussion

The long-term objective of our research is to investigate the usefulness of pNF-H as a diagnostic parameter for the presence and severity of neurologic disorders in the horse. The use of pNF-H as a diagnostic marker for neurologic disorders, i.e., amyotrophic lateral sclerosis (ALS), or brain injuries in humans, has proven to be useful (Chen et al., 2016; Gaiani et al., 2017; Gendron et al., 2017; Poesen et al., 2017; Shibahashi et al., 2016). Studies have linked the concentrations of these proteins to certain neurologic conditions in humans and in horses (Idland et al., 2017, Takei, 1992). Most of the aforementioned neurologic conditions, in humans or animals, are age related and it is not clear whether the observed association with pNF-H was confounded by the age of the study units—either humans or animals. Hence, it is imperative to investigate whether an association between serum concentrations of pNF-H and the likelihood of neurologic disorders in horses is likely to be confounded by the age of the horse.

The potential confounding effect of age is plausible. It is common knowledge that the neurons degenerate and die with age, so as a consequence it is reasonable to expect a proportionate increase of the concentration of NFs with age. Neurofilaments are found in both the central and peripheral nervous system (Petzold, 2015). As a consequence of neuronal or axonal damage associated with the aging process or trauma, NFs are believed to be released into the extracellular space increasing the concentration of pNF-H in the CSF and serum (Petzold, 2015; Steinacker et al., 2016b, 2016a). Several studies have used this finding to develop biomarkers for neurodegenerative diseases and traumatic conditions in humans and experimental animals (Intan-Shameha et al., 2017; Kirkcaldie and Collins, 2016; Yilmaz et al., 2017).

This study showed that although there was a positive change in the relationship between age and serum pNF-H with age, the degree of change was not high. Reports in humans have demonstrated similar association based upon examination of CSF (Bjerke et al., 2014; Steinacker et al., 2016b, 2016a). The difference between our study and the aforementioned studies is that in the human studies the concentrations of pNF-H were measured in the CSF and not serum.

To the authors' knowledge, this is the first study to investigate the association between serum concentration of NFs and age in the horse. Since CSF and serum pNF-H concentrations have a direct proportional relationship, we looked at previous studies that evaluated the association between serum pNF-H concentrations and age in human patients. Although several studies demonstrated that the concentration of pNF-H in the CSF was associated with age-related neurodegeneration in cognitively healthy adults, other studies were not able to make similar conclusions (Idland et al., 2017; Vågberg et al., 2015). Most of these human studies examined the relationship between age and the CSF-NFs concentrations by assessing the deterioration in the whole-brain (Bjerke et al., 2014; Steinacker et al., 2016b, 2016a; Vågberg et al., 2015; Zetterberg, 2017). The consensus among those studies was that there are age-related changes in the human brain tissue that reflect the ageing process and that concentration of the NFs measured in the CSF demonstrated high correlation between the NF-L and NF-H. Whereas in two studies the age was biased towards elderly individuals, other studies patients' age range was skewed (Idland et al., 2017; Steinacker et al., 2016a, Takasaki et al., 2002). Interestingly, the human study populations included only mature subjects ranging from 20 to 70 years of age (Vågberg et al., 2015; Zetterberg, 2017). In our study, horse age ranged from 0.58 to 31 years of age (11.65 mean) and included juvenile individuals.

The vitamin E concentrations in serum are known to be associated with aging in several neurologic disorders in animals and humans (Divers et al., 2006; Hamishehkar et al., 2016). The criteria for including horses in the normal category included a cutoff point for vitamin E of concentrations > 1.5 ug/mL. This cut-off point was based both upon our clinical experience and experimental findings (Divers et al., 2006). In the final analysis for assessing the association between age and concentrations of vitamin E, only horses with vitamin E values recorded in the medical record were included in the study. Since there is an association between the NF concentrations and age, it is not unreasonable to hypothesize that the NF serum concentrations might be associated with serum vitamin E concentration. However, in our study population there was no significant association between serum concentrations of NF and serum vitamin E among the healthy horses. To the authors' knowledge there is no previous study that examined this association in blood samples from human or animals. The only study that indirectly investigated the relationship between vitamin E and pNF-H, did so by examining histopathological changes and concluded that there was no significant association (Takei, 1992). That observation is consistent with the findings of this study.

Our study demonstrated significant differences in the concentration of pNF-H among horses performing different levels of activity. Horses undergoing heavy exercise (Thoroughbred racing horses) had higher serum pNF-H concentration than horses undergoing light or moderate (draft, pleasure riding, event horses). Although there are no previous studies in animals that demonstrated an association between serum pNF-H concentrations and levels of activity, several studies in human subjects showed that the serum pNF-H concentrations are increased among competitive athletes

(Oliver et al., 2016; Shahim et al., 2017). These studies attributed the increase of pNF-H concentrations among performing athletes to increased likelihood of trauma, concussion, or injury. Given the relatively high level of training activity experienced by Thoroughbred racehorses, it is reasonable to suggest that the significantly higher serum levels of pNF-H found in Thoroughbred racehorses in this study may reflect an increased exposure to exercise-related trauma in comparison to pleasure or draft horses.

Recent studies have promoted the use of NFs as a diagnostic biomarker for neurologic conditions in animals and humans (Disanto et al., 2017; Intan-Shameha et al., 2017; Nishida et al., 2014; Steinacker et al., 2016b, 2016a; Toedebusch et al., 2017). In our study we explored the potential use of serum pNF-H concentrations to complement clinical observations and conventional diagnostic tests in the diagnosis of EMND and EPM in the horse. A definitive diagnosis for horses afflicted with these conditions requires histopathological examination of the spinal cord to detect pathognomonic lesions (Divers, T.J.; Mohammed, H.O.; Cummings, J.F.; Valentine, B.A.; De Lahunta, A.; Jackson, C.A.; Summers, 1994; Reed et al., 2016). Both conditions affect the neurons in the CNS and associated axons leading to the release of neurofilaments in the serum. This study demonstrated the value of using elevated concentrations of serum pNF-H as a biomarker to predict the probability of the diagnosis of EMND and EPM in neurologic horses.

Previous studies of the prognostic value of the neurofilament concentrations had proposed positive cut-off points for the diagnosis of the respective neurologic condition (Nishida et al., 2014; Steinacker et al., 2016b, 2016a). Unlike the previous studies, the authors propose the use of a probability approach for the interpretation of neurofilaments concentrations in the diagnosis of EMND or EPM. This approach is based upon two premises: First, both of these neurologic conditions are progressive in nature and may have subclinical and clinical phases in which the serum concentrations of neurofilaments would likely differ. The probability of the disease would be associated with the specific level of the serum pNF-H value for a particular patient. Second, it is envisaged that serum concentrations of neurofilaments would be only one of the parameters a clinician would use in the diagnostic work-up, including medical history and clinical examination to make a specific diagnosis of EMND or EPM.

Finally, it can be difficult for equine practitioners to make a differential diagnosis between hind leg lameness and neurologic disease, i.e., EPM. The inclusion of serum neurofilament concentrations in the diagnostic work-up of hind leg lameness of performance horses has the potential to aid the clinician in making an accurate differential diagnosis between EPM and a hindlimb orthopedic lameness, thus enabling evidence-based treatment of the condition.

In conclusion, our results showed that although serum concentrations of pNF-H increased slightly with the age of the horse, the degree of this increase was not statistically significant. Serum pNF-H concentrations were not affected by the concentration of vitamin E in the serum, nor did they vary with the sex of the horse. Finally, the serum pNF-H concentration did vary with the activity of the horse, with horses undergoing heavy activities had significantly higher pNF-H values in comparison to light and moderate activities.

Declaration conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Table 1. Distribution of serum phosphorylated neurofilament H (pNF-H) concentration among the different activities and factors investigated in healthy control horses (169).

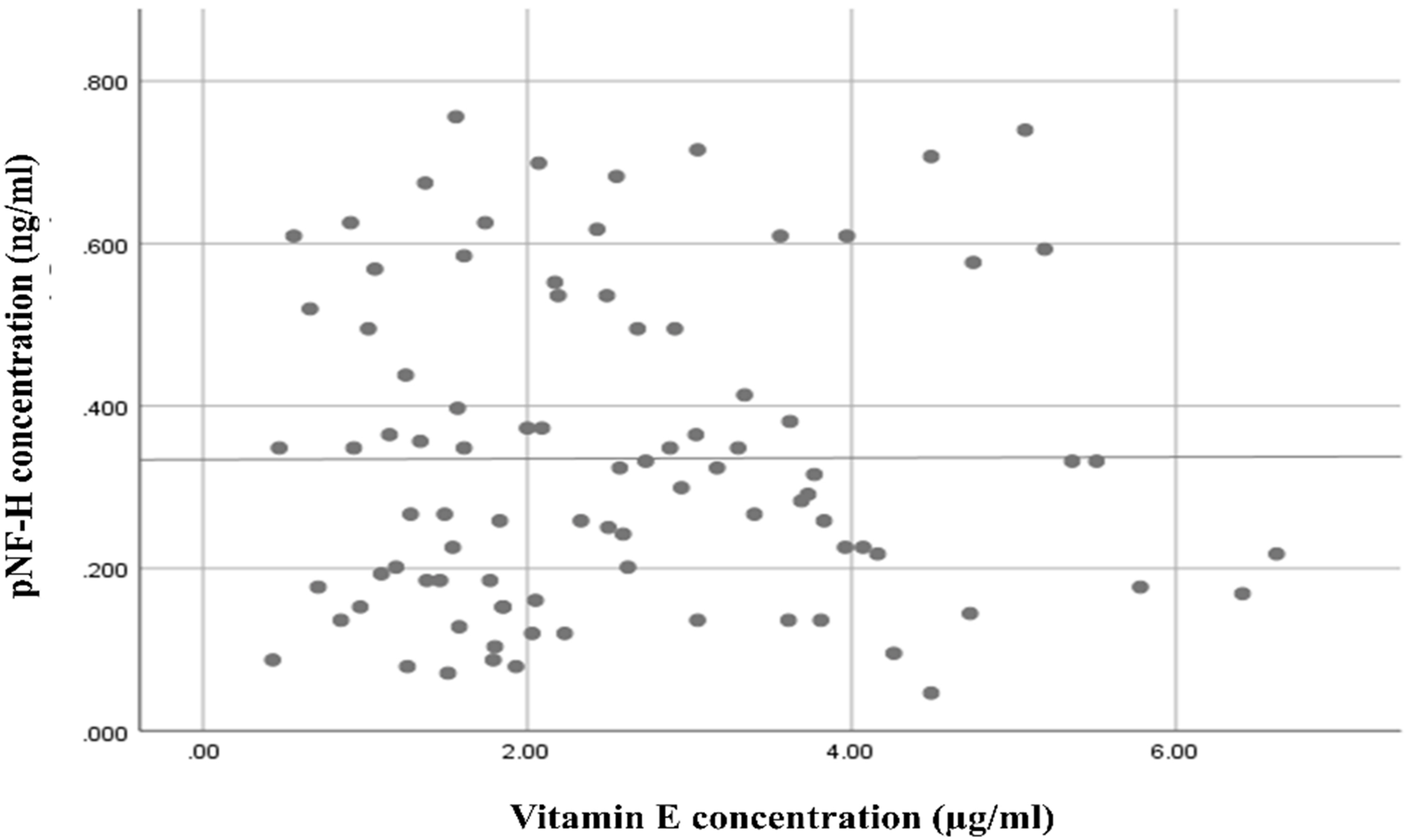
Table 2. Regression analysis results between the relationship of the horse age and the serum phosphorylated neurofilament H (pNF-H) concentration in healthy control horses.

Figure 1. The distribution of serum phosphorylated neurofilament H (pNF-H) concentration in horses without neurologic signs and the age of the horse.

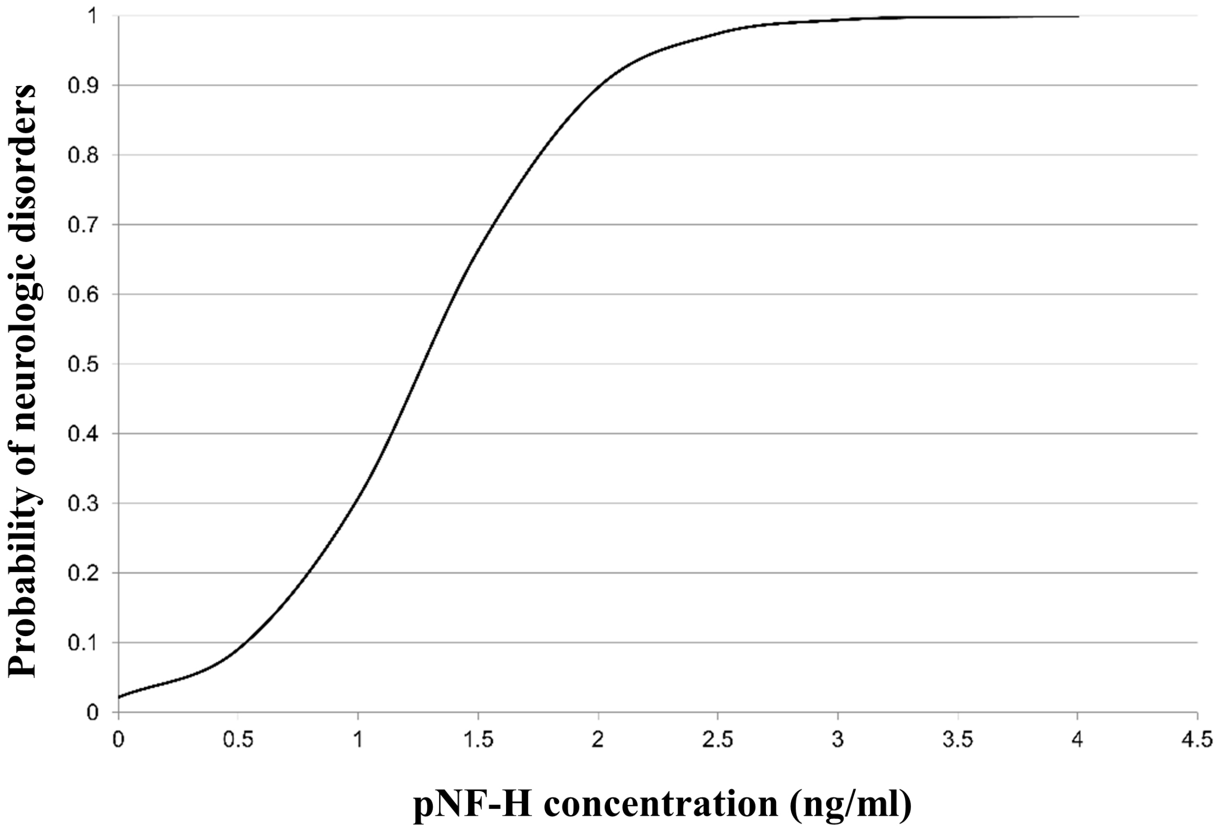
Figure 2. The distribution of serum phosphorylated neurofilament H (pNF-H) in horses without neurologic signs and vitamin E concentration.

Figure 3. The relationship between serum phosphorylated neurofilament H (pNF-H) concentration and the probability of neurologic disorders (EMND and EPM) as computed in the logistic regression analysis.

pNF-H concentration (ng/ml)
.800
.600
.400
.200
.000
.00
10.00
20.00
30.00
40.00
Age of horse in years



.800
.600
.400
.200
.000
.00
2.00
4.00
6.00
pNF-H concentration (ng/ml)
Vitamin E concentration (µg/ml)



Probability of neurologic disorders
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
pNF-H concentration (ng/ml)

Table 1. Distribution of serum phosphorylated neurofilament H (pNF-H) concentration among the different activities and factors investigated in healthy control horses (169).

Factors	**Mean***	**Standard error**	**95% Confidence interval**
pNF-H concentration (169)	0.278	0.015	0.248, 0.308
Type of horse activity/level of work			
Light (Pleasure)[ϕ] (124)	0.270[a]	0.017	0.236, 0.304
Moderate (Draft)[€] (27)	0.242[a]	0.041	0.162, 0.342
Heavy (Thoroughbred)[§] (18)	0.386[b]	0.015	0.278, 0.494
Sex of the horse			
Mare (71)	0.285	0.023	0.239,0.330
Gelding (89)	0.273	0.022	0.229, 0.317
Intact male (9)	0.271	0.060	0.133, 0.410
Vitamin E (μ/ml) (93)	2.565	1.496	2.268, 2.862
Age (years) (169)	11.65[¥]	0.52	0.58, 31.0

*: Means with different superscript letter are significantly different from each other
ϕ: Pleasure or trail horses
€ : Working draft horses or low-level eventer
§: Racing thoroughbred horses
¥: Mean age of the horses in the study

Table 2. Regression analysis results between the relationship of the horse age and the serum phosphorylated neurofilament H (pNF-H) concentration in healthy control horses.

Factor	**Regression coefficient**	**Standard error**
Type of horse activity/level of work		
Light (Pleasure)[ϕ] (124)	-0.130	0.041
Moderate (Draft)[€] (27)	-0.170	0.050
Heavy (Thoroughbred)[§] (18)	0.0	
Age*	0.002	0.002
Constant	0.359	0.055

*: Age was forced in the model
ϕ: Pleasure or trail horses
€: Working draft horses or low-level eventer
§: Racing thoroughbred horses