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STUDY ON NGF AND VEGF DURING THE EQUINE PERINATAL PERIOD

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ABSTRACT

The importance of trophic factors, such as nerve growth factor (NGF), vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF), during the perinatal period is now emerging. Through their functional activities of neurogenesis and angiogenesis, they play a key role in the final maturation of the nervous and vascular systems. Neonatal Encephalopathy (NE) may be caused by hypoxic ischemic or inflammatory insults and modified by innate protective or excitatory mechanisms. Understanding the underlying pathophysiology is important in formulating a rational approach to diagnosis. Part 1 of the study aims to: (i) evaluate NGF and VEGF levels obtained at parturition from the mare, foal and umbilical cord vein plasma, as well as in amniotic fluid; (ii) evaluate NGF and VEGF content in the plasma of healthy foals during the first 72 h of life (T0, T24 and T72); (iii) evaluate NGF and VEGF levels at parturition in relation to the selected mares' and foals' clinical parameters; (iv) evaluate the relationship between the two trophic factors and the thyroid hormone levels (TT3 and TT4) in the first 72 h of life; (v) assess mRNA expression of NGF, VEGF and BDNF and their cell surface receptors in the placenta. Part 2 of the study aims to clinically characterize a population of foals spontaneously affected by NE, and then to: (i) evaluate NGF and VEGF levels in plasma samples obtained in the affected population at parturition from the mare's jugular vein, umbilical cord vein and foal's jugular vein, as well as in amniotic fluid; (ii) evaluate NGF and VEGF content in the plasma of foals affected by NE during the first 72 h of life/hospitalization; (iii) evaluate NGF and VEGF levels at birth/admission in relation to selected mare's and foal's clinical parameters; (iv) evaluate the relationship between the two trophic factors and thyroid hormone levels (TT3 and TT4) in the first 72 h of life/hospitalization; (v) assess the mRNA expression of NGF, VEGF and BDNF, and their cell surface receptors, in the placenta of mares that delivered foals affected by NE. In Part 1, fourteen Standardbred healthy foals born from mares with normal pregnancies and parturitions were included, whereas in Part 2, thirteen affected foals born from mares hospitalized for *peripartum* monitoring (group NE) and twenty affected foals hospitalized after birth (group exNE) were included. The dosage of NGF and VEGF levels was performed using commercial ELISA kits, whereas NGF, VEGF and BDNF placental gene expression was performed using semiquantitative real-time PCR. Part 1 - In foal plasma, both NGF and VEGF levels decreased significantly over time, from T0 to T24 ($p = 0.0066$ for NGF; $p < 0.0001$ for VEGF) and from T0 to T72 ($p = 0.0179$ for NGF; $p = 0.0016$ for VEGF). In foal serum, TT3 levels increased significantly over time from T0 to T24 ($p = 0.0058$) and from T0 to T72 ($p = 0.0013$), whereas TT4 levels decreased significantly over time from T0 to T24 ($p = 0.0201$) and from T0 to T72 ($p < 0.0001$). A positive correlation was found in the levels of NGF and VEGF in foal plasma at each time point ($p = 0.0115$; $r = 0.2862$). A positive correlation was found between NGF levels in the foal plasma at T0 and lactate ($p = 0.0359$; $r = 0.5634$) as well as between VEGF levels in the foal plasma at T0 and creatine kinase ($p = 0.0459$; $r = 0.5407$). VEGF was expressed in all fetal membranes, whereas NGF and its receptors were not expressed in the amnion. Part 2 - In group NE, NGF levels decreased significantly from T0 to T24 ($p = 0.0447$) and VEGF levels decreased significantly from T0 to T72 ($p = 0.0234$), whereas in group exNE, only NGF levels decreased significantly from T0 to T24 ($p = 0.0304$). Compared to healthy foals, a significant reduction of TT3 levels was observed in both NE (T24, $p = 0.0066$; T72 $p = 0.0003$) and exNE (T0, $p = 0.0082$; T24, $p < 0.0001$; T72, $p < 0.0001$) groups, whereas a significant reduction of TT4 levels was observed only in exNE group (T0, $p = 0.0003$; T24, $p = 0.0010$;

T72, $p = 0.0110$). In group NE, NGF levels were positively correlated with both TT3 ($p = 0.0475$; r = 0.3424) and TT4 levels ($p = 0.0063$; r = 0.4589). In the placenta, a reduced expression of NGF in the allantois ($p = 0.0033$) and a reduced expression of BDNF in the amnion ($p = 0.0498$) were observed. The close relationship between the two trophic factors in foal plasma over time and their fine expression in placental tissues under physiological conditions appear to be key regulators of fetal development and adaptation to extra-uterine life. The less pronounced decrease of the two trophic factors in compromised foals compared to healthy ones, their relationship with thyroid hormones over time, and the reduced expression of NGF and BDNF in placental tissues of mares that delivered affected foals, could be key regulators in the mechanisms of equine NE.

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PREFACE

The end of pregnancy, the birth and the sudden need for the fetus to adapt to the extra-uterine environment characterize the perinatal period, which is considered eventful in all species. The many functional and developmental changes that occur during this period are probably greater than in any other stage of life and the final maturation of the nervous and vascular systems plays a key role in the adaptation of the fetus to extra-uterine life.

In addition to the normal maturational processes, there are also changes in the intra-uterine development and perinatal adaptation of the neonate in response to adverse conditions that alter the environment in utero, leading to ischemia/hypoxia/inflammation in the *prepartum* period (maternal ill health, nutrient deprivation, placental dysfunction) or at parturition (premature placenta separation, dystocia, cesarian section). As a result, the newborn foal could develop many behavioral abnormalities and other signs of neurologic dysfunction including alterations in respiratory function, changes in muscle tone, changes in responsiveness, vestibular signs, and autonomic disturbances, classified under the term Neonatal Encephalopathy (NE). Surprisingly, the prognosis in the foal compared with the human infant, in terms of short- and long-term neurological damage, is good.

Neurotrophins, including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), are known to play an important role as neuroprotective factors in pre- and post-natal brain development, making them of great interest for the diagnosis and treatment of several neurological disorders. Vascular endothelial growth factor (VEGF) is a potent mitogen, morphogen and chemo-attractant for endothelial cells and is widely recognized among trophic factors as the most potent stimulator of vasculogenesis and angiogenesis. It is also involved in several acute neurological disorders, such as cerebral ischemia, where it can mediate positive effects.

This study is the first to identify trophic factors in the equine perinatal period and to examine their changes in healthy and compromised foals, then the role of these changes in both physiological adaptations to extra-uterine life and pathophysiology of NE, with particular emphasis on the diagnostic and prognostic value of these novel biomarkers in the course of the disease.

Preliminary results of the present study were presented as an oral presentation by the authors at the 74th national SISVet congress (Italian Society of Veterinary Sciences), held in Italy on June 23-26, 2021. The entire study was finally published in 2022 in the form of two scientific papers in the international journal Veterinary Sciences (Q1; IF 2.518), as part of the special issue "Neuropeptides: role and function in species of veterinary interest".

The study has a strong translational bias and treats a purely spontaneous pathology of the equine neonate, which is particularly representative of clinical practice in a neonatal intensive care unit. The perinatal period was explored by the authors according to a compartmental model that should reflect feto-placental physiology (fetal membranes, amniotic fluid and umbilical vein plasma) and the physiology of the mother/neonate (mare and foal plasma). As a result, a biobank of clinical and biological materials was established on which innovative biomolecular diagnostic techniques were

developed. The future goal of the study is to lay the foundation for the development of new therapeutic protocols aimed at stimulating self-repair in regenerative and translational medicine.

In the first part of the present thesis, published under the title "Study on NGF and VEGF during the equine perinatal period—Part 1: Healthy foals born from normal pregnancy and parturition", the NGF and VEGF levels were measured in the plasma of mares with normal pregnancy and parturition, in the amniotic fluid, in the umbilical vein and in the plasma of their healthy foals in the first 72 h of life. To obtain a more complete picture, the trend of serum thyroid hormones in the first 72 h of life and the gene expression of *NGF*, *VEGF*, *BDNF* and their receptors in the fetal membranes were evaluated.

In the second part of the present thesis, published under the title "Study on NGF and VEGF during the equine perinatal period—Part 2: Foals affected by Neonatal Encephalopathy", a population of foals spontaneously affected by NE was clinically characterized. NGF and VEGF levels were measured in the plasma of mares diagnosed with placental insufficiency and/or dystocia, in the amniotic fluid, in the umbilical vein, in the plasma of their foals affected by NE (in the first 72 h of life) and in foals affected by NE admitted within 24 h after birth (in the first 72 h of hospitalization). The trend of serum thyroid hormones in the first 72 h of life/hospitalization and the gene expression of *NGF*, *VEGF*, *BDNF* and their receptors in the fetal membranes were also evaluated.

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Preliminary study on nerve growth factor in equine perinatal period

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No plasma biomarker is in current clinical use for foals with neonatal encephalopathy (NE) [1] and the role of Nerve Growth Factor (NGF) in the equine neonatal life has not been elucidated yet, although it plays a protective role in perinatal brain development [2]. The aims of the study were: (i) to examine NGF levels at parturition in amniotic fluid (AF), umbilical cord vein (UV) and foals' jugular vein (JV); (ii) to discuss NGF trend in plasma of healthy foals and foals affected by NE during the first 72h of life; (iii) to establish its role as diagnostic biomarker. Hypothetically, NGF levels should differ in foals affected by NE. Data were recorded from foals born from attended parturition or hospitalized within 24h of life. AF was collected by needle puncture of the amnion. EDTA plasma samples were obtained from UV and JV at 0, 24 and 72h from birth/admission (T0, T24, T72). Samples were analyzed by ELISA (Horse NGF ELISA, MyBiosource). The population was divided into healthy foals (group 1; N=10) and foals affected by NE (group 2; N=18). Unpaired t-test was performed to compare same parameters between groups, whereas paired t-test to compare different parameters within the same group. Pearson or Spearman test, based on data distribution, was used to evaluate biomarker frequency distribution among groups. In group 1, NGF levels were 162.9 ±40.9 ng/mL in AF, 154.8 ±29.5 ng/mL in UV, 130.7 ±59.1 ng/mL in JV at T0, 97.2 ±55.2 ng/mL in JV at T24 and 108.3 ±47.1 ng/mL in JV at T72. NGF levels in JV at T0 were negatively correlated with mares' age (P=0.035) and parity (P=0.036) and positively correlated with NGF levels in UV (P=0.002). In group 2, NGF levels were 150.7 ±35.3 ng/mL in AF, 156.8 ±45.9 ng/mL in UV, 127.1 ±48.3 ng/mL in JV at T0, 93.0 ±51.3 ng/mL in JV at T24 and 98.0 ±32.7 ng/mL in JV at T72. NGF levels in JV at T24 were positively correlated with APGAR score at birth (P=0.02). In both groups, NGF levels in JV decreased significantly from T0 to T24 (P=0.008 and 0.045, respectively). NGF levels in group 2 were lower at each time point than in group 1, but not significantly. Presumably, NGF is either transported from the mare or is produced by the placenta and seems to cross the utero-placental barrier to reach the neonatal brain through UV in a dependent manner. The source of NGF in AF and the influence of mares' age and parity on neonatal NGF levels are unknown. Increased utilization or reduced synthesis of NGF appears to characterize the first 24h of life. From a translational standpoint, due to the immature blood-brain barrier in the perinatal period, circulating low levels of NGF may reflect CNS levels, as already hypothesized in infants who have suffered from intrauterine hypoxia [3]. Although NGF appears to be an ideal biomarker, since it is stable, measurable with a high-sensitivity technique in an easy-to-access biological fluid and reaches a peak concentration early in life, it did not discriminate NE in the examined population.

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PART 1 – HEALTHY FOALS BORN FROM NORMAL PREGNANCY AND PARTURITION

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Article

Study on NGF and VEGF during the Equine Perinatal Period—Part 1: Healthy Foals Born from Normal Pregnancy and Parturition

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SIMPLE SUMMARY

The end of pregnancy, the birth and the sudden need for the fetus to adapt to the extra-uterine environment make the perinatal period eventful in all species. Trophic factors, such as nerve growth factor and vascular endothelial growth factor, as well as thyroid hormones, take part in the processes associated with the final maturation of the fetus. Our aim is to evaluate the levels of trophic factors and thyroid hormones obtained at parturition from a population of healthy mares and foals and in the first 72 h of foal life, as well as the expression of trophic factors in fetal membranes. The levels of both trophic factors decreased over time in foal plasma and a positive correlation was found between their levels at each time point, but no correlation was found with the thyroid hormone levels. Vascular endothelial growth factor was expressed in all fetal membranes, while nerve growth factor and its receptors were not expressed in the amnion. The close relationship between the two trophic factors in foal plasma over time and their fine expression in placental tissues appear to be key regulators of fetal development and adaptation to extra-uterine life.

ABSTRACT

The importance of trophic factors, such as nerve growth factor (NGF), vascular endothelial growth factor (VEGF), and brain-derived neurotrophic factor (BDNF) during the perinatal period, is now emerging. Through their functional activities of neurogenesis and angiogenesis, they play a key role in the final maturation of the nervous and vascular systems. The present study aims to: (i) evaluate the NGF and VEGF levels obtained at parturition from the mare, foal and umbilical cord vein plasma, as well as in amniotic fluid; (ii) evaluate NGF and VEGF content in the plasma of healthy

foals during the first 72 h of life (T0, T24 and T72); (iii) evaluate NGF and VEGF levels at parturition in relation to the selected mares' and foals' clinical parameters; (iv) evaluate the relationship between the two trophic factors and the thyroid hormone levels (TT3 and TT4) in the first 72 h of life; (v) assess mRNA expression of NGF, VEGF and BDNF and their cell surface receptors in the placenta. Fourteen Standardbred healthy foals born from mares with normal pregnancies and parturitions were included in the study. The dosage of NGF and VEGF levels was performed using commercial ELISA kits, whereas NGF, VEGF and BDNF placental gene expression was performed using semiquantitative real-time PCR. In foal plasma, both NGF and VEGF levels decreased significantly over time, from T0 to T24 ($p = 0.0066$ for NGF; $p < 0.0001$ for VEGF) and from T0 to T72 ($p = 0.0179$ for NGF; $p = 0.0016$ for VEGF). In foal serum, TT3 levels increased significantly over time from T0 to T24 ($p = 0.0058$) and from T0 to T72 ($p = 0.0013$), whereas TT4 levels decreased significantly over time from T0 to T24 ($p = 0.0201$) and from T0 to T72 ($p < 0.0001$). A positive correlation was found in the levels of NGF and VEGF in foal plasma at each time point ($p = 0.0115$; $r = 0.2862$). A positive correlation was found between NGF levels in the foal plasma at T0 and lactate ($p = 0.0359$; $r = 0.5634$) as well as between VEGF levels in the foal plasma at T0 and creatine kinase ($p = 0.0459$; $r = 0.5407$). VEGF was expressed in all fetal membranes, whereas NGF and its receptors were not expressed in the amnion. The close relationship between the two trophic factors in foal plasma over time and their fine expression in placental tissues appear to be key regulators of fetal development and adaptation to extra-uterine life.

Keywords: neonatal foal; pregnancy; parturition; equine perinatal period; amniotic fluid; umbilical cord vein; placenta; nerve growth factor; vascular endothelial growth factor; brain-derived neurotrophic factor; thyroid hormones

1. Introduction

The end of pregnancy, the birth and the sudden need for the fetus to adapt to the extra-uterine environment make the perinatal period eventful in all species. The many functional and developmental changes that occur during this period are probably greater than in any other stage of life and the final maturation of the nervous and vascular systems plays a key role in the adaptation of the fetus to extra-uterine life.

Nerve growth factor (NGF) gained scientific preeminence as the founding and bestcharacterized member of the neurotrophin family. Neurotrophins, including NGF and brain-derived neurotrophic factor (BDNF), were originally indicated as neuroprotective factors, due to their effect in reducing apoptosis and promoting the survival and maintenance of specific populations of neurons in both the peripheral and central nervous systems during pre- and post-natal brain development. They are important for axon growth during development [1], neuronal function [2], developmental maturity of the cerebral cortex and synaptic plasticity, leading to the refinement of the connections [3], morphologic differentiation and neurotransmitter expression [4]. The biological functions of the neurotrophins are mediated through two classes of cell surface receptors, the tyrosine kinase receptors (TRK) and the p75 neurotrophin receptors (p75NTR). NGF sends its survival signals

through activation of TRKA and can induce cell death by binding to p75NTR [5]. BDNF, via the corresponding TRKB receptor, is primarily present in immune cells, such as T cells and macrophages/microglia, and the number of BDNF-immunoreactive cells correlates well with lesion demyelinating activity [6]. Several other functional activities have, however, been attributed to neurotrophins and specifically to NGF, as suggested by NGF synthesis and/or expression of the high-affinity TRKA receptor in many cell types other than neurons, such as immune [7] and endocrine cells [8], endothelial cells and keratinocytes [9], and cardiomyocytes [10].

Although the equine NGF sequence has recently been identified in peripheral blood cells, the identification of NGF in the equine perinatal period has never been reported. In the study performed by Amagai et al. [11], there were no polymorphisms among the samples analyzed, and the neurotrophin showed more than 90% homology to human, mouse, rat, dog and bovine NGF, indicating that NGF-encoding is a highly conserved gene [11].

Vascular endothelial growth factor (VEGF) is a potent mitogen, morphogen and chemoattractant for endothelial cells and is widely recognized as the most potent stimulator of vasculogenesis and angiogenesis [12]. VEGF, and its two main receptor molecules, fms-like tyrosine kinase 1 (FLT1; VEGFR1) and kinase insert-domain containing receptor (KDR; VEGFR2), have been shown to be expressed in the endometrium and placenta of mares during pregnancy [13]. Although originally described as a key angiogenic factor, it is now well established that VEGF also plays a crucial role in the nervous system. FLT1 has a weak tyrosine kinase activity, stimulates postnatal angiogenesis through intracellular signaling and evidence is now emerging that it also exerts neuroprotective effects [14]. KDR has strong tyrosine kinase activity, stimulates vascular permeability and stimulates survival of various neural cell types in the nervous system [15].

The hypothalamus–pituitary–thyroid (HPT) axis has specific functions, mostly related to metabolic activities, cell differentiation and development, but thyroid hormones also take part in processes associated with the central nervous system. From the beginning of fetal life, they regulate and stimulate the proliferation and growth of neurons, synaptic formation and myelination [16]. Although a possible interaction of thyroid hormones with members of the neurotrophin family and their functional receptors has been suggested [17], to the authors' knowledge, the relationship between trophic factors and thyroid hormones in the equine perinatal period has never been investigated.

The biological nature of equine NGF and VEGF at foaling and in the early stages of equine neonatal life under physiological conditions has not yet been clarified. The aims of the present investigation, as a pilot study, are: (i) to evaluate NGF and VEGF levels in plasma samples obtained at parturition from the mare's jugular vein, the foal's jugular vein and the umbilical cord vein, as well as in amniotic fluid; (ii) to evaluate NGF and VEGF content in the plasma of healthy foals during the first 72 h of life; (iii) to evaluate the NGF and VEGF levels at parturition/birth in relation to the selected mares' and foals' clinical parameters; (iv) to evaluate the relationship between the two trophic factors and the thyroid hormone levels (TT3 and TT4) in the first 72 h of life; (v) to assess the mRNA expression of NGF, VEGF and BDNF and their cell surface receptors (p75 neurotrophin receptor and tropomyosin receptor kinase A for NGF; kinase insert domain receptor and fms-related receptor

tyrosine kinase 1 for VEGF; tropomyosin receptor kinase B for BDNF) in the placenta. This study is based on the hypothesis that NGF and VEGF, as well as signaling through their specific receptors and interaction with the HPT axis, represent key neuroprotective and angiogenic factors in fetal adaptation to extra-uterine life. The first part of the present study focuses on understanding the biological significance of their expression under physiological conditions for the regulation of pre- and post-natal development in the equine species. The levels of trophic factors and thyroid hormones at the time of birth and in the first 72 h of life well represent the transition of the healthy equine neonate to extra-uterine life.

2. Materials and Methods

2.1. Population

Fourteen Standardbred healthy foals born from healthy mares with normal pregnancy and parturition hospitalized at the Perinatology and Reproduction Unit (Equine Clinical Service, Department of Veterinary Medical Sciences) of the University of Bologna during the 2018 to 2021 foaling seasons were included in the study.

The mares were hospitalized at about 310 days of pregnancy because the owners requested an attended parturition. They were housed in separate wide straw-bedded boxes, fed hay ad libitum and concentrates twice a day, and were allowed to go to pasture during the day. At admission, a complete clinical evaluation, including complete blood count (ADVIA 2120 analyzer, Siemens Healthcare srl, Milan, Italy) and transrectal ultrasonography, were performed. Subsequently, the mares were clinically evaluated twice a day and by ultrasonography every 10 days until parturition. After delivery, macroscopic and histopathological examination of the placenta was performed in all mares.

Mares with a diagnosis of high-risk pregnancy [18,19], dystocia [20] or placental insufficiency [21,22] were excluded from the study.

At birth, a complete clinical evaluation, including complete blood count and serum biochemistry (AU 400 analyzer, Olympus/Beckman Coulter, Lismeehan, Ireland), was performed in all foals. The foals were monitored throughout the hospitalization period by a clinical examination performed every 6 h.

The foals born from normal pregnancy and birth were classified as healthy when they had an Apgar score ≥ 9 [23] and a normal clinical evaluation during the course of hospitalization, including a complete blood count and serum biochemistry at birth and an IgG serum concentration > 800 mg/dL (by immunoturbidimetric method; DVM Rapid Test II, MAI Animal Health, Elmwood, WI, USA) at 24 h of life.

The foals classified as affected by neonatal disease at birth or during the hospitalization period were excluded from the study.

2.2. Clinical Data and Sample Collection

The following data were recorded for each mare: age (years), parity, gestation length (days), length of stage II parturition (min), placenta/foal weight ratio (%), macroscopic and histopathological evaluation of the placenta.

The following data were recorded for each foal: sex, weight (kg), Apgar score at birth [23], blood glucose at birth (mg/dL) (Medisense Optium, Abbott Laboratories Medisense Products, Bedford, MA, USA), umbilical cord vein (UV) and jugular vein (JV) lactate concentrations at birth (mmol/L) (Lactate SCOUT+, Leipzig, Germany) as well as hematobiochemical parameters.

All biological samples were harvested as part of the clinical program of *peripartum* monitoring; owners gave written consent to use samples for research.

The amniotic fluid (AF) was collected within 5 min of the appearance of the amniotic sac, through the vulva by needle puncture of the amnion, using a 60 mL sterile syringe. The samples were immediately stored at −20 ◦C and analyzed within 6 months.

As soon as the foal was born, the umbilical structures were identified both visually and via manual palpation. The UV sample was taken as close to the foal's body wall as possible, using a 21 g butterfly needle attached to a vacuum system as well as K3 EDTA and serum (clot activator) tubes (Vacutest Kima, Arzergrande, Italy).

Blood was collected in K3 EDTA and serum tubes by jugular venipuncture in all mares at parturition (TP) and in all foals at each time point (at birth: T0; at 24 h of life: T24; at 72 h of life: T72). All blood samples were centrifuged at 3000 g for 10 min at 4◦C and aliquots of supernatant plasma/serum were collected, immediately stored at −20 ◦C and analyzed within 6 months.

Immediately after expulsion, the fetal membranes were weighed and subsequently placed on their side in an F shape to perform a macroscopic evaluation. To ensure an appropriate comparison between the different subjects, one placenta sample that was uniform in size (2 × 2 cm) was collected from each area of fetal membranes segments: body, pregnant horn, nonpregnant horn, cervical pole and amnion [24]. Samples were formalinfixed and paraffin-embedded. Routine histological hematoxylin- and eosin-stained slides were obtained.

For molecular biology investigations, in 8 of the 14 mares, two 0.5 × 0.5 cm samples were collected from the amnion and the body of the placenta at the base of the pregnant horn, near the umbilical cord attachment. The latter was manually separated into two portions: chorion and allantois. Samples were washed with sterile saline, frozen in liquid nitrogen for 60 s, stored at −80 ◦C and analyzed within 6 months.

2.3. Measurement of NGF and VEGF by ELISA

Dosage of NGF and VEGF levels was performed using commercial ELISA kits (MyBiosource, San Diego, CA, USA), which detect equine NGF and VEGF (Horse NGF/VEGF ELISA; NGF, Cod. MBS040618; VEGF, Cod. MBS035093). All samples were centrifuged at 4000 g for 10 min and at 4 ◦C prior to the assay and then analyzed according to the manufacturer's indications. Briefly, undiluted samples and standard curves were added to the wells and immediately mixed with HRP-conjugate.

After 60 min of incubation at 37 ◦C, the wells were washed four times and two chromogens were added in sequence. The stop solution was added after a second incubation (15 min at 37 ◦C) and the plate read within 15 min at 450 nm. Optical density values were interpolated on a linear standard curve using GraphPad Prism v 6.0. NGF standard curve ranges from 15.6 ng/mL to 500 ng/mL, whereas VEGF standard curve ranges from 31.2 pg/mL to 1000 pg/mL.

2.4. Measurement of Thyroid Hormones

Basal thyroid hormone levels of total triiodothyronine (TT3) and total thyroxine (TT4) from the mare JV, UV and the foal JV at T0, T24 and T72 were determined using the Siemen's Immulite TT3 and TT4 kits (Immulite Canine TT3 and TT4; Siemens, Oakville, ON, Canada) validated for use with equine serum at the Endocrine Laboratory, Prairie Diagnostic Services, Saskatoon, SK [25,26]. The analytical sensitivity of the TT3 Immulite assay is 0.54 nmol/L. Specificity data from the manufacturer regarding the anti-TT3 antibody identified a 100% cross-reaction with triiodo-L-thyronine, 100% with triiodo-D-thyronine, 1.3% with tetraiodothyroacetic acid and 0.7% with triiodothyroacetic acid. The analytical sensitivity of the TT4 assay was 0.15 nmol/L. Specificity data regarding the anti-TT4 antibody from the manufacturer identified a 100% cross-reactivity with L-thyroxine, 55% with D-thyroxine, 16% with tetraiodothyroacetic acid and 3.2% with triiodo-L-thyronine.

2.5. Placental Gene Expression

Chorion, allantois and amnion tissues were homogenized, and total RNA isolation was performed using RNeasy Microarray Tissue Mini Kit (Qiagen, Hilden, Germany, Cod. 73404) by the automated extractor QIAcube Connect (Qiagen).

Total RNA was eluted in RNase Free Water, and using a spectrophotometer (Nanodrop 2000, Thermo Scientific, Waltham, MA, USA, absorbance values at 260, 280 and 320 nm were measured. For the reverse transcription to generate the cDNA, the iScript™ gDNA Clear cDNA kit (Biorad, Hercules, CA, USA, Cod. 1725035BUN) was used, while semiquantitative real-time PCR was performed using the CFX96 real-time PCR system (BioRad, Hercules, CA, USA). The reactions were performed in a final volume of 20 µL consisting of SYBR Green qPCR master mix (BioRad, Cod. 1725274), 0.4 µM forward and reverse primers and nuclease-free water. The no-RT control was processed in parallel with the others and tested by real-time PCR for every primer pair. No template controls were added for each gene expression analysis. All primers were designed using Primer Blast software (NCBI, Bethesda, MD, USA) and synthesized by IDT (Coralville, IA, USA). Specific sequences of primers are listed in Table 1.

Table 1. List of the gene-specific primer sequences.

Genes	Primer Sequences (5′–3′)
NGF *Nerve growth factor*	Forward: GGGCCCATTAACGGCTTTTC Reverse: CATTGCTCTCTGTGTGGGGT
P75NTR *p75 Neurotrophin receptor*	Forward: GAGCCAACCAGACTGTGTGT Reverse: GGTAGTAGCCATAGGCGCAG
TRKA *Tropomyosin receptor kinase A*	Forward: GGAGCTGAGAAACCTCACCAT Reverse: GCACAGGAACAGTCCAGAGG
VEGF *Vascular endothelial growth factor*	Forward: AACGACGAGGGCCTAGAGT Reverse: CAAGGCCCACAGGGATTTTCT
KDR *Kinase insert domain receptor*	Forward: GATGACAACCAGACGGACAGT Reverse: TTTTGCTGGGCATCAGTCCA
FLT1 *Fms-related receptor tyrosine kinase 1*	Forward: CTGGCATCCCTGTAACCACA Reverse: AGGGTGCTAGCCGTCTTATTC
BDNF *Brain-derived neurotrophic factor*	Forward: CATGTCTATGAGGGTCCGGC Reverse: CATGTCCACTGCCGTCTTCT
TRKB *Tropomyosin receptor kinase B*	Forward: CGGGAACACCTCTCGGTCTA Reverse: CTGGACCAACACCTTGTCTTGA

Thermal profile of PCR reactions consisted first of a denaturation step (98 ◦C, 3 min) and 40 cycles of amplification (95 ◦C for 10 s and 60 ◦C for 1 min). At the end of the amplification cycles, the dissociation curve was obtained by following a procedure consisting of first incubating samples at 95 ◦C for 1 min to denature the PCR-amplified products, then ramping temperature down to 65 ◦C and finally increasing temperature from 65 ◦C to 95 ◦C at the rate of 0.5 ◦C/s, continuously collecting fluorescence intensity over the temperature ramp.

2.6. Statistical Analysis

The one-way ANOVA test was used to evaluate significant differences in biomarker levels at different collection times. Matched multiple comparison *versus* T0 was performed.

To evaluate correlations between parameters, Pearson or Spearman correlation coefficients were calculated, with Gaussian or non-Gaussian distribution, respectively.

NGF and VEGF levels were correlated in the foal's JV plasma, between mares and foals and with the data recorded for each mare at TP (age, parity, gestation length and placenta/foal weight ratio) and the following clinical and hematobiochemical parameters were recorded for each foal at T0: weight at birth, UV and JV lactate concentration, serum creatine kinase, total bilirubin, blood urea nitrogen, creatinine, magnesium and serum amyloid A.

A $p < 0.05$ was considered statistically significant. All statistical analyses were carried out using commercial software GraphPad Prism version 6.00.

3. Results

3.1. Population Characterization

The clinical data collected from the mares and foals included in this study are shown in Table 2. The clinical examinations performed during the hospitalization period were within normal limits at all time points, with all foals exhibiting normal immediate post-foaling behavior, including the ability to stand, nurse and pass meconium and urine.

Table 2. Clinical data collected from mares and foals born from attended parturition. *n* = number of animals. Data are expressed as mean ± standard deviation (min–max).

Mare			
Age (Years)	**Parity**	**Gestational Length (Days)**	**Stage II Labor Length (min)**
10.4 ± 5.1 (5–20)	3.8 ± 3.4 (1–12)	339.6 ± 9.2 (325–355)	11.9 ± 5.4 (6–25)
Foal			
Sex	**Weight (kg)**	**Apgar Score (0–10)**	**Placenta/Foal Weight Ratio (%)**
Males *n* = 5 Females *n* = 9	48.2 ± 5.7 (38–55)	9.4 ± 0.6 (8–10)	10.5 ± 1.4 (7.8–12.2)

The results of the complete blood count and serum biochemistry, including JV glucose and UV and JV lactate at T0 (measured through rapid methods) are summarized in Table S1 [27–30] in the Supplementary Materials. Macroscopic and histopathological evaluations of the placenta were also within normal limits in all mares.

3.2. Biomarkers (NGF, VEGF, TT3 and TT4) in Biological Fluids

NGF, VEGF, TT3 and TT4 were dosed in JV at each time point analyzed (0, 24 and 72 h from birth). Trends in plasma levels of NGF and VEGF and serum levels of TT3 and TT4 in foals in the first 72 h of life are illustrated in Figure 1. In foal plasma, both NGF and VEGF levels decreased significantly over time, from T0 to T24 ($p = 0.0066$ for NGF; $p < 0.0001$ for VEGF) and from T0 to T72 ($p = 0.0179$ for NGF; $p = 0.0016$ for VEGF). In foal serum, the TT3 levels increased significantly over time, from T0 to T24 ($p = 0.0058$) and from T0 to T72 ($p = 0.0013$), whereas TT4 levels decreased significantly over time, from T0 to T24 ($p = 0.0201$) and from T0 to T72 ($p < 0.0001$).

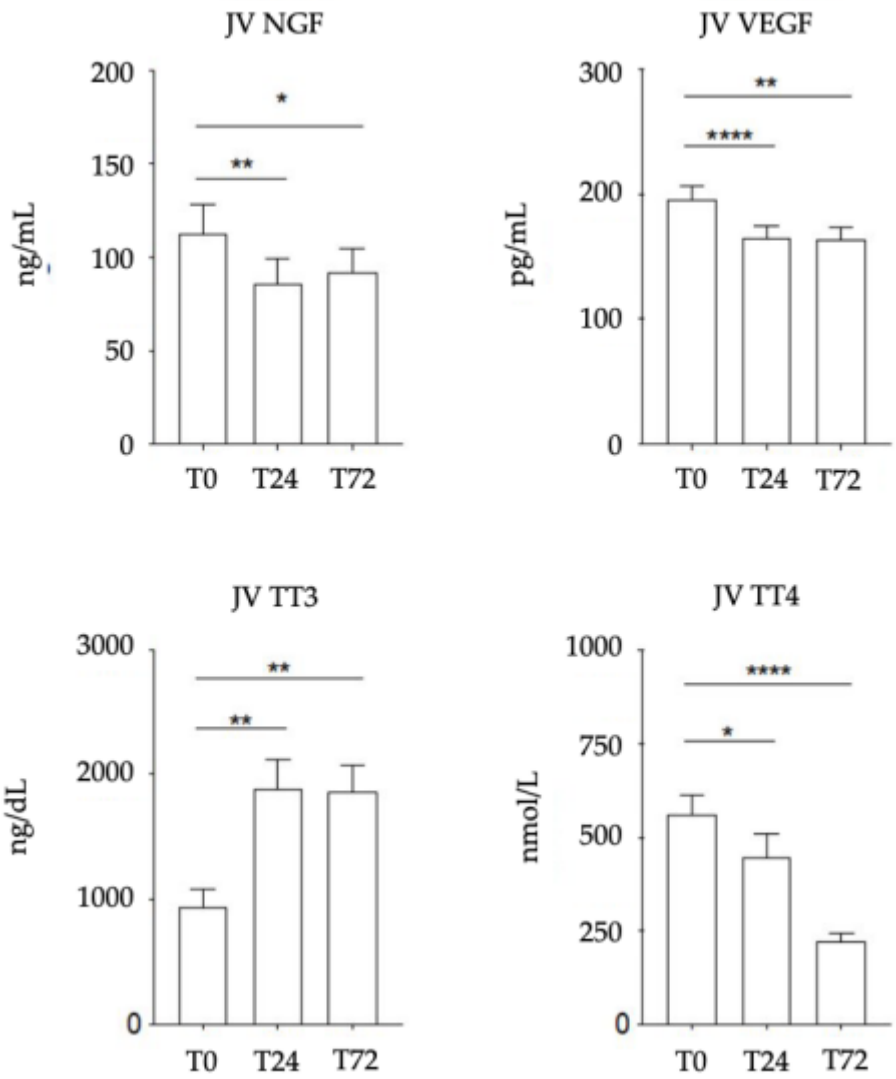

Figure 1. Time-dependent changes in plasma NGF–VEGF levels and serum TT3–TT4 levels in jugular vein (JV) of healthy foals. T0: birth; T24: 24 hours from birth; T72: 72 hours from birth. NGF, VEGF and TT4 levels decrease significantly from T0 to T24 and from T0 to T72, whereas TT3 levels increase significantly from T0 to T24 and from T0 to T72. Statistical analysis: one-way ANOVA, with the Geisser–Greenhouse correction. Adjusted *p* values JV NGF: * = 0.0179; ** = 0.0066; adjusted *p* values JV VEGF: ** = 0.0016; **** < 0.0001; adjusted *p* values JV TT3: ** = 0.0058; ** = 0.0013; adjusted *p* values JV TT4: * = 0.0201; **** < 0.0001.

Furthermore, as shown in Figure 2, a positive correlation was found in the plasma levels of NGF and VEGF in the foal's JV at each time point ($p = 0.0115$; $r = 0.2862$).

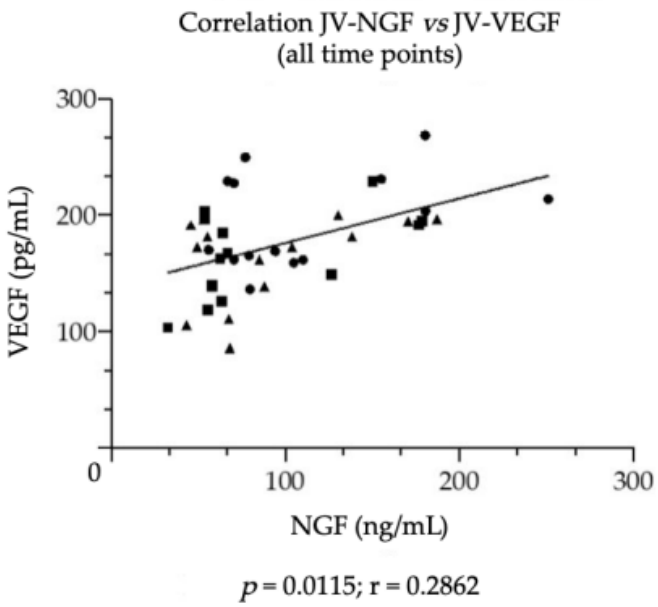

Figure 2. Correlation found between NGF and VEGF levels in plasma samples obtained from foal's jugular vein at three consecutive time points (round points T0: birth; square points T24: 24 h from birth; triangular points T72: 72 h from birth). Statistical analysis: non-parametric Spearman correlation. p = 0.0015; r = 0.2862.

Both NGF and VEGF were also dosed in all other matrices analyzed: amniotic fluid, plasma obtained from umbilical cord vein and plasma obtained from the mare's jugular vein at parturition. Overall, the results are summarized in Table 3.

Table 3. NGF–VEGF levels in amniotic fluid and in plasma samples obtained from umbilical cord vein, mare's jugular vein (JV) at parturition (TP) and foal's jugular vein (JV) at three consecutive time points (T0: birth; T24: 24 h from birth; T72: 72 h from birth); TT3–TT4 levels in serum samples obtained from umbilical cord vein, mare's jugular vein at parturition (TP) and foal's jugular vein at three consecutive time points (T0: birth; T24: 24 h from birth; T72: 72 h from birth). n = number of dosed samples; NA = data not available. Data are expressed as mean ± standard deviation (min–max).

	Amniotic Fluid	Umbilical Cord Vein	Mare's JV	Foal's JV		
			TP	T0	T24	T72
NGF (ng/mL)	146.8 ± 43.6 (95.4–233.0) (n = 14)	134.2 ± 46.8 (63.4–214.5) (n = 13)	101.9 ± 67.8 (38.3–208.4) (n = 8)	112.7 ± 57.5 (56.1–251.2) (n = 14)	85.9 ± 49.6 (32.6–178.8) (n = 14)	91.9 ± 47.6 (43.2–187.3) (n = 14)
VEGF (pg/mL)	268.1 ± 17.63 (239.6–294.2) (n = 12)	192.6 ± 32.0 (131.3–231.1) (n = 13)	134.0 ± 26.3 (80.8–162.9) (n = 8)	196.3 ± 40.7 (136.4–268.6) (n = 14)	164.9 ± 36.9 (103.5–229.4) (n = 14)	163.9 ± 38.2 (85.8–200.3) (n = 14)
TT3 (ng/dL)	NA	409.2 ± 102.8 (252.0–584.0) (n = 12)	59.8 ± 15.5 (40.0–87.8) (n = 8)	936.2 ± 550.1 (362.0–1828.0) (n = 14)	1888.1 ± 861.3 (552.0–3528.0) (n = 14)	1858.6 ± 755.7 (412.0–3128.0) (n = 13)
TT4 (nmol/L)	NA	585.9 ± 280.2 (336.0–1180.0) (n = 12)	15.5 ± 4.4 (12.9–20.6) (n = 8)	563.6 ± 180.2 (292.0–848.0) (n = 14)	446.8 ± 230.8 (198.0–951.0) (n = 14)	223.1 ± 71.5 (97.7–363.0) (n = 13)

No correlations were found between the NGF and VEGF levels in the amniotic fluid, the plasma obtained from the umbilical cord vein and the plasma obtained from the mare's jugular vein at parturition. Moreover, no correlation between NGF and VEGF mares and foals levels was found.

3.3. Equine NGF/VEGF and Clinical Data

As show in Figure 3, a positive correlation was found between NGF levels in the foal's JV at T0 and the lactate concentration at T0 ($p = 0.0359$; r = 0.5634) and between VEGF levels in the foal's JV at T0 and the serum creatine kinase at T0 ($p = 0.0459$; r = 0.5407).

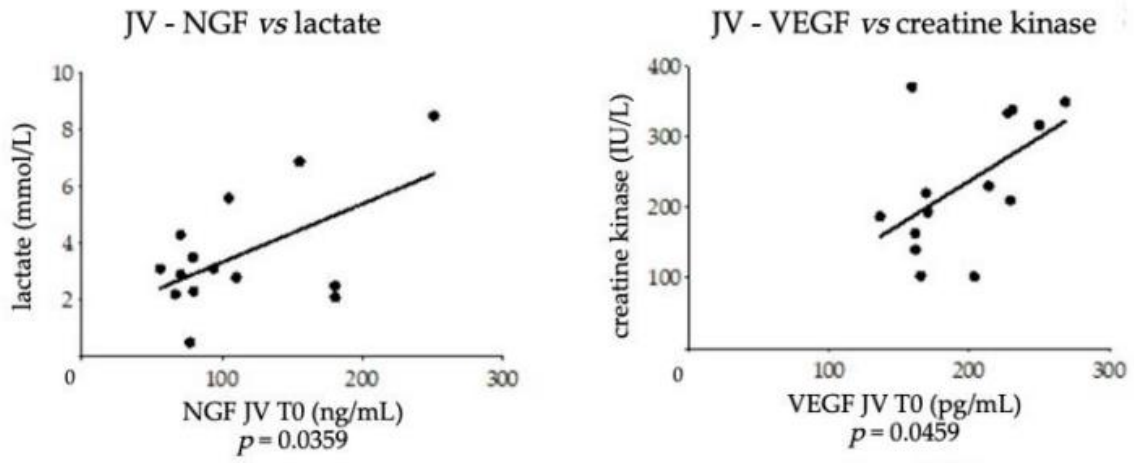

Figure 3. Significant correlations found between NGF and VEGF levels in plasma samples obtained from foal's jugular vein and hematobiochemical parameters at T0. Statistical analysis: parametric Pearson correlation (JV–NGF vs. lactate and JV–VEGF vs. creatine kinase).

3.4. Equine NGF, VEGF, BDNF and Their Receptors Gene Expression in the Placenta

The gene expression of NGF, VEGF and BDNF is shown in Table 4. In the chorion, the three neurotrophins NGF, VEGF and BDNF were expressed, as well as NGF and VEGF receptors, while only the BDNF receptor TRKB was not expressed. Additionally, in the allantois, the neurotrophins were all expressed, together with the NGF low-affinity receptor p75NTR and the two VEGF receptors KDR and FLT1, while the high-affinity NGF receptor TRKA and the BDNF receptor TRKB resulted not detectable. In the amnion, VEGF and both its receptors KDR and FLT1 were expressed, as well as BDNF; however, NGF and both its receptors p75NTR and TRKA, together with the BDNF receptor TRKB, were not expressed.

Table 4. Gene expression of NGF, VEGF, BDNF and their receptors in the three portions of placenta: chorion, allantois and amnion. Data are shown as gene expressed (exp) or not expressed (ne). Genes were indicated as expressed (exp) if they were amplified with a Cq ≤ 37. p75NTR = p75 neurotrophin receptor; TRKA = tyrosine kinase receptor A; KDR = kinase insert domain containing receptor; FLT1 = fms-like tyrosine kinase 1; TRKB = tyrosine kinase receptor B.

	Ligand	**Receptors**		**Ligand**	**Receptors**		**Ligand**	**Receptor**
	NGF	*p75NTR*	*TRKA*	*VEGF*	*KDR*	*FLT1*	*BDNF*	*TRKB*
Chorion	exp	exp	exp	exp	exp	exp	exp	ne
Allantois	exp	exp	ne	exp	exp	exp	exp	ne
Amnion	ne	ne	ne	exp	exp	exp	exp	ne

4. Discussion

Compared to the human species, little is known about the physiology of trophic factors in the equine perinatal period. NGF and VEGF are pleiotropic molecules which exert a wide variety of effects on different body districts and cell types, both during development and in adulthood. In the first part of this study, the NGF and VEGF levels were measured in the plasma of mares with normal pregnancy and parturition, in the amniotic fluid, in the umbilical vein and in the plasma of their healthy foals in the first 72 h of life. To obtain a more complete picture, the trend of serum thyroid hormones (TT3 and TT4) in the first 72 h of life and the gene expression of NGF, VEGF, BDNF and their receptors (TRKA, p75NTR, FLT-1, KDR, TRKB) in the fetal membranes were evaluated. The compartments explored in this study should reflect feto-placental physiology (fetal membranes, amniotic fluid and umbilical vein plasma) and the physiology of the mother/neonate (mare and foal plasma).

Higher amounts of NGF than VEGF were found in mare and foal plasma, umbilical vein plasma and amniotic fluid, but both trophic factors decreased significantly in the first 72 h of life in the plasma of the neonatal foals. This decline is most consistent for VEGF. Experimental and human clinical studies indicate that many body districts and cell types synthetize neurotrophic factors [7–10]; thus, it is reasonable to assume that circulating NGF and VEGF levels could be in part of fetal and neonatal origin. The most interesting aspect was perhaps the correlation that exists between the NGF and VEGF levels in the foal plasma at each time point. The close relationship between the two trophic factors has never been found in experimental or clinical studies before. The significance of this positive correlation should probably be investigated in light of the NGF and VEGF roles in the brain compartment. From a translational point of view, studies conducted on human perinatology showed that the blood levels of neurotrophins, including NGF and BDNF, are similar to those in the brain [31] and that the neural tube, from which the brain and spinal cord develop, becomes vascularized by a process involving VEGF [32]. In addition to its role in directing vessel sprouting, VEGF also regulates neuronal cell migration in the central nervous system in a mouse model [33].

Although the source of trophic factors in equine amniotic fluid is still unknow, explanatory fetal and placental mechanisms should be considered. Fetal urine can reasonably contain all molecules which cross the blood–brain barrier, including NGF, and changes to the brain can be reflected in urine [34].

Since fetal urine is a major component of the amniotic fluid during late gestation in equine species [35], it is reasonable to assume it may also be a source of NGF and VEGF in the amniotic fluid. This may seem especially true for NGF due to its lack of expression in the amnion.

The umbilical cord vein may contain blood components of placental and maternal origin; in fact, it is reasonable to assume that trophic factors such as NGF and VEGF cross the utero–placental barrier to reach the fetal compartment [36]. The lack of correlation between maternal, umbilical cord vein and neonatal plasma levels at parturition does not make it possible to confirm the possible transfer of these factors between the maternoplacental and neonatal circulation. This result is probably related to the participation of the fetus in the regulation of these pathways.

The few studies which have been performed on the human perinatal period under physiological conditions are related to the amniotic fluid and the maternal/neonatal plasma levels. It has been reported that NGF in the human amniotic fluid increases with gestational age [37], and that NGF is the only neurotrophin correlated to birthweight [31]. The maternal and umbilical cord plasma NGF levels, as well as the VEGF levels in the amniotic fluid, were correlated with fetal growth, with likely implications for postnatal neurodevelopment [38,39]. In the present study, no correlations were observed between plasma NGF and VEGF levels and the selected mares' and foals' clinical parameters, probably due to the low number of samples and high individual variability. In contrast to the reports in human literature, NGF is not correlated to birthweight in the equine species [31]. The correlation of plasma levels of NGF and VEGF in the foal with hematobiochemical parameters at birth, however, showed significant indices. From a clinical perspective, NGF correlated positively with blood lactate concentrations in healthy neonatal foals. Lactate could be evaluated to monitor the postpartum period and to indicate the need for prompt intervention, since it is the end-product of both aerobic and anaerobic glucose metabolism [28]. Although the blood lactate concentration may be physiologically elevated in the first 12 h of life, hyperlactatemia is produced in the event of hypoxia and poor tissue perfusion [40]. VEGF correlated positively with the serum creatine kinase (CK) level; this enzyme is present in the heart, skeletal muscles and brain. A mild elevation of CK level at birth is natural in neonatal foals, and the possible causes of this elevation are pressure in the birth canal and mild muscle damage from reactive oxygen [41]. However, hypothetical and speculative conclusions concerning correlations with blood parameters need to be critically evaluated in future investigations, since none of these parameters were highly correlated.

Although the thyroid function of the fetus improves steadily from mid-gestation, maternal thyroid hormones are required until the end of the pregnancy. Under physiological conditions, this study showed the serum TT3 and TT4 levels were several times higher in neonate foals at birth than in their mares, but similar to those of the umbilical cord vein. They cross the placental barrier to enter the fetal blood, and subsequently the blood–brain barrier (BBB) and blood–cerebrospinal fluid barrier [16]. Transplacental transport is mediated by transporters in the cell membrane and thyroid hormone-binding proteins in trophoblast cells, and during passage through the BBB they are captured by endothelial cells. T4 then enters astrocytes and is converted into T3 and transported to neurons [16]. The increasing trend in TT3 levels recorded in foal serum in the first 72 h of life is in agreement with a previous study that used the same time sampling to investigate thyroid function

[42]. In accordance with Pirrone et al. [42], the serum TT4 levels in foals decreased significantly in the first 72 h of life. Such high levels of thyroid hormones at birth can only be detected in neonate foals. They are higher than in any other species at any physiological stage and are responsible for their high thermogenic capacity and remarkable rapidity of growth during the perinatal period, especially of the nervous system [42]. From a translational perspective, several studies suggest the possible interaction of thyroid hormones with members of the neurotrophin family and their functional receptors, not only during brain maturation, but also during brain maintenance [17]. Although experimental studies have long since confirmed that thyroid hormones mediate direct effects on NGF-induced expression in the brain of neonatal mice [43] and in the cerebellum of perinatal rats [44], in the present study a relationship between trophic factors and thyroid hormones was not found in healthy foals.

Concerning fetal membranes, the mare exhibits a diffuse epitheliochorial placenta. At term, the extensive allantochorion consists of no more than a single thin layer of low columnar-to-cuboidal trophoblast cells which overlies and provides the essential structural framework for an incredibly densely packed mass of fetal capillaries supported in minimal amounts of allantoic mesoderm [13]. The amnion is the inner fetal membrane and consists of a cuboid epithelial layer that comes into direct contact with the amniotic fluid [45].

In the human species, NGF is reported to be present in the placenta [46] and mRNA expression has been demonstrated in the trophoblast, amnion/chorion and maternal decidua both early in gestation and at term [47]. In the equine species, NGF and its two main receptors, p75NTR and TRKA, are fully expressed in the chorion, whereas only the p75NTR receptor is expressed in the allantois. Surprisingly, they were not found to be expressed in the amnion, despite the NGF synthesis and/or expression of the high-affinity TRKA receptor in many cell types other than neurons, including endothelial cells [9]. In addition, a recent study also suggests that for a healthy human pregnancy, optimal NGF expression in the feto–maternal interface is essential [48], because it influences the process of angiogenesis as it exerts a potent angiogenic effect [49]. Prior to this, studies on neurotrophins in the placenta of domestic animals have never been conducted, and due to the profound differences between human and equine placenta, the authors cannot advance hypotheses on placental synthesis or excretion of NGF, but the main source of NGF in the amniotic fluid appears to be of fetal origin.

BDNF and its receptor TRKB were found in the uterus of many species, including the horse. They are co-expressed and co-localized in the glandular epithelium, luminal epithelium, vascular smooth muscle and myometrium of the mare during early pregnancy [50]. BDNF and TRKB have also been previously shown to activate the adhesion [51], angiogenesis [52], apoptosis [53] and proliferation [54] pathways, mainly in the brain and nervous system. Each of these pathways is also of paramount importance at the end of the pregnancy; however, little is known about the role of BDNF and TRKB in reproductive physiology. The results of the present study add limited but novel information on the topic. In the placenta of mares at term, BDNF was expressed in the chorion, allantois and amnion, whereas the TRKB receptor was not expressed in any of these portions. The lack of interaction between BDNF and the TRKB receptor observed in full-term mares may serve to inhibit the classic

BDNF–TRKB pathways, and also prevent nerve growth into placental tissue, which is, at the time of birth, soon degraded and removed in a cyclical manner. While the literature that supports BDNF expression during human pregnancy, particularly in the brain, is growing [55], its specific function is still unclear, but these results suggest that this signaling pathway is potentially important in normal equine pregnancy physiology.

VEGF, and its two major receptor molecules FLT1 and KDR, seem to be the principal vasculogenic and angiogenic factors. They are localized throughout most of the gestation on the two principal secretory cell types of the equine placenta, the glandular and luminal epithelia of the maternal endometrium and the trophoblast of the fetal allantochorion [13,56]. Our results also indicate that in the full-term mare, VEGF and its receptors are well expressed in the amnion. FLT1 stimulates postnatal angiogenesis through intracellular signaling and also exerts neuroprotective effects [14]. KDR stimulates vascular permeability as well as the survival of various types of neural cells in the nervous system [15]. Studies on pregnant sheep have shown that amniotic VEGF expression is regulated in vivo simultaneously with increased intramembranous uptake, qualifying VEGF as a candidate factor to influence amnion permeability [57]. It may therefore be reasonably postulated that, in the mare and in the fetus, VEGF, FLT1 and KDR together facilitate the development of maternal and fetal vascular and nervous networks for the interchange of nutrients and stimuli.

Some limitations of the study design should be noted. Neurotrophin signaling and regulation is really complex: each receptor can bind more than one ligand with varying affinity, multiple splice and transcript variants of ligands and receptors exist, several posttranslational modifications may be present, ligands are first translated as pro-proteins which bind receptors and ligands can exist as monomers or dimers. In addition, the circulating BDNF levels were not assessed in the population of mare and foal pairs but only in the placental tissues. Due to the lack of information on the physiology of trophic factors in the horses and the profound difference in terms of fetal development and placental structure between the equine and human species, references to other species are necessary to get a broader view of the topic.

5. Conclusions

The first part of the present study provided limited but novel insights into the role of NGF and VEGF in the equine perinatal period, considering that limited studies are available on humans as well. The close relationship between the two trophic factors in foal plasma over time and their fine expression in placental tissues are certainly key regulatory factors in fetal development and adaptation to extra-uterine life. The novel information obtained from the population of healthy mares and foals will now allow us to analyze the two trophic factors in sick neonates.

Supplementary Materials

Table S1. Foals complete blood cell counts, serum biochemistry, electrolyte concentrations and rapid determinations at birth. Data are expressed as mean ±standard deviation (min-max). For normal values refer to [27], [28], [29], and [30].

Haematology									
Haemoglobin g/dL	Haematocrit %	Erythrocytes 10^6/µL	Platelets 10^3/µL	Leucocytes 10^3/µL	Lymphocytes 10^3/µL	Monocytes cells/µL	Neutrophils 10^3/µL	Eosinophils cells/µL	Basophils cells/µL
16.0 ±0.8 (14.5-17.6)	48.3 ±2.1 (44.8-52.0)	11.2 ±0.6 (10.4-12.1)	197.8 ±39.8 (134-274)	8.3 ±1.1 (6.4-10.3)	1.4 ±0.4 (1.1-2.4)	218.6 ±71.6 (80-340)	6.6 ±1.0 (4.9-8.1)	15.7 ±12.2 (0-40)	82.9 ±153.4 (10-610)
Normal haematology values in one day old foals [27]:									
12.0-16.6	32-46	8.2-11.0	129-409	4.9-11.7	0.7-2.1	70-390	3.4-9.6	0-20	0-30
Serum biochemistry									
Creatine kinase IU/L	Total bilirubin mg/dL	Triglycerides mg/dL	Total protein g/dL	Albumin g/dL	Albumin / Globulin	Blood urea nitrogen mg/dL	Creatinine mg/dL	Fibrinogen g/L	Serum amyloid A µg/dL
232 ±93 (102-370)	2.4 ±0.8 (1.6-4.6)	11.1 ±6.3 (2-24)	4.1 ±0.2 (3.8-4.7)	3.3 ±0.3 (2.8-3.8)	4.3 ±1.2 (2.9-7.0)	35.2 ±4.7 (28.0-42.4)	2.6 ±0.4 (1.9-3.5)	1.6 ±0.2 (1.4-2.0)	7.9 ±7.0 (1-23)
Normal serum biochemistry values in one day old foals [28,29]:									
40-909	1.3-4.5	30-193	4.3-8.1	2.5-3.6		9-40	1.2-4.3	1-4	0-37
Electrolyte concentrations									
Phosphorus mg/dL		Calcium mg/dL		Sodium mg/dL	Potassium mg/dL		Chlorine mg/dL		Magnesium mg/dL
5.6 ±0.8 (4.1-6.9)		13.0 ±0.5 (12.3-14.2)		142.5 ±1.6 (140-145)	4.7 ±1.6 (4.0-5.7)		101.7 ±2.7 (96.9-104.9)		1.9 ±2.1 (1.7-2.1)
Normal electrolyte concentrations in one day old foals [28]:									
3.8-7.4		9.7-13.7		123-159	3.6-5.6		90-114		0.6-4.2
Rapid determinations									
		Jugular vein glucose mg/dL			Umbilical vein lactate mmol/L			Jugular vein lactate mmol/L	
		90.6 ±20.1 (51-127)			4.3 ±3.9 (0.5-15.1)			3.6 ±2.1 (0.5-8.5)	
		Normal determinations in foals at birth [28,30]:							
		67-99			3.2-4.7			2.3-5.0	

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PART 2 – FOALS AFFECTED BY NEONATAL ENCEPHALOPATHY

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Article

Study on NGF and VEGF during the Equine Perinatal Period—Part 2: Foals Affected by Neonatal Encephalopathy

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SIMPLE SUMMARY

Based on human medicine, Neonatal Encephalopathy is the term used by equine clinicians for newborn foals which develop a variety of non-infectious neurological signs in the immediate postpartum period. It has become the preferred term because it does not imply a specific underlying etiology or pathophysiology, as hypoxia and ischemia may not be recognized in all cases. Understanding the underlying pathophysiology is important in formulating a rational approach to diagnosis. Our aim is to clinically characterize a population of foals spontaneously affected by Neonatal Encephalopathy and to evaluate the levels of trophic factors, such as nerve growth factor and vascular epithelial growth factor, and thyroid hormones obtained at birth/admission from a population of affected foals and in the first 72 h of life/hospitalization, as well as the expression of trophic factors in the placenta of mares that delivered foals affected by Neonatal Encephalopathy. The less pronounced decrease of the two trophic factors compared to healthy foals, their close relationship with thyroid hormones over time, and the dysregulation of trophic factor expression in placental tissues, could be key regulators in the mechanisms of equine Neonatal Encephalopathy.

ABSTRACT

Neonatal Encephalopathy (NE) may be caused by hypoxic ischemic insults or inflammatory insults and modified by innate protective or excitatory mechanisms. Understanding the underlying pathophysiology is important in formulating a rational approach to diagnosis. The preliminary aim was to clinically characterize a population of foals spontaneously affected by NE. The study aimed to: (i) evaluate nerve growth factor (NGF) and vascular endothelial growth factor (VEGF) levels in plasma samples obtained in the affected population at parturition from the mare's jugular vein,

umbilical cord vein and foal's jugular vein, as well as in amniotic fluid; (ii) evaluate the NGF and VEGF content in the plasma of foals affected by NE during the first 72 h of life/hospitalization; (iii) evaluate NGF and VEGF levels at birth/admission in relation to selected mare's and foal's clinical parameters; (iv) evaluate the relationship between the two trophic factors and thyroid hormone levels (TT3 and TT4) in the first 72 h of life/hospitalization; and (v) assess the mRNA expression of NGF, VEGF and brain-derived neurotrophic factor (BDNF), and their cell surface receptors, in the placenta of mares that delivered foals affected by NE. Thirteen affected foals born from mares hospitalized for *peripartum* monitoring (group NE) and twenty affected foals hospitalized after birth (group exNE) were included in the study. Dosage of NGF and VEGF levels was performed using commercial ELISA kits, whereas NGF, VEGF, and BDNF placental gene expression was performed using a semi-quantitative real-time PCR. In group NE, NGF levels decreased significantly from T0 to T24 ($p = 0.0447$) and VEGF levels decreased significantly from T0 to T72 ($p = 0.0234$), whereas in group exNE, only NGF levels decreased significantly from T0 to T24 ($p = 0.0304$). Compared to healthy foals, a significant reduction of TT3 levels was observed in both NE (T24, $p = 0.0066$; T72 $p = 0.0003$) and exNE (T0, $p = 0.0082$; T24, $p < 0.0001$; T72, $p < 0.0001$) groups, whereas a significant reduction of TT4 levels was observed only in exNE group (T0, $p = 0.0003$; T24, $p = 0.0010$; T72, $p = 0.0110$). In group NE, NGF levels were positively correlated with both TT3 ($p = 0.0475$; r = 0.3424) and TT4 levels ($p = 0.0063$; r = 0.4589). In the placenta, a reduced expression of NGF in the allantois ($p = 0.0033$) and a reduced expression of BDNF in the amnion ($p = 0.0498$) were observed. The less pronounced decrease of the two trophic factors compared to healthy foals, their relationship with thyroid hormones over time, and the reduced expression of NGF and BDNF in placental tissues of mares that delivered affected foals, could be key regulators in the mechanisms of equine NE.

Keywords: placental insufficiency; dystocia; Neonatal Encephalopathy; nerve growth factor; vascular endothelial growth factor; brain derived neurotrophic factor; thyroid hormones

1. Introduction

Based on human medicine, Neonatal Encephalopathy (NE) is the term used by equine clinicians for newborn foals which develop a variety of non-infectious neurological signs in the immediate postpartum period. So far, the terms used included neonatal maladjustment syndrome, hypoxic-ischemic encephalopathy, hypoxic-ischemic syndrome, perinatal asphyxia syndrome, neonatal maladaptation syndrome, barkers, wanderers, convulsives, and dummies [1–11], but NE has become the preferred term because it does not imply a specific underlying etiology or pathophysiology [12], as hypoxia and ischemia may not be recognized in all cases.

The assessment of brain injury in newborn foals affected by NE currently relies on clinical examination. The disease in foals is recognized by many behavioral abnormalities and other signs of neurologic dysfunction including alterations in respiratory function, changes in muscle tone, changes in responsiveness, vestibular signs, and autonomic disturbances [13]. However, these tools are limited by their subjective nature and the expertise required for interpretation, and peripheral blood biomarkers which reflect end-organ injury may provide objective quantitative measures that

are free from these limitations [10]. Nevertheless, there are no blood-based biomarkers in current clinical use for foals with NE. Unlike in the human infant, the prognosis of foals with a single diagnosis of NE, in terms of short- and long-term neurological damage, is good [12].

Overall, equine NE can be the consequence of adverse *peripartum* events leading to ischemia/hypoxia/inflammation in the *prepartum* period (e.g., high-risk pregnancy, mares' systemic illness, placental insufficiency) and at parturition (e.g., premature placenta separation, dystocia, cesarian section) [12]. Causes of high-risk pregnancy in the equine species can be of maternal, fetal, or placental origin, and most placental conditions pose limited risks to the mare, but significant risks to the fetus [14]. Placental insufficiency is an ill-defined condition that has been identified in a large retrospective study as responsible for more than 60% of pregnancy losses due to abortion, stillbirth, and neonatal death [14,15]. Several factors contribute to placental insufficiency such as premature placental separation, placental villous hypoplasia, placental thickening, and placentitis [16–18].

Neurotrophins, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), are known to play an important role in pre- and post-natal brain development, making them of great interest for the diagnosis and treatment of numerous neurological disorders [19]. Vascular endothelial growth factor (VEGF) has also been implicated in several acute neurological disorders, such as in brain ischemia, where it can mediate positive effects according to three different mechanisms. VEGF can stimulate angiogenesis and modulate vascular permeability, exert direct neuroprotective effects, or promote neurogenesis [20]. In addition to its neuroprotective role, VEGF has also negatively been implicated in blood– brain barrier break-down after ischemia and in mediating inflammatory responses [21,22]. Recently, the authors measured NGF and VEGF levels in the plasma of mares diagnosed with normal pregnancy and parturition, in the amniotic fluid, in the umbilical cord vein, and in the plasma of their healthy foals in the first 72 h of life [23].

Reported laboratory abnormalities in foals affected by NE include low thyroid hormone concentrations [7]. Although not diagnostic, thyroid disfunction is a frequent condition in critically ill foals [24], and low thyroid hormones are associated with disease severity also in sepsis [25] and prematurity [26]. Although a possible interaction of thyroid hormones with members of the neurotrophin family has been investigated in the equine species under physiological conditions [23], to the authors' knowledge, the relationship between trophic factors and thyroid hormones in the equine NE is still under investigation.

As the NGF and VEGF levels at parturition and in the early stages of equine neonatal life under physiological conditions have been elucidated in the first part of the study [23], the role of the two trophic factors can now be investigated in a population of foals affected by NE. The preliminary aim of the present investigation, as a pilot study, was to clinically characterize a population of foals spontaneously affected by NE. Particularly (i) to evaluate NGF and VEGF levels in plasma samples obtained in the affected population at parturition from the mare's jugular vein, umbilical cord vein and foal's jugular vein, as well as in amniotic fluid; (ii) to evaluate NGF and VEGF content in the plasma of foals affected by NE during the first 72 h of life/hospitalization; (iii) to evaluate NGF and VEGF levels at birth/admission in relation to selected mare's and foal's clinical parameters; (iv) to evaluate the relationship between the two trophic factors and thyroid hormone levels (TT3 and TT4)

in the first 72 h of life/hospitalization; and (v) to assess the mRNA expression of NGF, BDNF, and VEGF and their cell surface receptors in the placenta of mares that delivered foals affected by NE. This study was based on the hypothesis that circulating NGF and VEGF levels, which theoretically reflect central levels and then cerebral status, should differ between healthy neonatal foals and foals affected by NE, and that trophic factors also play a key role in metabolic activities, cell differentiation and development through their interaction with the hypothalamus–pituitary–thyroid (HPT) axis.

2. Materials and Methods

2.1. Population

Thirteen sick foals born from mares hospitalized for *peripartum* monitoring and twenty sick neonatal foals hospitalized within 24 h after birth at the Perinatology and Reproduction Unit (Equine Clinical Service, Department of Veterinary Medical Sciences) of the University of Bologna during 2018–2021 foaling seasons were included in Part 2 of the study. The population of 14 healthy foals born from mares with normal pregnancy and parturition hospitalized for *peripartum* monitoring examined in Part 1 of the study [23] represented the healthy control group (group H).

Upon a pregnant mare's admission, a complete clinical evaluation, including blood count (ADVIA 2120 analyzer, Siemens Healthcare srl, Milan, Italy) and transrectal ultrasonography were performed. Based on the judgement of the clinician, transabdominal ultrasonography was performed when indicated. Subsequently, mares were clinically evaluated twice a day and by ultrasonography every 5–10 days until parturition. When an increase in the combined thickness of the uterus and placenta (CTUP) was observed, a cervical swab was performed to obtain a bacterial culture and a focused treatment was eventually started based on the clinician's judgment. After delivery, a macroscopic and histopathological examination of the placenta was performed in all mares.

High-risk pregnancy was defined as a history of premature udder development/lactation, an increase of CTUP, purulent/serosanguineous vulvar discharge, or the mare's systemic illness [15,18]. Dystocia was defined as any stage II impediment that could result from maternal or fetal causes or from the fetal membranes [27,28]. Diagnosis of placental insufficiency was performed retrospectively following macroscopic and histopathological examination of the placenta [14,18].

At birth, a complete clinical evaluation, including complete blood count and serum biochemistry (AU 400 analyzer, Olympus/Beckman Coulter, Lismeehan, Ireland), was performed in all foals born from attended parturition. Based on the judgment of the clinician, blood culture and arterial blood gas analysis (from dorsal metatarsal artery by CO-oximetry using the blood gas analyzer ABL 800 FLEX, Radiometer Medical ApS, Copenhagen, Denmark) were performed when indicated. In all sick foals admitted to the hospital after birth, a complete clinical evaluation, including blood culture, complete blood count, serum biochemistry, arterial blood gas analysis and serum IgG determination (by immunoturbidimetric method; DVM Rapid Test II, MAI Animal Health, Elmwood, WI, USA) were performed. The foals were monitored throughout the hospitalization period by a clinical examination performed every 2–6 h, depending on the severity of the existing conditions.

Sick foals were born from attended parturition or referred to the hospital after birth. The inclusion criteria were: (i) age at admission less than 24 h; and (ii) diagnosis of NE requiring level 1–3 of intensive care, based on the classification proposed by Koterba [29].

Sick foals were classified as affected by NE based on their history and clinical signs, especially those of neurological dysfunction [30], with the exclusion of other neurological diseases such as meningitis or trauma. Typical historical events included high-risk pregnancy, dystocia, and/or placental insufficiency, and common clinical signs included loss or absence of the suckle reflex, inappropriate teat-seeking behavior, dysphagia, hyperreactivity, and weakness [7]. Foals affected by NE and other diseases (i.e., sepsis, prematurity/dysmaturity, neonatal isoerythrolysis) were excluded from the study.

The population was then divided into two groups: 13 foals affected by NE born at the Perinatology and Reproduction Unit (group NE), and 20 foals affected by NE hospitalized within 24 h of life (group exNE).

2.2. Clinical Data and Sample Collection

The following data were recorded for each mare: breed, age (years), parity, clinical signs, ultrasonographic findings, cervical swab culture, *prepartum* treatment, gestation length (days), type of parturition (eutocic/dystocic), length of stage II parturition (min), placenta/foal weight ratio (%), macroscopic and histopathological evaluation of the placenta.

The following data were recorded for each foal born from attended parturition: breed, sex, weight (kg), Apgar score at birth [31], blood glucose at birth (mg/dL) (Medisense Optium, Abbott Laboratories Medisense Products, Bedford, MA, USA), umbilical cord vein (UV) and jugular vein (JV) lactate concentration at birth (mmol/L) (Lactate SCOUT+, Leipzig, Germany), hematobiochemical parameters, clinical signs of NE, organ dysfunctions associated with NE, level of intensive care [29], length of hospitalization (days), and outcome. The same data were recorded for the foals hospitalized after birth, except for the Apgar score and UV lactate concentration.

All biological samples were harvested as part of the clinical monitoring program of the Unit; owners gave written consent to use samples for research.

The amniotic fluid (AF), UV blood, and plasma/serum sampling from the mare's JV at parturition (TP) and from the foal's JV at each time point (at birth/admission: T0; at 24 h from birth/admission: T24; at 72 h from birth/admission: T72), as well as the macroscopic and histopathologic evaluation of the placenta, were collected as described in Part 1 of the study [23]. In 5/13 mares of group NE, placenta samples were collected for molecular biology investigations.

2.3. Measurement of NGF and VEGF by ELISA

As described in Part 1 of the study [23], dosage of NGF and VEGF levels was performed using commercial ELISA kits (MyBiosource, San Diego, CA, USA), which detect equine NGF and VEGF (Horse NGF/VEGF ELISA; NGF, Cod. MBS040618; VEGF, Cod. MBS035093). According to the kit's instructions, samples were centrifuged, incubated with primary antibody, and conjugated with microplate, with HRP-conjugate and two chromogens added in sequence. A sequence of four

washings was carried out before adding chromogens. Finally, the stop solution was distributed, and the plate read within 15 min at 450 nm.
Quantification was performed by interpolating the optical density values on a linear standard curve using the GraphPad Prism v 6.0. NGF standard curve ranges from 15.6 ng/mL to 500.0 ng/mL, whereas VEGF standard curve ranges from 31.2 pg/mL to 1000.0 pg/mL.
Each clinical group (H, NE and exNE) was represented in each analytical session.

2.4. Measurement of Thyroid Hormones

As described in Part 1 of the study [23], basal thyroid hormone levels of total triiodothyronine (TT3) and total thyroxine (TT4) from the mare JV, UV, and the foal JV at T0, T24, and T72 were determined using the Siemen's Immulite TT3 and TT4 kits (Immulite Canine TT3 and TT4; Siemens, Oakville, ON, Canada) validated for use with equine serum at the Endocrine Laboratory, Prairie Diagnostic Services, Saskatoon, SK [32,33]. The analytical sensitivity was 0.54 nmol/L for the TT3 assay and 0.15 nmol/L for the TT4 assay.

2.5. Placental Gene Expression

As described in Part 1 of the study [23], chorion, allantois, and amnion tissues were homogenized, and total RNA isolation was performed using the RNeasy Microarray Tissue Mini Kit (Qiagen, Hilden, Germany; Cod. 73404) by the automated extractor QIAcube Connect (Qiagen).
Total RNA was eluted in RNase Free Water and quantified through a spectrophotometer (Nanodrop 2000, Thermo Scientific, Waltham, MA, USA) measuring absorbance values at 260, 280, and 320 nm. cDNA was produced using the cDNA the iScript™ gDNA Clear cDNA kit (Biorad, Hercules, CA, USA; Cod. 1725035BUN).
A semi-quantitative real-time PCR was performed using the CFX96 real-time PCR system (BioRad, Hercules, CA, USA). The reactions were performed in a 20 µL final volume including SYBR Green qPCR master mix (BioRad, Cod. 1725274), 0.4 µM of forward and reverse primer mix, and nuclease-free water. The no-RT control was processed in parallel with the others and tested by the real-time PCR for every primer pair to check for eventual genomic DNA contamination, and no-template controls were also added for each gene expression analysis. All primers were designed using the Primer Blast software (NCBI, Bethesda, MD, USA) and synthesized by IDT (Coralville, IA, USA). Specific sequences of primers are listed in Table 1 in Part 1 of the study [23]. For the semi-quantitative analysis, the expression of the genes was normalized on the housekeeping gene β-actin (ACTB; NM_001081838.1).
The thermal profile of PCR reactions consisted first of a denaturation step (98 ◦C, 3 min) and 40 cycles of amplification (95 ◦C for 10 sec and 60 ◦C for 1 min), followed by the melting curves (55 ◦C to 95 ◦C, $\Delta t = 0.5$ ◦C/s).
Primer efficiency values for all primers were 95–100%, therefore the $2-\Delta\Delta$ Ct method was used to perform the analysis.

2.6. Statistical Analysis

In order to compare the population affected by Neonatal Encephalopathy (NE and exNE groups) with the healthy population (H group), all biomarkers were expressed as a percentage of the mean of the results obtained in the healthy group at each time point and a t-test was performed to reveal significant differences between the groups. When the biomarker levels of the three groups were compared, based on age at T0, only foals in the exNE group with 0–12 h at admission were compared with those in the H and NE groups.

To assess the correlations between the parameters, Pearson or Spearman correlation coefficients were calculated with Gaussian or non-Gaussian distributions, respectively.

NGF and VEGF levels were correlated in foals JV-plasma, between mares and foals and with the data recorded for each mare at TP (age, parity, gestation length, placenta/foal weight ratio) and the following clinical and haematobiochemical parameters, recorded for each foal at T0: weight at birth, UV and JV lactate concentration, serum creatine kinase, total bilirubin, blood urea nitrogen, creatinine, magnesium, and serum amyloid A.

A $p < 0.05$ was considered statistically significant.

All statistical analyses were carried out using commercial software GraphPad Prism version 8.00.

3. Results

3.1. Population Characterization

The clinical and histopathological data collected from mares hospitalized for attended parturition that delivered foals affected by NE (group NE) are shown in Table 1. Four/thirteen mares had a high-risk pregnancy associated with increased transrectal CTUP (three/four mares) and both increased transrectal CTUP and systemic illness (surgical colic at 282 days of gestation; one/four mares). In 8/13 mares, a diagnosis of placental insufficiency was reached on the basis of macroscopic and histopathological placenta evaluation. Specifically, placental insufficiency was associated with placental villous hypoplasia in 5/8 mares. Macroscopically, an extensive transition area between the normal chorionic surface and the hypoplasic/discolored surface of the chorioallantois was observed. The histological preparation of the chorioallantois stained with hematoxylin–eosin showed severe hypoplasia of the chorionic villi. In 3/8 mares, placental insufficiency was associated with placental edema. Macroscopically, generalized edematous and heavy fetal membranes, with an increased placenta/foal weight ratio, were observed. The histological section of the chorioallantois showed hyperemia and edema of the chorionic connective lamina associated with mild to severe hypoplasia of the chorionic villi. In 4/13 mares, the clinical condition of the neonate was associated with a dystocic parturition and in 1/13 mares the pregnancy, parturition, and placenta evaluation were apparently normal. As shown in the table, 3/13 mares (23%) from group NE were treated for increased transrectal CTUP with a negative cervical swab, using flunixin meglumine, 1.1 mg/kg, iv, q12h, and pentoxifylline, 8.5 mg/kg, po, q12h, until CTUP returned to normal. In addition, 1/10 mares from group NE received treatment for both increased transrectal CTUP with a negative cervical swab and surgical colic (sodium ampicillin, 20 mg/kg, iv, q8h; gentamicin sulfate, 6.6 mg/kg,

iv, q24h; flunixin meglumine, 1.1 mg/kg, iv, q12h; pentoxifylline, 8.5 mg/kg, po, q12h; altrenogest, 0.088 mg/kg, po, q24h).

Table 1. Clinical and histopathological data collected from the 13 mares hospitalized for attended parturition (group NE). SB = Standardbred; QH = Quarter Horse; WB = Warmblood; SD = Saddlebred; US = ultrasonographic; CTUP = transrectal combined thickness of the uterus and placenta; NA = data not available. Data are expressed as mean ± standard deviation (min-max).

Breed	Age (Years)	Parity	US Findings	Cervical Swab	Prepartum Treatment (Y/N)	Gest. Length (Days)	Dystocia (Y/N)	Stage II Labor Length (min)	Placenta/Foal Weight Ratio (%)	Macroscopic Placenta Evaluation	Histopathologic Placenta Evaluation	Mare's Diagnosis
SB (n = 10) QH (n = 1) WB (n = 1) SD (n = 1)	10 ± 3.7 (4–16)	2 ± 1.9 (1–7)	Normal (n = 9) Increased CTUP (n = 4)	Neg (n = 4) NA (n = 9)	Y (n = 4) N (n = 9)	337 ± 10.3 (326–359)	Y (n = 5) N (n = 8)	19 ± 13.5 (7–55)	11.1 ± 2.4 (8.2–16.7)	Villous hypoplasia (n = 4) Extensive edema (n = 3) Meconium-stained amnion (n = 1) Normal (n = 5)	Chorionic epithelium hypoplasia (n = 5) Severe edema (n = 3) Normal (n = 5)	Placental insufficiency (n = 8) Dystocia (n = 4) Apparently normal (n = 1)

The clinical data collected from 13 affected foals of group NE born from attended parturition are summarized in Table 2. The clinical data collected from 20 affected foals of group exNE hospitalized within 24 h of life, with an average age at admission of 10 h, are summarized in Table 3. Three/thirteen foals (23%) of NE group and three/twenty foals (15%) of exNE group were born from red bag delivery (premature placenta separation). Overall, 10/33 foals (30.3%) required level three of intensive care [29], which is provided to severely affected neonates. Foals were unable to stand and/or unable to nurse from the mare and need round-the-clock care; this level of care usually involves separation of the foal from the dam, oxygen therapy, parenteral nutrition, inotropes/vasopressor therapy, insulin therapy. Based on the level of intensive care and outcome, foals in the exNE group presented a more severe clinical condition.

Overall, these foals developed a wide range of neurological signs in the immediate postpartum period. Among the most frequent clinical signs of NE, the authors documented: depression (20/33 foals, 60.6%), hypoventilation (16/33 foals, 48.5%), lack of suckle reflex (16/33 foals, 48.5%), lack of affinity with the mare (14/33 foals, 42.4%), severe and prolonged dysphagia (11/33 foals, 33.3%), lateral recumbency (10/33 foals, 30.3%), tongue protrusion (7/33 foals, 21.2%), coma (4/33 foals, 12.1%), and hyperexcitability (4/33 foals, 12.1%). These signs were frequently accompanied by gastrointestinal (abdominal distension, ileus, gastric reflux, gastric ulceration, meconium retention; 13/33 foals, 39.4%), respiratory (abnormal respiratory patterns, dyspnea, hypoxemia; 13/33 foals, 39.4%), renal (oliguria; 11/33 foals, 33.3%), cardiovascular (hypotension, dysrhythmia; 10/33 foals, 30.3%), and metabolic (hypo/hyperglycemia; 6/33 foals, 18.2%) dysfunctions.

The results of the complete blood count, serum biochemistry and arterial blood gas analysis, including JV glucose and UV and JV lactate at T0 (measured through rapid methods) performed in both groups are shown in Table S1 [34–38] in the Supplementary Materials.

Table 2. Clinical data collected from 13 foals affected by Neonatal Encephalopathy born from attended parturition (group NE). SB = Standardbred; QH = Quarter Horse; WB = Warmblood; SD = Saddlebred; Sv = survived to hospital discharge; NSv = not survived; *n* = number of animals. Data are expressed as mean ± standard deviation (min-max).

Breed	Sex	Weight (kg)	Apgar Score (0–10)	Clinical Signs of NE	Organ Dysfunctions Associated with NE	Care Level (1–3)	Hosp. Length (Days)	Outcome
SB (*n* = 10) QH (*n* = 1) WB (*n* = 1) SD (*n* = 1)	Males (*n* = 10) Females (*n* = 3)	45 ± 9.7 (17–55)	7 ± 2.4 (1–10)	Depression (*n* = 7); severe and prolonged dysphagia (*n* = 7); hypoventilation (*n* = 5); lateral recumbency (*n* = 4); lack of affinity with the mare (*n* = 4), lack of suckle reflex (*n* = 4); hypothermia (*n* = 2); weakness (*n* = 2); disorientation (*n* = 1); tongue protrusion (*n* = 1)	Cardiovascular (*n* = 1) Respiratory (*n* = 2) Gastrointestinal (*n* = 5) Renal (*n* = 2) Metabolic (*n* = 2) None (*n* = 6)	Level 1 (*n* = 7) Level 2 (*n* = 4) Level 3 (*n* = 2)	12 ± 8.6 (4–30)	Sv (*n* = 12) NSv (*n* = 1)

Table 3. Clinical data collected from 20 foals affected by Neonatal Encephalopathy hospitalized within 24 h after birth (group exNE). SB = Standardbred; QH = Quarter Horse; SD = Saddlebred; AH = Arabian Horse; Sv = survived to hospital discharge; NSv = not survived; NA = data not available; *n* = number of animals. Data are expressed as mean ± standard deviation (min-max).

Breed	Sex	Age at Adm. (Hours)	Weight (kg)	Mare's History				Clinical Signs of NE	Organ Dysfunctions Associated with NE	Care Level (1–3)	Hosp. Length (Days)	Outcome
				Age (Years)	Parity	Gest Length (Days)	Other					
SB (*n* = 10) QH (*n* = 6) SD (*n* = 3) AH (*n* = 1)	Males (*n* = 12) Females (*n* = 8)	10 ± 6.4 (1–24)	42 ± 8 (17–56)	12 ± 5.2 (4–21)	3 ± 1.7 (1–6)	334 ± 12.5 (320–357)	Dystocia (*n* = 9) NA (*n* = 9) Placental insuff. (*n* = 2)	Depression (*n* = 13); lack of suckle reflex (*n* = 12); hypoventilation (*n* = 11); lack of affinity with the mare (*n* = 10); lateral recumbency (*n* = 6); tongue protrusion (*n* = 6); coma (*n* = 4); severe and prolonged dysphagia (*n* = 4); hyperexcitability (*n* = 4); head pressing (*n* = 3); convulsions (*n* = 2); barking (*n* = 2); chewing movements (*n* = 2); wandering (*n* = 2); hypothermia (*n* = 1); weakness (*n* = 1); facial hemiplegia (*n* = 1); sialorrhea (*n* = 1); tremors (*n* = 1); anisocoria (*n* = 1)	Cardiovascular (*n* = 9) Respiratory (*n* = 11) Gastrointestinal (*n* = 8) Renal (*n* = 9) Metabolic (*n* = 4) None (*n* = 5)	Level 1 (*n* = 2) Level 2 (*n* = 10) Level 3 (*n* = 8)	11 ± 5 (4–20)	Sv (*n* = 15) NSv (*n* = 5)

3.2. Biomarkers (NGF, VEGF, TT3 and TT4) in Biological Fluids

The NGF and VEGF plasma levels and the TT3 and TT4 serum levels found in the foal JV at 0, 24, and 72 h from birth, AF, UV, and mare JV at TP in group NE, and in the foal JV at 0, 24, and 72 h from admission in group exNE are shown in Table 4 (a,b,c). In group NE, NGF levels decreased significantly from T0 to T24 (p = 0.0447), VEGF levels decreased significantly from T0 to T72 (p = 0.0234) and TT4 levels decreased significantly from T0 to T72 (p = 0.0007). In group exNE, NGF levels decreased significantly from T0 to T24 (p = 0.0304) and TT4 levels decreased significantly from T0 to T24 (p = 0.0055) and from T0 to T72 (p = 0.0044).

Figures 1 and 2 represent the comparison of biomarkers *versus* healthy group. Data are expressed as a percentage of the mean of the results obtained in the healthy group at each time point and in each biological fluid analyzed. A significant reduction of TT3 levels was observed in both NE (T24, p = 0.0066; T72 p = 0.0003) and exNE groups (T0, p = 0.0082; T24, p < 0.0001; T72, p < 0.0001), whereas a significant reduction of TT4 levels was observed only in exNE group (T0, p = 0.0003; T24, p = 0.0010; T72, p = 0.0110).

Among the different biological fluids analyzed, no correlation was found in either the NE or exNE group, but in NE group NGF levels were positively correlated with both TT3 (p = 0.0475; r = 0.3424) and TT4 levels (p = 0.0063; r = 0.4589), as shown in Figure 3.

Table 4. Biomarker levels in: **a**; group NE—samples obtained from foal's jugular vein at three consecutive time points (T0: birth; T24: h from birth; T72: h from birth); **b**; group NE— samples obtained from amniotic fluid, umbilical cord vein and mare's jugular vein at parturition (TP); **c**; group exNE—samples obtained from foal's jugular vein at three consecutive time points (T0: admission; T24: h from admission; T72: h from admission). Data are expressed as mean ± standard deviation (min-max). *n* = number of samples analyzed; NA = data not available. * Asterisks indicate significant differences from T0, specific adjusted *p* values are listed below: *[1] $p = 0.0447$; *[2] $p = 0.0234$; ***[3] $p = 0.0007$; *[4] $p = 0.0304$; **[5] $p = 0.0055$; **[6] $p = 0.0044$.

a. NE	**T0**	**T24**	**T72**
NGF (ng/mL)	114.9 ± 47.9 (54.6–204.8) (*n* = 13)	93.0 ± 37.7 *[1] (39.8–181.2) (*n* = 12)	97.5 ± 39.8 (46.5–177.4) (*n* = 12)
VEGF (pg/mL)	199.3 ± 34.9 (150.2–258.4) (*n* = 13)	188.6 ± 54.8 (92.1–261.8) (*n* = 12)	179.8 ± 43.7 *[2] (95.9–231.1) (*n* = 12)
TT3 (ng/dL)	850.0 ± 826.4 (140.0–2172.0) (*n* = 12)	867.5 ± 830.9 (162.0–2324.0) (*n* = 11)	638.7 ± 618.8 (78.6–2008.0) (*n* = 11)
TT4 (nmol/L)	511.4 ± 220.0 (220.0–860.0) (*n* = 12)	386.0 ± 250.8 (123.0–776.0) (*n* = 11)	227.1 ± 98.5 ***[3] (66.0–440.0) (*n* = 11)
b. NE	**Amniotic fluid**	**Umbilical cord vein**	**Mare's jugular vein (TP)**
NGF (ng/mL)	139.4 ± 40.2 (61.2–201.9) (*n* = 10)	127.5 ± 57.0 (65.5–214.5) (*n* = 9)	102.2 ± 74.4 (43.5–193.4) (*n* = 5)
VEGF (pg/mL)	264.0 ± 48.4 (138.9–328.4) (*n* = 10)	218.1 ± 33.4 (151.5–261.8) (*n* = 8)	132.8 ± 26.5 (106.0–161.6) (*n* = 5)
TT3 (ng/dL)	NA	350.3 ± 145.0 (110.0–540.0) (*n* = 10)	69.3 ± 34.1 (40.0–118.0) (*n* = 4)
TT4 (nmol/L)	NA	501.4 ± 220.2 (221.0–800.0) (*n* = 10)	18.7 ± 11.5 (12.9–36.0) (*n* = 4)
c. exNE	**T0**	**T24**	**T72**
NGF (ng/mL)	82.6 ± 36.8 (56.8–176.9) (*n* = 13)	57.6 ± 14.6 *[4] (34.6–77.7) (*n* = 12)	59.0 ± 14.0 (42.8–84.7) (n = 9)
VEGF (pg/mL)	174.6 ± 15.0 (165.4–197.0) (*n* = 4)	150.6 ± 48.3 (88.3–204.6) (*n* = 4)	169.2 ± 56.4 (89.6–222.2) (*n* = 4)
TT3 (ng/dL)	368.1 ± 470.0 (60.3–1856.0) (*n* = 13)	279.8 ± 151.3 (99.7–552.0) (*n* = 13)	279.0 ± 111.4 (157.0–505.0) (*n* = 10)
TT4 (nmol/L)	302.5 ± 136.7 (57.8–500.0) (*n* = 13)	196.1 ± 75.2 **[5] (56.1–292.0) (*n* = 13)	142.0 ± 65.8 **[6] (46.6–282.0) (*n* = 10)

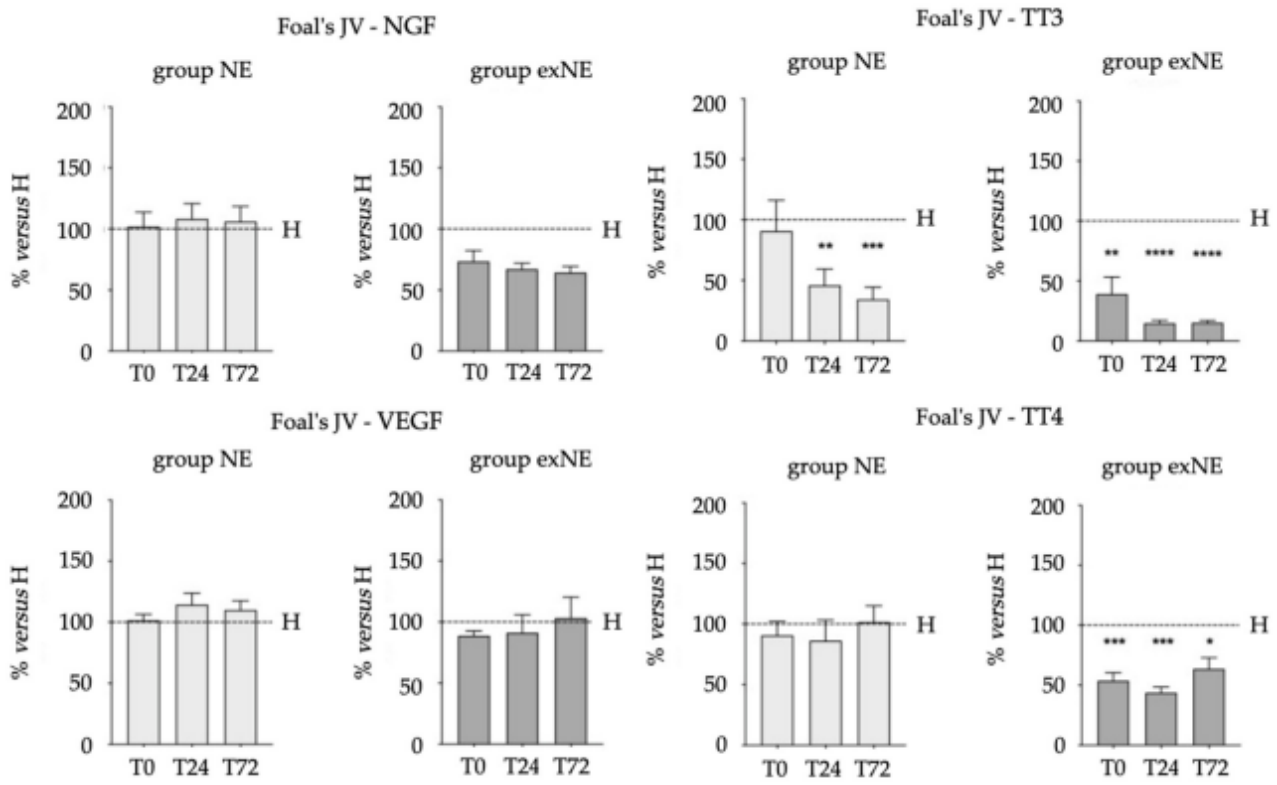

Figure 1. Biomarkers comparison between NE and exNE groups *versus* H group. Levels in samples obtained from foal's jugular vein at three consecutive time points (T0: birth/admission; T24: h from birth/admission; T72: h from birth/admission) are expressed as percentage of the mean of the results obtained in the healthy group at each time point. * p < 0.05; ** p < 0.01; *** p < 0.005; **** p < 0.001. Vet. Sci. 2022, 9, 459 13 Figure 1. Biomarkers comparison between NE and exNE groups *versus* H group. Levels in samobtained from foal's jugular vein at three consecutive time points (T0: birth/admission; T24: hbirth/admission; T72: h from birth/admission) are expressed as percentage of the mean of the reobtained in the healthy group at each time point. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$;

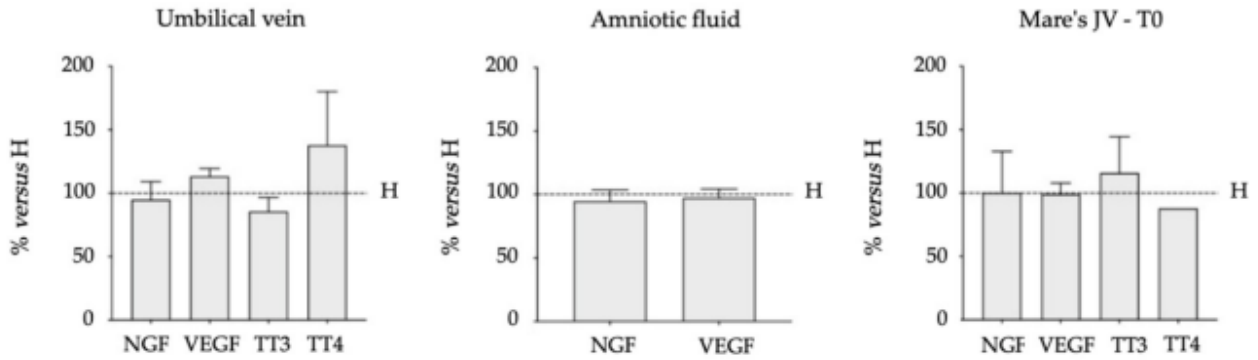

Figure 2. Biomarkers comparison between NE group *versus* H group. Levels in samples obtained from umbilical cord vein, amniotic fluid and mare's jugular vein (JV) at parturition (TP) are expressed as percentage of the mean of the results obtained in the healthy group (H) at each time point. No differences were observed between groups.

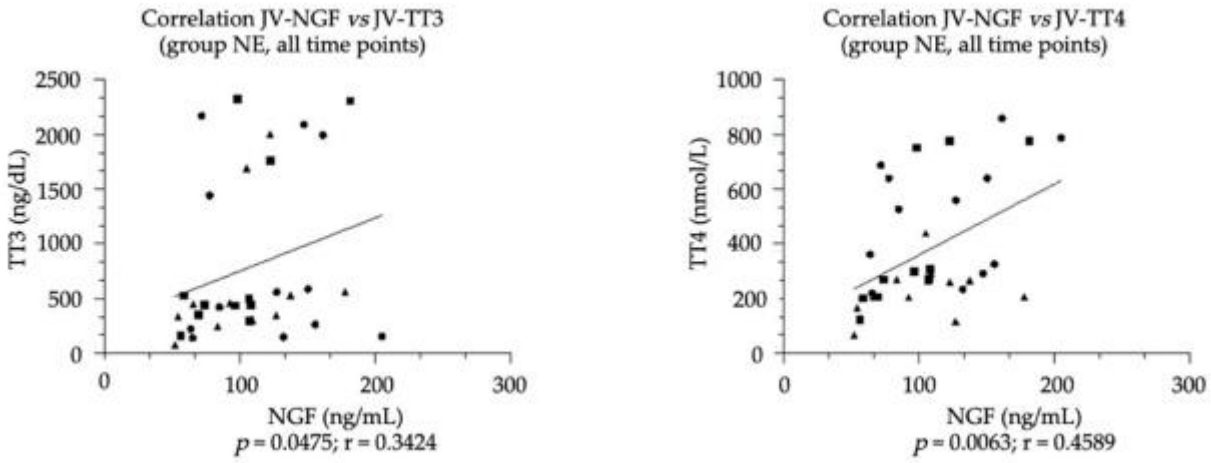

Figure 3. Correlation found in NE group between NGF plasma levels and TT3-TT4 serum levels in samples obtained from foal's jugular vein at three consecutive time points (T0: birth; T24: h from birth; T72: h from birth). The correlation between NGF and TT3 levels was calculated using Spearman's non-parametric correlation, whereas the correlation between NGF and TT4 levels was calculated using Pearson's parametric correlation.

3.3.Equine NGF/VEGF and Clinical Data

No significant correlations were found between NGF levels in the foal's JV at T0 and the data recorded for the mares at TP and the selected foal's clinical parameters at T0, but a significant negative correlation was found between VEGF levels in the foal's JV at T0 and the lactate concentration at T0 ($p = 0.0500$; r = −0.6444), as shown in Figure 4.

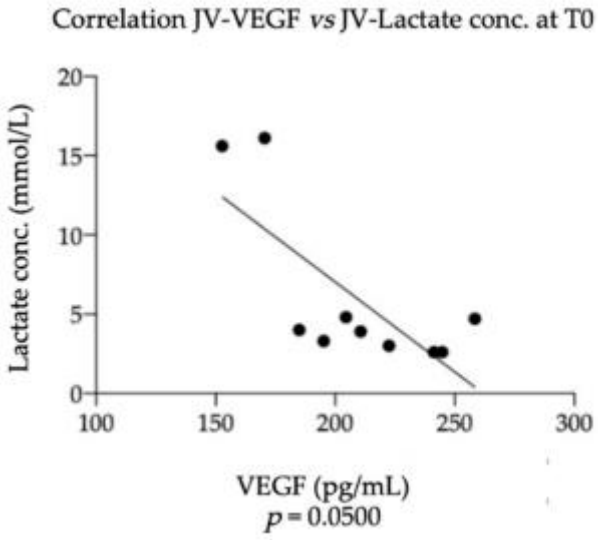

Figure 4. Significant correlation found between Vascular endothelial growth factor (VEGF) levels inplasma samples obtained from foal's jugular vein and the lactate concentration at T0 ($p = 0.0500$; r =−0.6444). Statistical analysis: nonparametric Spearman correlation.

3.4. Equine NGF, VEGF, BDNF and Their Receptors Gene Expression in the Placenta

In 5/13 mares in the NE group diagnosed with placental insufficiency, the fetal membranes were subjected to molecular biology investigations. Among all the genes analyzed, in the chorion, no significant differences were found between H and NE groups in terms of the expression of trophic factors and their receptors. In the allantois, a decreased NGF expression was observed in NE group ($p = 0.0033$), while no differences in the expression of P75NTR and TRKA receptors were observed. In the amnion, a decreased BDNF expression was observed in NE group ($p = 0.0498$), with no differences in TRKB receptor expression. Only those genes that showed significant differences between the two groups were included in Figure 5.

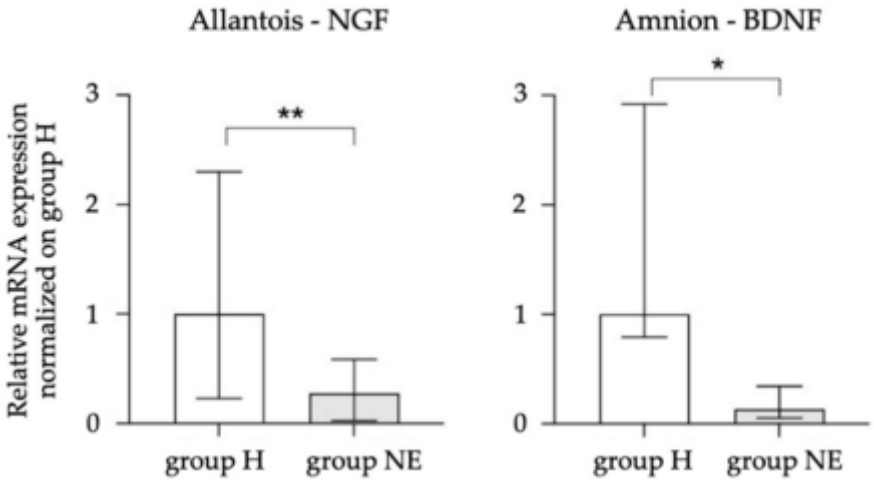

Figure 5. Differences in nerve growth factor (*NGF*) and brain-derived neurotrophic factor (*BDNF*) relative mRNA expression normalized on healthy foals between H and NE groups in allantois and amnion, respectively. * $p < 0.05$; ** $p < 0.01$.

4. Discussion

In the present study, NGF and VEGF levels were measured in the plasma of mares diagnosed with placental insufficiency and/or dystocia, in the amniotic fluid, in the umbilical vein, in the plasma of their foals affected by NE (in the first 72 h of life—group NE), and in foals affected by NE admitted within 24 h after birth (in the first 72 h of hospitalization—group exNE). The trend of serum thyroid hormones (TT3 and TT4) in the first 72 h of life/hospitalization and the gene expression of *NGF*, *VEGF*, *BDNF*, and their receptors (*TRKA*, *p75NTR*, *FLT-1*, *KDR*, *TRKB*) in the fetal membranes were also evaluated.

The first aim of the present study was to clinically characterize a population of foals spontaneously affected by NE. This is a complex disease recognized across different species, characterized by neurologic dysfunction, but often leading to multiorgan dysfunction [39]. Reviews, case reports, and ongoing investigations demonstrate how the understanding of the disease in foals is constantly evolving [6,11,40,41]. Identification of blood markers of brain injury would be crucial for the

diagnosis and the prognosis of NE in foals, and the role of trophic factors requires ongoing investigation, since it is likely to be pivotal.

A specific pathogenesis related to hypoxia, ischemia, and asphyxia may not always be recognized in all cases [11,12,42]. In the present study, a history of placental insufficiency based on the histopathological placenta evaluation, dystocic parturition, including premature placenta separation, was evident in most of the cases reported, but not in all, especially in foals hospitalized after birth, where information related to pregnancy and parturition was often lacking. In group NE, in which pregnancies were monitored and parturition attended, the Apgar score provided a semi-quantitative assessment of the severity of signs occurring in response to *peripartum* asphyxia, and the following score was assigned: <6 (severe asphyxia), 6-8 (mild asphyxia), or 8-10 (normal foals) [31]. Unfortunately, sick foals are usually referred several hours after birth, when the Apgar score can no longer be performed.

In the present study, NE in foals was clinically recognized by many behavioral abnormalities and the most common signs of neurological dysfunction, include alterations in mental status and in respiratory function, changes in muscle tone, changes in responsiveness, vestibular signs, and autonomic disturbances. Behavioral abnormalities included lack of affinity with the mare, disorientation and abnormal udder seeking, lack of suckle reflex and tongue incoordination, and less frequently abnormal vocalization. In many cases, clinical signs of NE followed very predictable patterns; however sometimes foals appeared normal at birth, but developed behavioral or neurological signs a few hours later, as already described [12]. The foals of exNE group presented more clear and severe neurological signs and, generally, more severe clinical conditions. Differently from group NE, foals born from assisted delivery were hospitalized after birth and did not receive prompt intervention and supportive treatment. Furthermore, the clinical signs most frequently associated with NE have been reported in the affected population, devoid of typical concomitant neonatal diseases, and respiratory, gastrointestinal, renal, and cardiovascular dysfunction appear to be the most common.

Laboratory findings in foals with NE are non-specific and often reflect secondary systemic disease or varying degrees of organ dysfunction associated with NE. In both groups, foals could be hypoxic, hypercapnic, had acid-base and electrolyte disorders, azotemia, and poor glucose control. Hypermagnesemia may be the result of severe tissue damage with cell injury or death, and release of the intracellular magnesium, or related to acidosis and hypoxia [8]. Elevated creatinine at birth is commonly observed in foals that have experienced fetal distress or placental dysfunction. The allantoic fluid contains high concentrations of creatinine, and under pathological conditions there is redistribution of fetal fluids to the fetus, resulting in higher blood creatinine levels [43,44]. Foals born from dystocia showed increased creatine kinase activity because they are likely to be affected by muscle damage [45].

Treatment of NE in foals is largely supportive and directed at controlling neurologic dysfunction and addressing associated multiple organ dysfunction. Goals of therapy should be aimed at supporting perfusion and oxygen delivery via fluid therapy, inopressors and oxygen administration, controlling seizures, assessing renal function, supporting metabolic function

through careful nutritional management and blood glucose regulation, and preventing sepsis. The prognosis of foals with a unique diagnosis of NE in this study is in line with those proposed previously [10,12,13], with a survival rate of 80%. In the present study, it was not possible to investigate the prognostic value of NGF and VEGF levels at birth/admission, due to the low number of non-surviving foals; therefore, further investigations in larger cohorts are required.

The decreasing trend of TT4 levels recorded in the serum of NE and exNE foals in the first 72 h of life/hospitalization is in agreement with a previous study conducted on foals aged less than 12 h affected by NE [7]. In the present study, serum TT3 levels were lower in both affected foals born at the Unit and those hospitalized after birth compared to healthy subjects, while TT4 levels were lower only in foals hospitalized after birth compared to healthy ones. Perinatal asphyxia could trigger effects on the neonatal HPT axis, causing the lower levels of thyroid hormones found in affected foals in this study, which aggravate both neurological symptoms and multi-organ dysfunction. The decrease in thyroid hormone levels in foals with NE found in this study could be an expression of altered transplacental transport due to a condition of placental insufficiency, or an expression of non-thyroidal illness syndrome (NTIS) developed after birth [46]. This is a well-recognized syndrome described in patients with severe non-thyroidal illness characterized by low T3 associated with normal or low T4 [47]. NTIS probably represents an adaptive response to a systemic illness with a suppressive effect on the HPT axis and a decreased metabolism preventing organ dysfunction or death. Initially, the conversion of T4 to T3 in peripheral tissues decreases, then, as the severity of illness progresses, T4 concentration also decreases, suggesting dysfunctions at the hypothalamic, pituitary, or thyroid gland level [47]. It should be noted that in this study, reduced TT4 levels were observed in group exNE foals hospitalized after birth, which had a more severe clinical condition.

The few studies on NGF which have been performed in the human perinatal period under pathological conditions are mainly related to intrauterine growth restricted (IUGR) fetuses and neonatal plasma levels [48], preeclamptic women [49], and infants born preterm [50]. It has been reported that circulating NGF is significantly lower in IUGR neonates than in appropriate for gestational age ones [48]. Maternal plasma NGF levels in preeclamptic women are lower than those in normotensive ones [49], while maternal and cord plasma NGF levels were reported to be significantly reduced in women who deliver preterm, suggesting a correlation between reduced cord NGF levels and fetal growth, with likely implications for post-natal neurodevelopmental disorders [50]. Otherwise, foals with NE did not differ significantly from healthy control subjects in terms of plasma NGF and VEGF levels, as well as in terms of umbilical cord vein plasma and amniotic fluid levels. Notably, NGF plasma levels are around 1000 times higher in foals compared to human neonates [48]. Under pathological conditions, no correlations were observed between plasma NGF and VEGF levels and the selected mare's and foal's clinical parameters, probably due to the low number of samples and high individual variability. However, a negative correlation was found at T0 between VEGF levels in the foal plasma and lactate concentration. On the contrary, an inverse correlation between NGF levels and symptom severity (as assessed by Glasgow Coma Scale score) has been reported in human children with traumatic brain injury [51]. The pathophysiologic principles of CNS ischemia and hypoxia are shared by animals and humans. Compared to human

infants with hypoxic ischemic encephalopathy, foals appear to respond rapidly or more successfully to hypoxia, also in view of the better prognosis of the disease in the newborn foal [12]. It can be postulated that high endogenous levels of NGF may exert a default neuroprotective role in foals.

Although hyperlactatemia does not provide diagnostic information, it does indicate the severity of the disease and the need for early and aggressive intervention or closer monitoring, as it occurs during hypoxia and poor tissue perfusion [52]. VEGF can stimulate angiogenesis and modulate vascular permeability [20], and it is also involved in mediating inflammatory responses [21,22]. Although speculative conclusions on this correlation need to be further evaluated, it cannot be excluded that in affected foals, VEGF is able to restore normal tissue perfusion and oxygenation, leading to a decrease in blood lactate concentrations. Differently from that observed in the healthy population, in NE and exNE foals, NGF plasma levels decreased significantly only in the first 24 h of life/hospitalization, whereas VEGF plasma levels decreased only in group NE. Starting from comparable plasma values at birth, in healthy foals the levels of the two trophic factors drop more markedly than in affected foals. Another interesting result was the lack of the positive correlation that exists in healthy foals between plasma levels of NGF and VEGF at each time point. These findings are not diagnostic, but it cannot be ruled out that a failure to decrease circulating trophic factors in the first 72 h of life may be related to the extent of brain damage. Increased segregation of NGF in the brain of these foals cannot be excluded, and the authors speculate that this change primarily reflects the brain compartment, as suggested by studies conducted in human perinatology, showing that blood levels of neurotrophins are similar to those in the brain [48], changing in infants with neurological disorders due to clinical states of prolonged perinatal hypoxia [49]. In vitro and in vivo animal models have also shown that hypoxia-induced cell death is preceded by a period of NGF up-regulation, suggesting that NGF plays a protective role [53]. In the same direction, clinical studies conducted on infants with hypoxic-ischemic brain injury and treated with intraventricular NGF infusion showed a significant clinical improvement in their neurological condition [54,55], and increased NGF and VEGF segregation in asphyxiated foals may protect neurons against protracted injury.

The second interesting aspect was the correlation that exists in group NE foals between NGF and TT3-TT4 levels at each time point. The close relationship between the trophic factor and thyroid hormones has not been found in healthy foals [23], but experimental studies have long confirmed that thyroid hormones mediate direct effects on NGF-induced expression in neonatal mice [56], and also modulate NGF expression in the cerebellum of perinatal rats [57]. The significance of this positive correlation should be investigated in light of NGF and thyroid-hormones roles in the brain compartment. From a translational point of view, several studies suggest the possible interaction of thyroid hormones with members of the NGF family of neurotrophins and their functional receptors, not only during brain maturation, but also during its maintenance. Thyroid hormones are known to regulate endogenous NGF synthesis under physiological conditions [58,59] and their administration promotes NGF synthesis and increased NGF content in the brain, depending on brain region and post-natal age [60–62]. Although further studies are needed to investigate the functional consequences of the interaction between thyroid hormones and NGF during NE, the increased NGF

segregation and the relationship observed with TT3-TT4 could indicate a neuronal distress and the need for protection from ischemic damage.

In the NE group of mares, the histological preparation of the chorioallantois stained with hematoxylin–eosin showed varying degrees of hyperemia and edema of the connective lamina associated with mild to severe hypoplasia of the chorionic villi. These findings may indicate an inability of the placenta to meet the increasing metabolic demands of the fetus as pregnancy progresses. Conditions affecting the utero-placental unit, such as placental hypoplasia and placental edema, can cause a decrease in the nutrients and oxygen supplied to both the fetus and placenta, and any deficiency in placental structure and function may be reflected in a corresponding deficit in fetal growth and maturity, leading to a manifestation of NE in the neonate [44]. Despite the low number of samples, the reduced expression of NGF in the allantois and the reduced expression of BDNF in the amnion seems to characterize the fetal membranes of mares with placental insufficiency that delivered foals affected by NE. Neurotrophins are defined as "angioneurins" as they also regulate angiogenesis in the placenta [63] by contributing to the maintenance, survival, and function of endothelial cells [64]. The allantois represents the essential structural framework for an incredibly dense mass of fetal capillaries supported by minimal amounts of allantoic mesoderm [65], whereas the amnion consists of a cuboid epithelial layer that comes into direct contact with AF [66]. Neurotrophins such as NGF and BDNF play an important role in placental development and maturation, acting through autocrine–paracrine mechanisms [67,68]. In women, NGF is reported to be synthetized in the placenta [69], while a recent study also suggests that optimal NGF expression at the feto-maternal interface is essential for a healthy pregnancy [70], influencing the process of angiogenesis [63]. In the present study, the reduced expression of the two neurotrophins could be related to dysregulation of adhesion [71], angiogenesis [72], apoptosis [73], and proliferation [74] pathways. It can therefore be reasonably postulated that, in the mare and in the fetus, decreased expression of *NGF* and *BDNF* in placental tissues may imply the development of impaired maternal and fetal vascular and nervous networks for the interchange of nutrients and stimuli, leading to a clinical manifestation of NE in the neonate.

The main limitation of the study design is that the population of foals affected by NE was not perfectly homogeneous. In some cases, the disease was associated with a "chronic hypoxia" due to placental insufficiency, whereas in others it was associated with an "acute hypoxia" due to dystocic parturition. Nevertheless, the population offered a pure and spontaneous model of equine NE, devoid of typical concomitant diseases such as bacteremia, local infections, sepsis, or prematurity/dysmaturity.

5. Conclusions

The present study provides limited but novel information on the role of trophic factors in the early stages of equine neonatal life, when the diagnosis of NE is reached through careful clinical examination. Foals with NE do not differ significantly from healthy control subjects in terms of plasma NGF and VEGF levels, although the levels of the two trophic factors decrease less markedly than in healthy foals. A positive correlation between NGF and both thyroid hormones appears to

characterize the first 72 h of life in affected foals, as their fetal membranes seem to be characterized by the dysregulation of NGF and BDNF expression.

Overall, these results provide a starting point for a better understanding of the role of NGF and VEGF during the equine perinatal period under pathological conditions, although they require confirmation by further studies on a wider population of affected foals.

Nevertheless, no blood biomarkers are yet in current clinical use for foals with NE. In the authors' opinion, the ideal biomarker for identifying equine NE would be stable, measurable during the first hours of life with a high-sensitivity technique, and in an easyto-access biological sample. Although NGF and VEGF were unable to detect the disease in the population examined, they appear to be promising candidates, which warrants investigation in wider cohorts.

Supplementary Materials

Table S1. Foals complete blood cell counts, serum biochemistry, electrolyte concentrations, rapid determinations and arterial blood gas analysis at birth (group NE) or at admission (group exNE). Data are expressed as mean ±standard deviation (min-max). For normal values refer to [34], [35], [36], [37], and [38].

Haematology									
Haemoglobin g/dL	Haematocrit %	Erythrocytes 10^6/µL	Platelets 10^3/µL	Leucocytes 10^3/µL	Lymphocytes 10^3/µL	Monocytes cells/µL	Neutrophils 10^3/µL	Eosinophils cells/µL	Basophils cells/µL
group NE									
15.4 ±1.7 (12.3-19.8)	48.0 ±4.3 (40.2-58.4)	10.5 ±0.9 (8.9-12.9)	180.6 ±28.6 (137-242)	7.5 ±2.0 (4.6-12.3)	1.5 ±0.4 (0.8-2.2)	150.8 ±87.5 (30-280)	5.8 ±2.1 (2.5-10.7)	23.6 ±25.8 (10-80)	90.8 ±127.6 (10-380)
group exNE									
14.0 ±2.8 (7.5-18.9)	42.4 ±8.3 (22.4-55.3)	9.8 ±1.8 (5.0-12.6)	175.3 ±61.8 (61-276)	7.7 ±4.2 (0.6-13.9)	1.2 ±0.8 (0.1-3.2)	106.5 ±73.4 (10-260)	6.3 ±4.4 (0.5-12.8)	12.5 ±15.2 (0-60)	56.5 ±54.3 (0-190)
Normal haematology values in one day old foals [34]:									
12.0-16.6	32-46	8.2-11.0	129-409	4.9-11.7	0.7-2.1	70-390	3.4-9.6	0-20	0-30
Serum biochemistry									
Creatine kinase IU/L	Total bilirubin mg/dL	Triglycerides mg/dL	Total protein g/dL	Albumin g/dL	Albumin / Globulin	Blood urea nitrogen mg/dL	Creatinine mg/dL	Fibrinogen g/L	Serum amyloid A µg/dL
group NE									
300 ±260 (132-1136)	2.5 ±0.7 (1.3-3.6)	22 ±18 (7-60)	4.2 ±0.3 (3.4-4.5)	3.2 ±0.3 (2.6-3.4)	3.5 ±0.5 (2.7-4.2)	41 ±11 (29-62)	3.6 ±3.1 (1.1-13.1)	1.8 ±0.4 (1.4-2.6)	6 ±7 (1-24)
group exNE									
5511 ±11818 (92-51050)	4.0 ±1.2 (2.0-6.1)	76 ±82 (26-257)	4.5 ±0.4 (3.8-5.1)	3.0 ±0.3 (2.4-3.5)	2.3 ±1.0 (1.3-4.6)	46 ±15 (19-73)	4.0 ±3.3 (0.7-14.8)	2.2 ±0.5 (1.7-3.1)	101 ±170 (1-620)
Normal serum biochemistry values in one day old foals [35,36]:									
40-909	1.3-4.5	30-193	4.3-8.1	2.5-3.6		9-40	1.2-4.3	1-4	0-37

Electrolyte concentrations					
Phosphorus	Calcium	Sodium	Potassium	Chlorine	Magnesium
mg/dL	mg/dL	mg/dL	mg/dL	mg/dL	mg/dL
group NE					
8.7 ±3.8 (5.0-14.6)	13.6 ±2.2 (9.2-15.7)	143.8 ±2.6 (141-149)	5.2 ±0.9 (4.3-6.8)	98.6 ±4.2 (92.3-102.6)	2.1 ±0.2 (1.8-2.5)
group exNE					
5.2 ±2.1 (4.0-9.8)	11.9 ±0.6 (11.0-12.8)	143.1 ±3.3 (139-147)	3.6 ±0.4 (3.1-4.0)	99.3 ± 5.6 (90.0-105.1)	2.4 ±0.6 (1.4-3.9)
Normal electrolyte concentrations in one day old foals [35]:					
3.8-7.4	9.7-13.7	123-159	3.6-5.6	90-114	0.6-4.2

Rapid determinations			
	Jugular vein glucose mg/dL	Umbilical vein lactate mmol/L	Jugular vein lactate mmol/L
group NE			
	92 ±17 (75-109)	3.5 ±0.7 (3.0-4.0)	6.4 ±6.6 (2.7-22.5)
group exNE			
	96 ±64 (32-160)	/	9.1 ±7.0 (2.5-23.4)
Normal determinations in foals at birth [35,37]:			
	67-99	3.2-4.7	2.3-5.0

Arterial blood gas analysis									
pH	Oxygen partial pressure mmHg	Carbon dioxide partial pressure mmHg	Oxygen saturation %	Potassium mmol/L	Sodium mmol/L	Chlorine mmol/L	Anion gap mmol/L	Bicarbonate mmol/L	Acid base excess mmol/L
group NE									
7.37 ±0.04 (7.32-7.41)	48.0 ±10.9 (34.2-59.9)	43.8 ±4.3 (39.4-47.7)	87.9 ±5.1 (82.7-93.9)	3.6 ±0.5 (3.3-4.4)	140.3 ±1.3 (139-142)	99.8 ±4.4 (95-104)	19.3 ±7.8 (9.8-28.7)	24.2 ±3.6 (20.2-28.2)	-0.2 ±4.2 (-5.0-4.4)
group exNE									
7.36 ±0.04 (7.31-7.41)	66.3 ±17.5 (48.5-96.8)	43.7 ±6.9 (36.6-54.1)	92.9 ±3.5 (87.4-97.2)	2.9 ±0.1 (2.6-3.0)	141.4 ±4.3 (135-147)	102.4 ±4.3 (93-107)	17.9 ±3.7 (12.6-22.6)	23.5 ±1.6 (21.7-26.4)	-0.9 ±1.9 (-3.2-2.5)
Normal arterial blood gas values in foals at birth [38]:									
7.36-7.44	64.2-68.8	46.0-49.4	97-100	3.6-5.6	123-159	90-114	9.5-31.7	24.1-25.9	-0.1-1.9

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GENERAL CONCLUSIONS

The importance of trophic factors during the equine perinatal period is now emerging. Through their functional activities of neurogenesis and angiogenesis, they play a key role in the final maturation of the nervous and vascular systems. In the study model proposed by the author, the compartments explored and sampled reflected feto-placental pathophysiology (fetal membranes, amniotic fluid and umbilical vein plasma) and the phatophysiology of the mother/neonate (mare and foal plasma), and the levels of trophic factors at the time of birth and in the first 72 h of life well represented the transition of the healthy or compromised equine neonate to extra-uterine life. The authors also proposed a new fetal membranes sampling model for use in molecular biology investigations. Once a biobank of biological materials was obtained, innovative biomolecular diagnostic techniques were developed, including the plasma assay of trophic factors by ELISA and their placental gene expression by semiquantitative real-time PCR.

Studies conducted on human perinatology showed that the blood levels of neurotrophins, including NGF and BDNF, are similar to those in the brain and that the neural tube, from which the brain develops, becomes vascularized by a process involving VEGF. The significant decline in the NGF and VEGF content and their close relationship in foal plasma over time under physiological conditions appeared to be key regulators in directing vessel sprouting and regulating neuronal cell migration in the CNS. Due to the lack of expression of NGF and its receptors in the amnion, fetal urine, which is a major component of amniotic fluid during late gestation in the equine species and reasonably contains all molecules that cross the blood-brain barrier, could be the sourse of NGF in amniotic fluid.

A population of foals spontaneously affected by Neonatal Encephalopathy was clinically and finely characterized. The disease appeared to be the consequence of adverse *peripartum* events leading to ischemia/hypoxia/inflammation in the *prepartum* period or at the time of parturition, and the most common behavioral abnormalities, signs of neurological disfunction, and organ dysfunction associated with the disease reported in the study represent a possible guide for clinicians in their diagnostic-therapeutic process. Perinatal asphyxia triggers effects on the neonatal hypothalamus-pituitary-thyroid axis, causing the lower levels of thyroid hormones found in affected foals, which aggravate both neurological symptoms and multi-organ dysfunction. The decrease in thyroid hormone levels in foals with Neonatal Encephalopathy found in this study could be an expression of altered transplacental transport due to a condition of placental insufficiency, or an expression of non-thyroidal illness syndrome developed after birth. Overall, the less pronounced decrease of the two trophic factors compared to healthy foals, their relationship with thyroid hormones over time, and the dysregulation/reduced expression of NGF and BDNF in placental tissues of mares with placental insufficiency, could be key regulators in the mechanisms of equine Neonatal Encephalopathy, due to the development of impaired maternal and fetal/neonatal vascular and nervous networks for the interchange of nutrients and stimuli.

In the equine species, NGF showed more than 90% homology with human NGF, indicating that NGF-encoding is a highly conserved gene. Surprisingly, NGF plasma levels are around 1000 times

higher in foals compared to human neonates, and in human medicine an inverse correlation between NGF levels and symptom severity has been reported in children with traumatic brain injury. The pathophysiologic principles of CNS ischemia and hypoxia are shared by animals and humans. Compared to human infants with hypoxic-ischemic encephalopathy, foals appear to respond rapidly or more successfully to hypoxia, also in view of the better prognosis of the disease in the newborn foal. The authors hypothesized that high endogenous levels of NGF may exert a default neuroprotective role in foals.

Identification of blood markers of brain injury would be crucial for the diagnosis and the prognosis of Neonatal Encephalopathy in foals, but the role of trophic factors requires ongoing investigation, since it is likely to be pivotal. Overall, this study provides a starting point for a better understanding of the role of NGF and VEGF in the equine perinatal period under pathological conditions, laying the foundation for the future development of new therapeutic protocols in regenerative and translational medicine.

INSTITUTIONAL REVIEW BOARD STATEMENT

Ethical review and approval were waived for this study, because opportunistic sample harvesting was applied.
Written informed consent was given by the owner for each animal. This has been confirmed by the ethical committee.

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To whom it may concern

***Re**: Dott. Aliai Lanci's research entitled "Study on NGF and VEGF during the equine perinatal period" – ID 4396*

This is to acknowledge that dott. Aliai Lanci has sent her manuscript entitled "Study on NGF and VEGF during the equine perinatal period" to the Animal Welfare Committee of the University of Bologna for evaluation.

The research described in this manuscript does not fall within Directive 63/2010 of the European Parliament and of the Council on the protection of animals used for scientific purposes (transposed into Italian law by Legislative Decree 26/2014) and thus doesn't require any authorization from the national competent Authorities.

The Animal Welfare Committee of the University of Bologna acknowledges that oral informed consent was given by the owner for each animal.

Prof. Luca Lorenzini, DVM, PhD
Chair of the Committee