THESIS

PROTEOMIC ANALYSIS OF EQUINE MELANOCYTIC NEOPLASM IN THE GREY COAT COLOR

PARICHART TESENA

GRADUATE SCHOOL, KASETSART UNIVERSITY
Academic Year 2021

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THESIS

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PARICHART TESENA

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Animal Health and Biomedical Sciences)
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Academic Year 2021

Parichart Tesena : Proteomic Analysis of Equine Melanocytic Neoplasm in the Grey Coat Color . Doctor of Philosophy (Animal Health and Biomedical Sciences), Major Field: Animal Health and Biomedical Sciences, Faculty of Veterinary Medicine.
Thesis Advisor: Assistant Professor Attawit Kovitvadhi, Ph.D.
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Equine melanocytic neoplasm (EMN) is cutaneous neoplasm and frequently detects in aged grey horses. This study aimed to examine the profiles proteins expression of EMN in serum and feces samples. This study reveals the potential proteins in less to non-invasive approach on EMN using proteomics from serum and feces samples. All of the samples were collected from twenty-five grey horses which were assigned into three groups containing normal (free of EMN; *n*=10), mild EMN (*n*=6) and severe EMN (*n*=9) in pony, Lusitano and Thoroughbred breeds. The grey horses were collected the blood and feces. Subsequently, the Liquid Chromatography-Tandem Mass spectrometry were used for analysis and identification of the protein. According the differentially expressed proteins DEPs, the significant proteins were categorized involved in metabolism and non-metabolism functions according to KEGG database. The serum results showed that BRCA1, phosphorylase b kinase regulatory subunit: PHKA1, tyrosine-protein kinase receptor: ALK and rho-associated protein kinase: ROCK1 correlating to EMN progress were revealed at high intensity especially in mild and severe groups. In feces results presented that DAGK, diacylglycerol kinase: MAML2, mastermind like transcriptional coactivator 2: SMC4, structural maintenance of chromosomes 4: TGM2, Transglutaminase 2 were high level in EMN stages both of mild and severe. Interestingly, FOSL1: FOS like 1 can be found only in mild EMN group. Moreover, both of serum and feces samples, the majority of proteins involved in lipid metabolism which plays crucial role for EMN growth, invasive, proliferative and progression. In conclusion, this study demonstrated the occurrence of EMN in Thailand and the specific proteins to diagnostic EMN was discovered.

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Student's signature Thesis Advisor's signature

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CHAPTER I
INTRODUCTION: THE IMPORTANCE AND RATIONALE OF RESEARCH

Equine Melanocytic Neoplasm (EMN) is the abnormality of melanin metabolic rate that commonly occurred on the skin of the grey horses (Rowe & Sullins, 2004). The etiology is abnormality transformed of melanin metabolism that affects melanoblasts overproduction located in the epidermis (Patterson-Kane et al., 2001). Unlike the human, melanomas in horses are not induced by the ultraviolet radiation exposure (Scott & Miller, 2011). Melanocytic neoplasm can found in tertiary rank of tumors in horses inferior to sarcoid and squamous cell carcinoma, respectively (Hewes & Sullins, 2009). However, it is the most common and highly prevalence approximately 80% in the older grey horses (greater than 15 years old) (Rieder et al., 2000) but less common in the solid color horses (Johnson, 1998). Besides, the common breed including Arabians, Lipizzaner and Percheron are the most that can found its very regularly (Scott & Miller, 2003) Moreover, it is not limited in sexes (male and female) (Seltenhammer et al., 2003). However, the occurrence or the prevalence detail had not reported in Thailand yet. EMN could high impact the health status, economic benefits for medical or surgical or chemotherapy treatment costs and occupied performance of the horses in Thailand.

By reason of the EMN progresses very slowly (MacGillivray et al., 2002) consequently the health status is normal, resulting in the treatment is gradually to support the symptoms. At one point, the progression of the disease was severe or metastasize to the internal organ that could affect the life threatening. There is a various way to treat EMN such as surgical excision that limited in size not to over 2 cm of diameter (Hewes & Sullins, 2009) combined with the intralesional chemotherapy by using cisplatin (Theon et al., 2007). Cimetidine, a histamine H2-receptorantagonist dosage 2.5 mg/kg q8h duration 4-12 months (Goetz et al., 1990) can be help for regulation of angiogenesis, inhibit of the immune response (Moriarty et al., 1988) and decreased cell extravasation by inhibit E-selectin expression (Kubecova et al., 2011). In addition, acupuncture is the alternative method that used

to treat melanocytic neoplasia (Longo, 2017). Although the vaccination are used to prevent the neoplasm status but still uncertainly of the result and under the trailed (Metcalfe et al., 2013). There are several methods to diagnose the EMN including determined from the physical inspection, which the specific appearance such as dome shaped, smooth surface, hairless on masses, black or grey color, firm to hard in consistency, usually greater than 1 lump, very many sizes between 0.2-2 cm of diameters and unpainful on palpate the masses. Moreover, underneath the tail, perineum region include vulva or penis or scrotum, perianal region, around the muzzle both of upper and lower lips, around the eyelids are the commonly specific locations of the tumors (Fleury et al., 2000). Besides, there are less commonly sites such as parotid gland, head and neck (Rodríguez et al., 1997a). However, the regularly method to confirm is histological but using in immunohistology can indicated between benign and malignant stage by the expression markers of Ki-67 and PCNA (proliferating cell nuclear antigen) (Seltenhammer et al., 2004). Moreover, receptor for activated C kinase 1 (RACK1) was the marker of malignant stage that could be used for diagnosis in mammalian including human, pig and horses (Adams et al., 2011). Interestingly, the proteomic study showed 4 of the protein's expression in human of the cutaneous malignant melanoma (CMM) composed with the cathepsin inhibitor cystatin B (CSTB), myo-inositol 1-phosphate synthase (ISYNA1), the coagulation factor F13A1 and the calcium binding protein S100A13 (Azimi et al., 2014). Additionally, the expression of S100A13 is the crucial protein marker to identify and predicted the responding of the treatment (Azimi et al., 2014). Even though the emerging of contribution the proteomics or phosphoproteomics in melanoma study reasonably debauched and widespread but the limited in research and pattern of the methods are still different (Sengupta & Tackett, 2016). Wherewith, EMN has not been accomplished about proteomics yet. Moreover, there is no successful treatment also non-invasive of diagnosis pattern of EMN.

Therefore, the rational of this study is to identify the relationships of proteins expression and pathogenesis in the serum and the feces sample between the EMN grey horses compared with the normal grey horses. Moreover, the proteins expression in each group can be compared the differential protein expression levels (PELs)

across different EMN stages for identify the functional character of proteins. Furthermore, the significant differentially expressed proteins (DEPs) associated metabolic and non-metabolic roles can be attended for considerate of pathophysiology pathway of EMN in horses. Moreover, the unique significant potential protein in mild or severe stages might be used for establish EMN diagnosis and treatments in the equine industry.

Accordingly, the study on EMN in horses has not established in Thailand. In addition, EMN could be impact directly on their health status, activity performance including economic benefits. As a result, the discovery of candidate proteins can be used for therapeutic treatment administration which there is none of effective treatment yet. Proteomics may be used in developing treatment knowledge in the future especially natural field EMN and proteomics has never been reported before. Proteomics is a crucial tool in the investigation for biomarkers, new therapeutic targets including these solve the issues of pathogenesis by determining which proteins manifested at what stage of disease development, both at the beginning and during the disease. This makes determining where to develop proteomics as an important tool to discover biomarkers and also new therapeutic targets. All of these could be led to more accurate diagnosis, better treatment method as well as more effective disease prevention. These biomarkers could be used to measure the effects of research during clinical trials. In addition, the candidate proteins can be identified early changes in beginning stage of EMN that indicate and monitor the severity of EMN. This statement could be built on two foundations to straight the horse and equine research society especially the horses from natural field and toward the true problem. Furthermore, this research analysis highlights the need for the interactions between pathogenesis across of EMN stages (normal, mild EMN and severe EMN) and potential proteins expression outcomes using bioinformatic analysis as relevant to the process may be universal.

CHAPTER II
LITERATURE REVIEW

2.1 Anatomy of equine skin and etiology of EMN

Equine melanocytic neoplasm (EMN) is the uncontrolled or abnormal growth of melanin metabolic rate that occurred frequently on the skin of the grey horses (Rowe & Sullins, 2004). It is perceived that the cellular originate melanocytes are the dendritic cells derivative from neuroectodermal melanoblasts. They are principally located in the basal layer on the skin and outer root sheath of hair follicles (Smith et al., 2002) (Figure 1). Naturally of the grey horses, they are maybe born any base color for example bay, chestnut or roan (dark hair and dark skin). Afterward, their hairs begin lighter that can be appear or shortly after birth. Continuously, they are become progressively white hair with the horse ages but skin still dark (Seltenhammer et al., 2004). Melanin is the pigment that produced by melanocytes in the basal layer of the epidermis. Normal effect how to change the hairs color in morphology appearance, start with the melanocytes produces the melanosomes by using the activator the tyrosinase enzyme (the enzyme stimulated melanogenesis). Consequently, the enzyme tyrosinase corrective generated the melanin to the melanin granule. However, melanin granule cannot include in the hair follicle led to the hair to eventually become unpigmented. Remarkably, the etiology of melanoma formation is abnormality transformed of melanin metabolism that affects melanoblasts overproduction located in the epidermis of the horses (Patterson-Kane et al., 2001). Certainly, caused of hyperplasia of melanoblasts leading to accumulation of melanin on the horse skin.

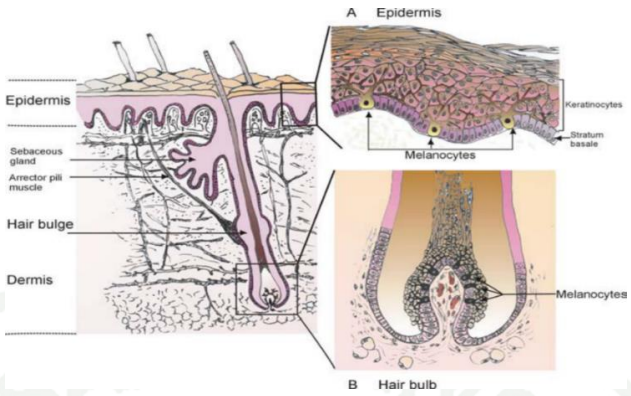

Figure 1: Anatomy of the skin show the melanocytes located in the basal layer on the skin and outer root sheath of hair follicles (Cichorek et al., 2012).

In the genetic appearance of the normal effect. Initially, the melanocortin 1 receptor (*MC1R*), also known as melanocyte-stimulating hormone receptor (MSHR), melanin-activating peptide receptor or melanotropin receptor play a role in melanocyte-stimulating hormone receptor activity and melanocortin receptor activity. It is activated by binding with the α-MSH or ACTH, appearing in enhance of the cyclic-AMP (*cAMP*) levels and intensify of microphthalmia-associated transcription factor (*MITF*) expression. *MITF* is function as the melanoma progression that related the regulation of genes about migration, invasion and metastasis of melanoma (Teixeira et al., 2013). Resulting of increasing *MITF* expression is principle to melanin synthesis, stimulant of tyrosinase leading to eumelanin pigment production and also increased transcription nuclear receptor subfamily 4 group A member 3 (*NR4A3*) (Sundström et al., 2012). *NR4A3* is the mediator of cell cycle regulation cell proliferation and cell apoptosis in consequence of cyclin D2 (*CCND2*) action (Teixeira et al., 2013). In the other hand, the genetic mutation of gene Syntaxin-17 (*STX17*) converted duplication which is responsible for the vesicle transportation of malignant cell melanosome and nuclei (Rosengren et al., 2008). As a result, mutation of CIS-acting regulatory has been occurred. There is also an abnormality of the Agouti signaling protein (ASIP) which has contributed to increase the melanocortin-1-receptor pathway signaling (Rosengren et al., 2008) (Figure 2). Unlike the human,

ultraviolet radiation exposure is not caused of melanomas in horses (Scott & Miller, 2011).

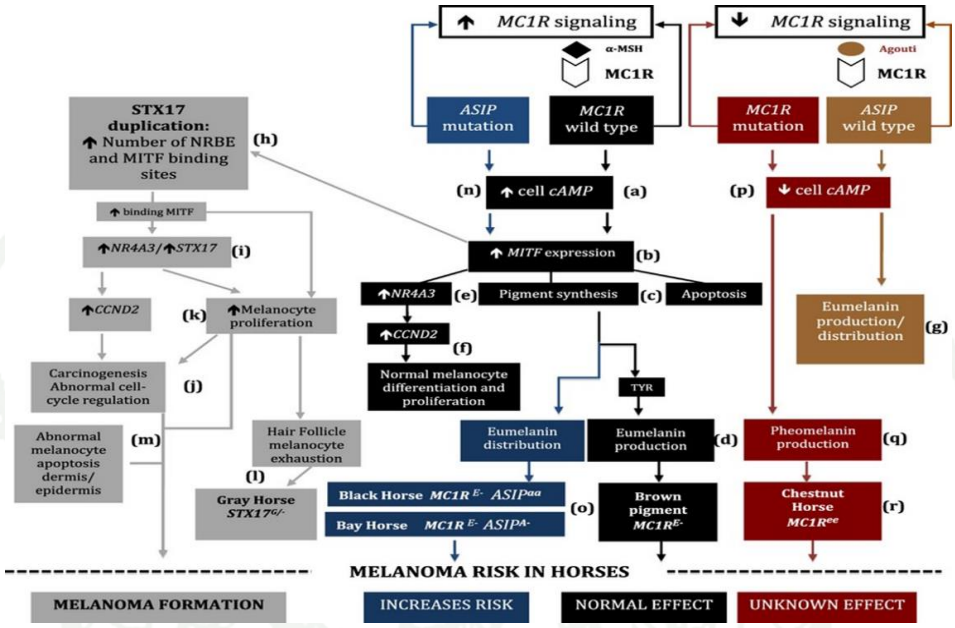

Figure 2: Diagramed of equine melanoma formation (Rosengren et al., 2008).

2.2 Incidence of EMN

melanocytic neoplasm can be found in tertiary rank of tumors in horses inferior to sarcoid and squamous cell carcinoma, respectively (Hewes & Sullins, 2009). Metcalfe et al. (2013) and his colleagues reported that melanocytic neoplasm had been showed characterize up to 18.7% of all equine cutaneous neoplasms. In the same way, Phillips and Lembcke (2013) have been reported melanocytic neoplasm with identified genetic predisposed in the grey coat approximately 3-15% of all cutaneous neoplasms in the horses. The prevalence of melanocytic neoplasm approximately 80% in the older grey horses (greater than 15 years old) (Rieder et al., 2000) but less common in the solid color horses (Johnson, 1998). Therefore, it is hypothesis that the high prevalence of melanoma in grey horses had been associated with to these age related pigment alterations (Seltenhammer et al., 2004). In addition, it cannot found melanocytic neoplasm in horse under 2 years old (Foley et al., 1991).

However, congenital EMN (Cox et al., 1989) and congenital melanotic papilloma (Cotchin, 1960) have been reported including pigs and cows. The common breed including Arabians, Lipizzaner, Percheron and Thoroughbreds can found its very frequently (Scott & Miller, 2003). Moreover, it is not limited in sexes (male and female) (Seltenhammer et al., 2003) according to many researcher have been shown no sexes predisposes as well (Pulley & Stannard, 1990). However, Foley et al. (1991) and his colleagues have been shown the highly incidence of melanocytic neoplasm in the male horses.

2.3 Clinical sign of EMN

The suspected of melanocytic neoplasia lesions including underneath the tail, perineum region along with the vulva or penis or scrotum, perianal region, around the muzzle both of upper and lower lips, around the eyelids are the commonly specific locations of the tumors (Fleury et al., 2000). Besides, there are less sites such as parotid gland, head and neck (Rodríguez et al., 1997a). MacGillivray et al. (2002) have been reported that more than 79% of melanocytic neoplasm had the tumor present in the perineal area and ventral tail area. The primary organ of EMN metastasis were regional lymph nodes and lungs (Pulley & Stannard, 1990). The spleen, liver, blood vessels and heart were the others internal organ system affected (Traver et al., 1977). There are 4 kinds different tumor patterns were recognized composed with melanocytic nevi, dermal melanoma, dermal melanomatosis and anaplastic malignant melanoma (B. Valentine, 1995).

2.3.1 Melanocytic nevi; this type can be found in young aged or less than 5 years old of any color. It is the benign appearance that involve to superficial dermis and/or the epidermal-dermal junction. The common sites can be found in legs and chest which it is not in the typical site as usually.

2.3.2 Dermal melanoma; This kind tumor can be found only the grey horses, average

13 years old of age. Solitary established, hemispheric nodules appearance involving deep dermis. It is usually benign that can be develop to be malignant tumor and regularly metastasizes.

2.3.3 Dermal melanomatosis; This type is common in aging (approximately 17

years old) and only in the grey horses. There is plaque with or without associated nodules involving deep dermis. The histologically similarity with dermal melanoma but difference in clinical features. The tumor features are large, coalescing and multiple masses at under the tail, in the perineum, on external genitalia and parotid salivary gland. It is the malignant tumor and frequently metastasizes that rapid spread in all major organs and body cavities. The effect organ composes with gastrointestinal tract that can be limiting defecation and fecal impaction finally the horse showed the colic sign, kidney can be found the blood and melanocyte in urine sample, the spleen can show the bleeding into the abdomen, the guttural pouch showed the interfere blood vessel leading to bleeding and interfere nerves can be showed difficulty swallowing and tongue weakness.

2.3.4 Anaplastic malignant melanoma; It is the aggressive tumor that commonly in older horses and can be found in all color. The feature is large, neighboring invasive, habitually poorly pigmented. This tumor metastasizes quickly.

2.4 Diagnosis of EMN

Diagnosis of melanocytic neoplasm: start with signalmen that refers to breed, age and sex along with the horse inspection, history taking, physical condition and the stage of lesion were assessed the impact of genetics on melanoma development (Seltenhammer et al., 2003). In addition, there are several methods to diagnose the EMN including determination from the physical inspection, which the specific appearance such as dome shaped, smooth surface, hairless on masses, black or grey color, firm to hard in consistency, usually greater than 1 lumps, very many sizes between 0.2-2 cm of diameters and unpainful on palpate the masses (Seltenhammer et al., 2003). There are no reliable hematological or blood biochemistry available to encourage in diagnosis of EMN but might be essential to find the other complication. The fine needle aspiration (Maurus et al.) is commonly used to diagnose a various skin tumor in many companions' animal. In the term of EMN can be found neoplastic melanocyte and melanophages from mass. It is the rapidity of result, accuracy diagnose and may be no requirement for sedation or anesthesia (Zachar et al., 2016). In addition, the FNA is easy, not too expensive but the limited of this technique still

counting invasive and the diagnose revenue were poor. Moreover, the skin biopsy is the optimal pathologic evaluation of melanocytic lesions. This technique requires the complete excision that combined the full of lesion tumor conjugated with the intact skin (Sober et al., 2001). Likewise, partial biopsy by punch needle can be organize but these techniques are associated with increased risk of adverse clinical effects. In addition, it is the invasive procedure, requires sedation and minor surgery. However, histopathology is the regularly way to confirmed melanocytic neoplasm. It had can be found atypical melanocytes in sheets, nests, or cords that associated with epitricial sweat glands and hair follicles (Scott & Miller, 2003). In the same way, Pulley and Stannard (1990) had reported microscopic examination can be helpful classified between benign and malignant of melanoma. Additionally, the radiographic findings can be made using gross and anatomic description, pattern recognition or probability of occurrence. Radiography of the thorax might be necessary to find a suspicion diagnosis (Cavalleri et al., 2014). Similarly, the ultrasonography necessary in case with the tumors has metastasized widely, the horse might be the clinical signs for unspecific such as weight loss, colic or exercise intolerance. In addition, a full work-up, including ultrasonography of the thoracic and abdominal can be find for thought diagnosis. Also, the rectal palpation with or without a transrectal should be performed in cases which the tumors occur in the perianal region are involved in the disease progress also the horse present unspecific finding to recognize. The rectal palpation might be identifying the tumors abdominal metastases to evaluate the interference of tumor with defecation. Besides, endoscopic examination can be used for evaluated the tumor at guttural pouch and upper respiratory airway. Interestingly, computed tomography (CT) is regularly used in practice to evaluate the equine head even there are less reported of the appearance of melanomas in this location (Dixon et al., 2016). However, CT scan should be identified that had undergone CT of the head, with a diagnosis of melanoma based on cytology, histopathology, or visual assessment of black tissue.

2.5 Treatment of EMN

Treatment of melanocytic neoplasm: there are many way to manage these tumors however, treatment indicated should be considers about the severity, location, amount, the growth rate, the equipment, facilities and treatment cost (Foy et al., 2002). Due to the melanocytic neoplasm progresses very deliberately (MacGillivray et al., 2002) consequently the health status was normal so the treatment is steadily proceed. At any point, the progression of the disease was severe or metastasize to the internal organ that could affect the life threatening. Therefore, initial management should be done in small size tumor is highly recommendation.

2.5.1 medical treatment, Cimetidine, a histamine H2-receptor antagonist is the biological response modifier or immunomodulation effect that used for clinical controlling of progressive. The action can be block the histamine receptors on suppressor T cells that can be help regulation of angiogenesis, inhibit of the immune response (Moriarty et al., 1988) and decreased cell extravasation by inhibit E-selectin expression (Kubecova et al., 2011). On the other hand, there are various research that had reported cimetidine usage were not effective on many experimental in the different type of melanoma. Goetz et al. (1990) and co-worker reported that partial to complete remission in 3 horses following administration of oral cimetidine in dosage 2.5 mg/kg every 8 hour for 4-12 months in 3 horses and conclude that the tumors still progressive, the tumor size also the amount not decreasing on investigation. Laus et al. (2010) and other investigators have not documented this clinical effect, possibly as the drug seems to be most effective in rapidly developing tumors.

2.5.1 surgical treatment, the surgical excision is the simple method but limited in size not to over 2 cm of diameter (Hewes & Sullins, 2009) with or without adjunctive treatment and the tumors not more than 15 lumps. The large tumors which incomplete removal lead to increase development or recurrence and metastasis (Rowe & Sullins, 2004). In addition, surgical excision can be performed with other methods such as CO2 laser and cryosurgery had been reported (Hewes & Sullins, 2009). Besides, the local invasion of tumor in parotid gland should be considering because it is difficult to surgical approach and the horse must be in under the general anesthesia.

2.5.2 chemotherapy, cisplatin is the platinum-based chemotherapy drug that used in

many tumors (Dasari & Tchounwou, 2014). It is the local intra-lesional therapy and suitable for early lesions prior to metastasis and the mechanism of action is bifunctional alkylating agent leading to DNA crosslinking at interstrand also intrastrand by adjacent with guanine molecules results in local denaturation of DNA (Noll et al., 2006). As a result, the decrease tumor sized and prevent the side effect can be minimal quantity of drug. In addition, the tissue absorption had the limiting so the drug should be performed after surgical debunking with 4 treatment every 2 weeks interval (Theon et al., 1999). The complication can be found after injection including post-injection inflammation, swelling and pain (Theon et al., 2007).

2.5.4 vaccination, In the United States there is a DNA plasmid vaccine, encoding human tyrosinase (HuTyr), licensed for the treatment of canine melanoma. This xenogenic xenogenic vaccine utilizes the 92% homology of human and canine tyrosinase to produce a tyrosinase-specific anti-tumor response. The equine tyrosinase sequence has a 90% homology to the human sequence therefore cross-reactivity of the HuTyr DNA vaccine is an area warranting further research. Although the vaccination are used to prevent the neoplasia status but still uncertainly of the result and under the trailed (Metcalfe et al., 2013).

2.5.5 alternative treatment, acupuncture is an alternative method that used to treat melanocytic neoplasia (Longo, 2017). Moreover, radiation therapy is the high energy radiation from x-ray, neutrons, protons usage for treating large or deeply seated tumors. There are 2 kinds including; 1. Teletherapy the horse under general anesthesia in lateral decumbency position when the linear accelerator radiotherapy and total prescribed dose is delivered in a multiple treatment. The radiation source is a long distance (80–100 cm) from the tumor and cobalt-60 unit providing high-energy x-rays. The limitation is the horse requires multiple under general anesthetic for the typical 6–9 treatments every 3–4 weeks and very expansive treatment (Theon, 1998). 2. Brachytherapy the total prescribed dose is delivered in a single or small treatment. This allows for a high dose of radiation to be applied directly to the tumor. Because the radiation only penetrates within 2 mm of the probe, lesions must be superficial. The limitation is the horse requires under general anesthetic and suitable with the small lesion such as the tumor of the eye (Frauenfelder et al., 1982). Furthermore,

hyperthermia is the applied technique that increases the skin temperature to 50°C. The tumor cells are more sensitive to the heat more than the normal one. It is apply to used alone or/and combine with chemotherapy but the treatment have to repeated multiple times (Hoffman et al., 1983).

2.6 Biomarkers of EMN

Biomarker in equine melanoma is relatively less research when compared with the duration of time of this tumor has been occurred. All of the biomarkers are the way to resolve the solution to answer the tumor between benign and malignant. As the result, the collection of the biomarker characteristics obtained from the immunohistology and immunofluorescence in the equine industry. MacGillivray et al. (2002) had made some histological observations and found the large, round or ovoid and euchromatic nuclei with distinct nucleoli. In addition, they were spindloid to stellate in shape, and surrounded by melanophages of different sizes also mitotic figures were hardly detected. However, the regular method used to confirm is histological but immunohistochemmistry can be indicated between benign and malignant stage by the expression markers of dermal Ki67 and proliferating cell nuclear antigen (PCNA) (Seltenhammer et al., 2004). In addition, microphthalmia-associated transcription factor (MITF) and paired box 3 (PAX3) were used for the markers by using immunofluorescence (Campagne et al., 2012). Moreover, receptor for activated C kinase 1 (RACK1) was the marker of malignant stage that could be used for diagnosis in mammalian species including human, pig and horses (Adams et al., 2011). Furthermore, in cell-culture had shown the expressed of Malan-A, MEGA-1, MEGA-3 and PCNA in highly rapidity (Chapman et al., 2008). Correspondingly, EMN and mass spectrometry (MS) has been established as exceptional method for identification and investigation of protein study. Interestingly, the proteomic study showed 4 of the proteins expression in human of the cutaneous malignant melanoma (CMM) composed with the cathepsin inhibitor cystatin B (CSTB), myo-inositol 1-phosphate synthase (ISYNA1), the coagulation factor F13A1 and the calcium binding protein S100A13 (Azimi et al., 2014). Additionally, the expression of S100A13 is the crucial protein marker to identify and predicted the responding of the treatment (Azimi et al., 2014). Moreover, saliva proteomics of canine oral melanoma showed

the expression of sentrin-specific protease 7 (SENP7) and Toll-like receptor 4 variant 1(TLR4) are the potential proteins candidate by matrix-assisted laser desorption/ ionization with time-of-flight mass spectrometry (MALDI-TOF MS) coupled with liquid chromatography-tandem MS (LC-MS/MS) technique (Ploypetch S et al., 2019). In similarity way but different method of saliva proteome in late stage of oral melanoma in dogs found that increasing expressed of protein tyrosine phosphatase non-receptor type 5 (PTPN5) and had associated with p53 when compared with the control group by an in-gel digestion coupled with mass spectrometry (GeLC-MS/MS) (Ploypetch et al., 2020). Phosphoproteomics had been accepted as influential as in clarification of signaling partway. Jacob A Galan et al. (2014) and his colleagues defined that the RSK (p90 ribosomal s6 kinase) phosphoproteome in melanoma cell tumor suppressor PDCD4 (programmed cell death protein 4) leading to determine the therapeutic target of melanoma. Even though the emerging of contribution the proteomics or phosphoproteomics in melanoma study reasonably debauched and widespread but the limited in research and pattern of the methods are still different (Sengupta & Tackett, 2016). Wherewith, EMN has not been accomplished about proteomics yet.

Presently, the study of protein in proteomic research had been popular and widespread. Mass spectrometry (MS) is an analytical technique which measures the stream of light of the electrons moving with different mass/charge (m/z) ratios in magnetic or electrical fields (Shushan, 2010). MS has been admitted method for identification and examination of protein. There are two MS techniques that regularly usage in project composed with the matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). MALDI-TOF MS including 2 processes, MALDI: the crystallized matrix, the simplify ionization process are mixed with the samples and activated adjacent the plate. Afterward, the laser used to blast directly to the mixed sample (Shushan, 2010). The associated matrix can be absorbed the energy in the direction of through to the gas phase. The sample fragment is exterminated in the vacuum and the advance high voltage is applied stimulated the sample portion. Additionally, TOF MS: the ionized fragment will be moved to the ion detector afterthought the flight time assessment will be determined the masses molecules. The

peptide mass fingerprint (PMF) is the result of MALDI-TOF MS method (Aebersold, 2003). In part of LC-MS/MS or tandem MS is the MS that they are two or more MS combination to enhance the capability to evaluate the sample. Firstly, the ionized particles are separated by the mass-to-charge in the initially MS until they are split in to fragment ion. Secondly, the particles are separated by the mass-to-charge ratio again then the particle are detected by mass analyzer. The peptide sequences are the result of LC MS/MS that can be identified proteins by matching with the database on computer program.

CHAPTER III
MATERIALS AND METHODS

There were 4 processes for achieving the objectives.

3.1 Experimental horses

The experimental protocol was approved by Institution Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Science, Kasetsart University, Bangkok, Thailand (ACKU63-VET-046).

All of the sample were collected from International Riding School, Horseshoe Point, Pattaya, Chonburi, Thailand. Approximately 15 melanocytic neoplasia grey horses and 10 normal healthy skin in grey horses (n=25) in all breeds including Thoroughbred, Lusitano and pony, both sexes (stallion, mare, and gelding) with the range of 3-31 years old and body weight of 400-500 kg were used. In addition, History and type of work were noted. Afterward, the clinical melanoma examination were classified by (Desser et al., 1980) (Table 1).

Table 1: The clinical classification of melanoma

Grade	Description
0	Free of melanoma
1	The early stage the nodule 0.5 cm diameter
2	Several nodules with 0.5-2 cm diameter
3	One or several nodules with 5 cm diameter
4	Extensive confluent melanoma cover with skin; signs of destruction (necrosis, ulceration) metastasis
5	Exophytic growth of tumor with the metastasis

After examined the lesions of neoplasm on the typical site, three veterinarian investigators were grading the lesions of 25 experiment grey horses. Then, the grade was put into 3 components depend on the lesions for categorization. In normal group

had not seen any nodule on the typical site. Mild group had seen 1-2 small nodule on the typical site. The severe group had found obviously nodule especially on the typical site. The clinical classification of melanoma had shown in Table 2.

Table 2: The clinical classification of EMN for categorization (modified from Desser et al., 1980)

Grade	Description	Category
0	Free of melanoma	normal
1	The early stage the nodule 0.5 cm diameter	mild
2	Several nodules with 0.5-2 cm diameter	severe
3	One or several nodules with 5 cm diameter	severe
4	Extensive confluent melanoma cover with skin; signs of destruction (necrosis, ulceration) metastasis	severe
5	Exophytic growth of tumor with the metastasis	severe

The inclusion criteria for experiment were used the grey horses, age 3-31years old. The gender including stallion, gelding and mare were used. The exclusion criteria for experiment were the grey horses below 3 years old and over 31 years old, the solid color horses.

3.1.1 Sample collection procedure

Firstly, all the grey horses were taken to the stall for measuring the body weight and the body condition score were assessed. Subsequently, the physical examination including heart rate, respiratory rate, gut sound, capillary refilling time (CRT), mucous membrane, hydration status and rectal temperature were investigated. In addition, regional lymph nodes such as inguinal area, sub-mandibular area and retropharyngeal area were palpated. Afterward, the grey horses were inspected and palpation the neoplasia along with count the amount of the lumps and measured each of the lumps. Moreover, the position of the lumps was recorded. History illness were taken note and the other's abnormality were examined.

3.1.2 Tissue biopsy collection

The grey horses were injected with 0.5-1.1 mg/kg of xylazine hydrochloride intravenously. The lesion margin approximately 0.5-1 cm were excised (Hewes & Sullins, 2009). The lump was located and will be shaved prepare for the completely removal excisional. Afterthought, the surgical region was scrubbed by the chlorhexidine and betadine diluted. Local anesthesia was applied by 2% lidocaine hydrochloride around the excised area. The surgical blade was used for removed the lump and simple continuous suture pattern were used to pull mucosal get closed by vicryl#2/0 and simple interrupted suture pattern were used in submucosal layer by supramid#2/0. After surgery, the horse was treated with antibiotic drug, penstep® LA injection that composed with penicillin G benzathine 100,000 I.U., penicillin G procaine 150,000 I.U. and dihydrostreptomycin sulfate 200 mg dosage 25,000-50,000 mg/kg twice per day intramuscularly for 7 days. In addition, the horse was given the nonsteroidal anti-inflammatory drugs (NSAIDs), phenylbutazone 2.2-4.4 mg/kg twice per day orally for 7 days. The tissue biopsy was kept in 10% formalin buffer then they were examined by histopathology.

3.1.3 Blood sample collection

The grey horses were collected the blood from the jugular vein. The blood samples 10 ml were kept in 2 plain tubes and 3 ml in 1 EDTA tube. All of the blood sample were kept refrigerated at 4^{o}C. Afterward, the blood samples in the plain tubes were centrifuged for collected the serum and they were kept frozen at –80^{O}C before analyses. The blood samples in the EDTA were tested for complete blood count (CBC) and serum chemistry profiles such as Blood urea nitrogen (Koss et al.), creatinine, aspartate aminotransferase (AST) and gramma-glutamyl transpeptidase (GGT).

3.1.4 Feces sample collection

The grey horse was collected the feces by rectal palpation. Then the feces were contained in Eppendorf. Afterward, the samples were kept in liquid nitrogen at -196^{0}C. Next, they were kept frozen at –80^{O}C until analyses.

3.2 Laboratory procedure

3.2.1 The sample preparation

All of the sample including the blood and the feces were extracted the protein. Afterwards, each all the sample were determined the total protein concentration by according to the method of Lowry and his colleagues (Lowry et al., 1951) with bovine serum albumin (BSA) were used as the standard. Approximately 5 microgram of protein samples were subjected to in-solution digestion. Samples were completely dissolved in 10 mM ammonium bicarbonate (AMBIC), reduced disulfide bonds using 5 mM dithiothreitol (DTT) in 10 mM AMBIC at 60ºC for 1 hour and alkylation of sulfhydryl groups by using 15 mM Iodoacetamide (IAA) in 10 mM AMBIC at room temperature for 45 mins in the dark. The mixed samples were measured for the net absorbance for calibration standards, and reference standard at 750 nm by RT-2100C microplate reader. Afterward the net values for the standards versus protein concentration were prepared a calibration plot by graphing. For digestion, samples were mixed with 50 ng/µl of sequencing grade trypsin (1:20 ratio) (Promega, Germany) and incubated at 37ºC overnight. Prior to LC-MS/MS analysis, the digested samples must be dried and protonated with 0.1 % formic acid before injection into LC-MS/MS.

3.2.2 Liquid Chromatography-Tandem Mass Spectrometry

The tryptic peptide samples were prepared for injection into an Ultimate3000 Nano/Capillary LC System (Thermo Scientific, UK) coupled to a HCTUltra LC-MS system (Bruker Daltonics Ltd; Hamburg, Germany) equipped with a Nano-captive spray ion source. Briefly, five microlitre of peptide digests were enriched on a µ-Precolumn 300 µm i.d. X 5 mm C18 Pepmap 100, 5 µm, 100 A (Thermo Scientific, UK), separated on a 75 µm I.D. x 15 cm and packed with Acclaim PepMap RSLC C18, 2 µm, 100Å, nanoViper (Thermo Scientific, UK). The C18 column will be enclosed in a thermostatted column oven set to 60 °C. Solvent A and B containing 0.1% formic acid in water and 0.1 % formic acid in 80% acetonitrile, respectively were supplied on the analytical column. A gradient of 5–55% solvent B were used for elute the peptides at a constant flow rate of 0.30 µl/min for 30 min. Electrospray

ionization will be carried out at 1.6kV using the CaptiveSpray. Nitrogen was used as a drying gas (flow rate about 50 l/h). Collision-induced-dissociation (CID) product ion mass spectra were obtained using nitrogen gas as the collision gas. Mass spectra (MS) and MS/MS spectra were obtained in the positive-ion mode at 2 Hz over the range (m/z) 150–2200. The collision energy will be adjusted to 10 eV as a function of the m/z value. The LC-MS analysis of each sample were performed in triplicate.

3.2.3 Quantification and identification of proteins

Intended for the quantification of proteins, DeCyder MS Differential Analysis software (DeCyderMS, GE Healthcare) were used for quantify the proteins in individual samples while the Mascot search engine to correlate MS/MS spectra to the Uniprot mammal database. The Mascot's standard settings was performed: maximum of three miss cleavages, mass tolerance of 0.6 dalton for main search, trypsin as digesting enzyme, carbamidomethylation of cysteine as fixed modification, the oxidation of methionine as variable modifications and peptide charge state (1+, 2+ and 3+). The visualization and statistical analyses were conducted using the MultiExperiment Viewer (MeV) in the TM4 suite software.

In the direction of identification of the proteins, the selection of the raw proteomes data from the experimental grey horses each group of normal grey horses, mild EMN and severe EMN in the grey horses. Firstly, the list of protein names was checked the duplicates by remove data duplicates in Excel program and double checked by VLookup function program. Then, the maximum of intensity each protein was performed in two components composed with horses ID all groups (normal, grey horses, mild EMN and severe EMN) and protein name lists.

3.3 Bioinformatic based on protein annotation and interaction analysis

The raw protein data were used for illustrating between each group of experiment in normal, mild and severe then were showed their relationships by jvenn (http://bioinfo.genotoul.fr/jvenn/). A set diagram output indicating which protein best hit were generated at each intersection or unique to a certain list.

The particular of ID detail best hit were provided the scientific community with a comprehensive, high-quality and resource of protein sequence and functional information by uniprot (http://www.uniprot.org/).

The intensity of significant proteins database each group (serum candidate proteins and fecal candidate proteins) were annotate and generate heatmap by CLUE program (www.clue.io/command).

The database of the protein and predicted interactions between chemicals and proteins were stem from computational prediction and were predicted the knowledge transfer between organism and interaction aggregated by STITCH Version 5.0 (www.stitch.embl.de).

3.4 Statistical analysis

The data of horse's information including aged, gender and breed were presented as Arithmetic Mean. The data of blood parameters (hematology and blood chemistry) were presented as post-hoc testing a Duncan's Multiple Range Test using SigmaStat 3.5 (Systat Software Inc., California, USA) and performed to compare across EMN groups. An one-way ANOVA for multiple comparisons was performed to evaluate differences average in each group. For all statistical tests used, a *p–value* of 0.05 was considered significant.

The Wilcoxon rank-sum test and multiple testing via false discovery rate (FDR) correction were performed to identify statistically significant differences (adjusted p-value < 0.05) in cancer pathway in each group. Remarkably, the Wilcoxon rank-sum test were selected in this study because it is a nonparametric test that compare medians between 3 groups of independent samples. In the DEPs analysis between the normal, mild and severe groups, the Wilcoxon rank-sum test and FDR correction were also used to identify significant proteins (adjusted p-value < 0.05). For the main functional categories and metabolic functional annotation of DEPs were used base on the KEGG database for analyzed between each group.

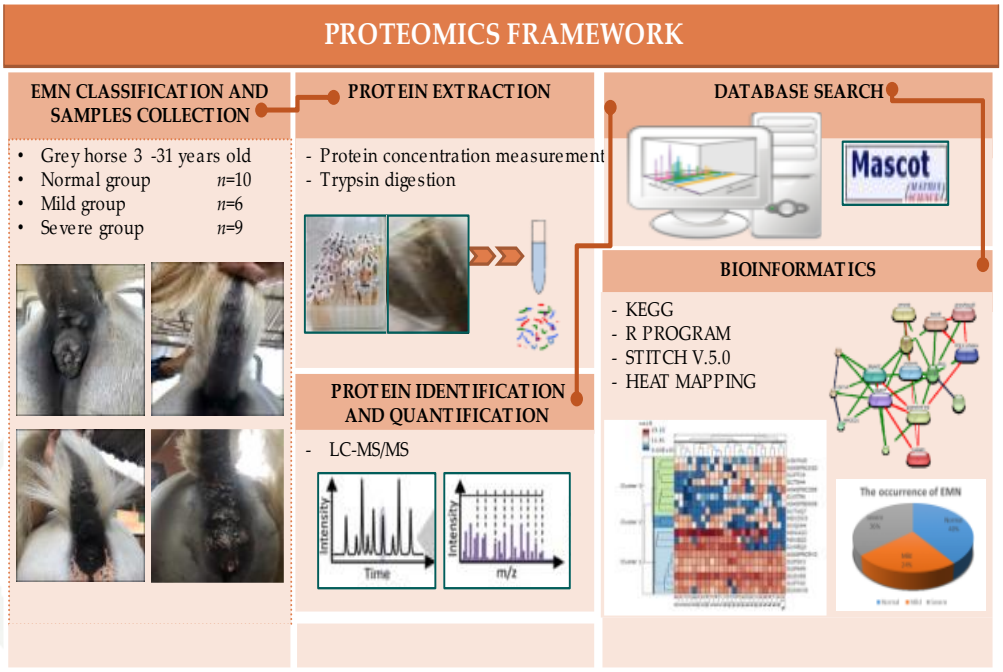

Figure 3: The proteomics framework of an experiment which entire process in serum and fecal of EMN samples then fundamental to protein identification and quantification. The data were analyzed by DeCyder MS Differential Analysis and the Mascot software (Matrix Science) was submitted for database using to search the mammalian database for protein identification. Bioinformatics were used for multidisciplinary for molecular biology.

CHAPTER IV
RESULTS

4.1 Clinical classification of the experimental grey horses

The data on physical inspection and the signalment of 25 experimental grey horses including age, sex and breed is shown in Table 3. In addition, the physical examination of horse cannot indicate the particular sign of EMN. The neoplasm appearance and lesion graded of the experimental grey horses is shown in Table 4. This study found that there were all groups of EMN including normal or free of EMN, mild EMN and severe EMN. Besides, in the location of the tumor lesion that can be found in the specific site including underneath of the tail, perianal region and perineum region both of vulva and penis. Likewise, the lumps can be found on inspection and palpation in the neck and shoulder as well. The location of the EMN is shown in Figure 4. Moreover, this research found that the experimental 10 grey horses (40%) in normal grey horse group cannot found the nodule especially on the typical site. Six grey horses in mild EMN (24%) had the 1-2 nodules on the typical site the appearance is smooth and grey color with a diameter less than 0.5 cm on underneath of the tail. The characteristic of the 9 grey horses (36%) in the severe EMN group including the several nodules with a diameter of 0.5-2 cm on underneath of the tail, perianal, perineum and diffused on the body. Therefore, the occurrence of EMN in this study approximately 60% and the occurrence of EMN pie chart is shown in Figure 5.

Table 3: The signalment data of the experimental grey horses

Horse name	Age	Sex	Breed	BW (kg)	H (hand)	HR (bpm)	RR (bpm)	Temp (Cocco et al.)	Gut sound	BCS	CRT (sec)	MM	HS
A=Sai Tip	15	M	TB	470	14.3	36	44	37.5	1+,2+ 2+,2+	3/5	<2	Pale pink	Normal
B=Safira	23	M	TB	506	16	54	60	37.3	2+,2+ 2+,2+	3/5	<2	Pale pink	Normal
C=Dimente	18	M	Lu	476	15.3	36	20	37.5	2+,2+ 2+,2+	3.5/5	<2	Pale pink	Normal
D=Venus	13	M	Lu	560	15.2	36	30	37.4	2+,2+ 2+,2+	4/5	<2	Pale pink	Normal
E=Piccaju	22	M	P	275	12.2	34	48	37.8	1+,2+ 2+,2+	3/5	<2	Pale pink	Normal
F=Popcicle	14	M	P	270	12.1	40	24	36.8	1+,2+ 1+,2+	3/5	<2	Pale pink	Normal
G=Karena	26	M	Lu	432	15.0	52	60	37.8	2+,1+ 2+,1+	2.5/5	>2	Pale pink	Normal
H=Zagalita	19	M	Lu	425	15.2	36	42	37.2	2+,2+ 2+,2+	3/5	>2	Pale pink	Normal
I=Mochitaru	22	M	P	307	11.3	44	20	37.3	1+,1+ 1+,2+	3.5/5	<2	Pale pink	Normal
J=Mu-Lhane	23	M	Li	447	15.0	39	24	37.8	1+,2+ 1+,2+	3/5	<2	Pale pink	Normal
K=Querida	19	M	Lu	470	15.0	36	48	37.4	2+,2+ 1+,2+	3.5/5	<2	Pale pink	Normal
L= Zetta	23	M	Lu	532	14.3	36	48	37.4	1+,2+ 1+,2+	4.5/5	<2	Pale pink	Normal
M=Robinhood	26	G	P	221	12.0	36	68	37.0	2+,1+ 2+,1+	3/5	<2	Pale pink	Normal
N=San Dee	10	G	TB	432	15.3	40	68	38.6	2+,2+ 2+,2+	2.5/5	<2	Pale pink	Normal
O=Estoryl	24	M	Lu	420	14.3	40	48	37.4	2+,2+ 2+,2+	3/5	<2	Pale pink	Normal
P=Ausawin	26	S	P	191	11.1	28	48	37.6	2+,2+ 2+,2+	2.5/5	<2	Pale pink	Normal
Q=Goliate	13	G	Lu	447	15.2	36	40	37.3	2+,2+ 1+,2+	3.5/5	<2	Pale pink	Normal
R=Ruby	12	M	Lu	506	16.1	40	28	37.3	2+,1+ 1+,2+	3/5	<2	Pale pink	Normal
S=Sky	14	M	Lu	425	14.3	40	28	37.5	1+,1+ 1+,2+	3.5/5	<2	Pale pink	Normal
T=Tovatory	18	M	Lu	485	15.0	33	32	37.5	1+,2+ 1+,2+	3/5	<2	Pale pink	Normal
U=Mario	17	G	Lu	461	16.1	44	68	37.4	1+,1+ 1+,1+	3/5	<2	Pale pink	Normal
V=Vandy	26	M	P	280	11.3	36	28	37.4	1+,2+ 2+,1+	4/5	<2	Pale pink	Normal
W= Carrusso	14	G	Lu	470	17.0	36	32	37.0	1+,2+ 1+,2+	3/5	<2	Pale pink	Normal
X=Mustrado	29	S	Lu	412	16.3	30	12	37.4	2+,2+ 2+,2+	3/5	<2	Pale pink	Normal
Y=Veena	31	M	P	260	12.0	40	36	37.0	2+,2+ 2+,2+	3/5	<2	Pale pink	Normal
Z=Princess	18	M	P	421	13.3	38	40	38.0	1+,1+ 1+,1+	3.5/5	<2	Pale pink	Normal
Average	19			398.7	14.24	38.30	40.15	37.44					
SD	5			98.9	1.71	5.52	15.52	0.352					

*TB=Thoroughbred, Li=Lipizzaner, Lu=Lusitano, P=Pony, G=Gelding, S=Stallion, M=Mare, BW=Body weight, H=Height, HR=Heart rate, RR=Respiratory rate, bpm=beat per minute, Temp=Temperature, ^{O}C=degree Celsius, CRT=Capillary refilling time, Gut sound= (-) absent, (1+) hypomotility, (2+) normal motility, BCS=body condition score, MM=mucous membrane, HS=Hydration status

Table 4: Neoplasm appearance of the experimental grey horses for categorization (modified from Desser et al., 1980)

Horse ID	Neoplasm appearance and location	Categorization
1; Ruby	Not found	Grade 0=normal
2; Venus	Not found	Grade 0=normal
3; Goliate	Not found	Grade 0=normal
4; Popsicle	Not found	Grade 0=normal
5; Sky	Not found	Grade 0=normal
6; Sai Tip	Not found	Grade 0=normal
7; Tovatory	Not found	Grade 0=normal
8; Kreda	Not found	Grade 0=normal
9; Zagalita	Not found	Grade 0=normal
10; Robinhood	Not found	Grade 0=normal
1; Carrusso	1-2 nodules below the tail with a diameter less than 0.5 cm	Grade 1=mild
2; Princess	1-2 nodules below the tail with a diameter less than 0.5 cm	Grade 1=mild
3; Damante	1-2 nodules below the tail with a diameter less than 0.5 cm	Grade 1=mild
4; Mochitaroo	1-2 nodules below the tail with a diameter less than 0.5 cm	Grade 1=mild
5; Estoryl	1-2 nodules below the tail with a diameter less than 0.5 cm	Grade 1=mild
6; Vena	1-2 nodules below the tail with a diameter less than 0.5 cm	Grade 1=mild
1; Sandee	several nodules with a diameter of 0.5-2 cm below the tail and perianal	Grade 2=severe
2; Mario	several nodules with a diameter of 0.5-2 cm below the tail and perianal	Grade 2=severe
3; Piccaju	several nodules with a diameter of 0.5-2 cm below the tail, perianal, vulva and diffuse on body	Grade 2=severe
4; Setta	several nodules with a diameter of 0.5-2 cm with the extensive confluent melanoma covered with skin; signs of destruction as the ulceration below the tail, perianal and vulva.	Grade 4=severe
5; Saphera	several nodules with a diameter of 0.5-2 cm below the tail, perianal, vulva.	Grade 2=severe
6; Vandy	several nodules with a diameter of 0.5-2 cm below the tail, perianal, vulva.	Grade 2=severe
7; Karena	several nodules with a diameter of 0.5-2 cm below the tail, perianal, vulva and the neck	Grade 2=severe
8; Awassawin	several nodules with a diameter of 0.5-2 cm below the tail and perianal	Grade 2=severe
9; Mustrado	several nodules with a diameter of 0.5-1 cm below the tail	Grade 2=severe

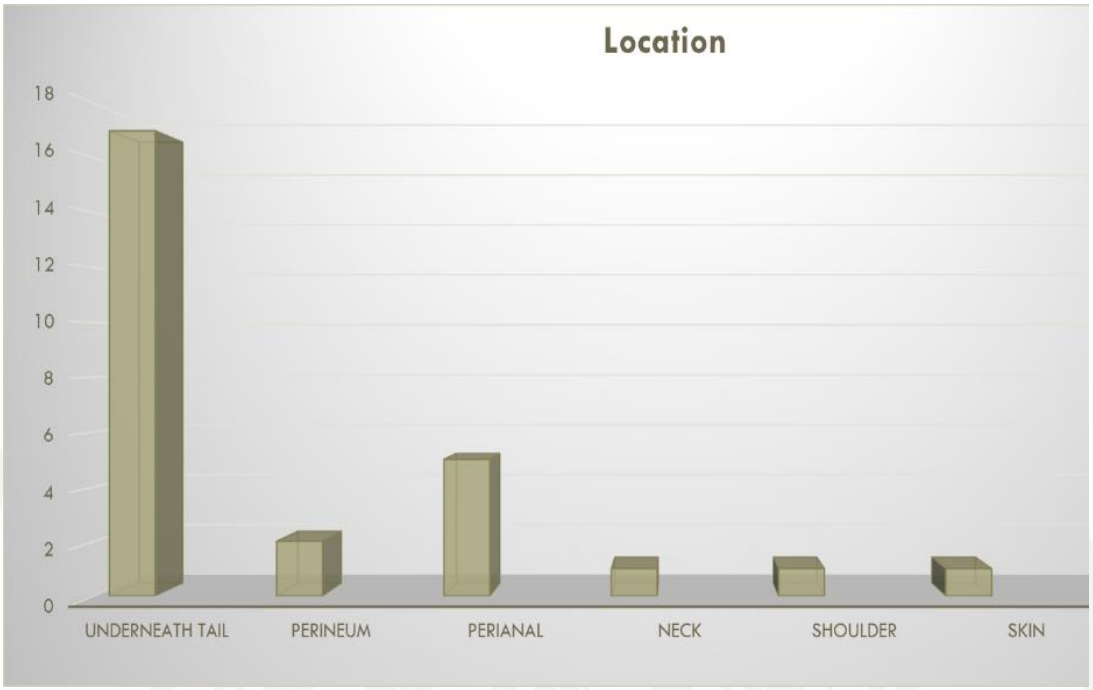

Figure 4: The location of EMN. The bar graph showed among of EMN in the typical sites and other sites on inspection and palpation including underneath of the tail, perineum region, perianal, neck, shoulder and skin.

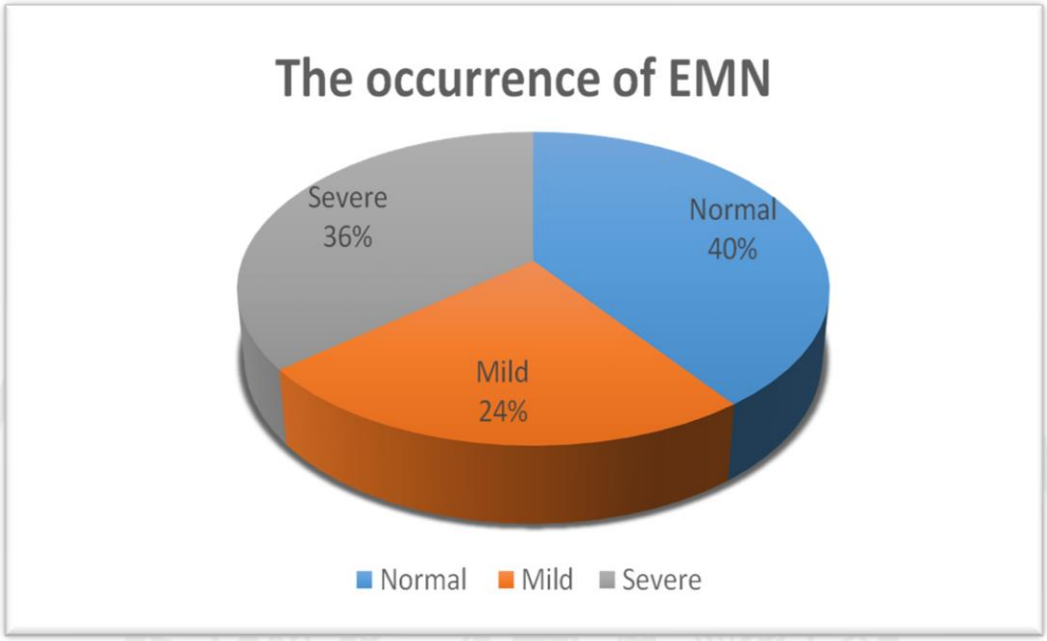

Figure 5: The occurrence of EMN (%). The pie chart showed the occurrence of EMN was 60% (15/25) that the normal grey horses was found 40% (10/25), mid EMN 24% (6/25) and severe EMN 36% (9/25).

4.2 The Complete Blood Count (CBC) and serum chemistry profiles in the experimental grey horses

The CBC results on raw data showed that 9 grey horses had higher lymphocyte level than the reference range. In addition, there were 14 grey horses had higher fibrinogen level than the reference range. Likewise, the serum chemistry profiles showed that there was 1 grey horse had high level in AST (SGOT) concentration. The CBC and serum chemistry on raw data analysis as shown in Table 5.

In addition, the platelet of mild EMN group was significantly lower than the control group ($p<0.05$). The CBC and serum chemistry were not difference between studied groups and the value still in the normal range. The CBC and serum chemistry on One-way ANOVA analysis as shown in Table 6.

Table 5: The Complete Blood Count (CBC) and serum chemistry profiles of the individual experimental grey horses

Hematology	**Reference Range**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**K**	**L**	**M**
CBC													
WBC (cll/mm^3)	5.6-12.10	11	15.4	10.2	9.9	12.6	12.6	11.4	14.1	10.3	13.1	9.9	12.2
Neutrophils	52-70%	60	58	50	52	67	39	30	35	54	45	59	40
Band neutrophils	0-1%	0	0	0	0	0	0	0	0	0	0	0	0
Lymphocyte	21-42%	34	37	47	45	26	58	66	60	44	52	38	54
Monocyte	0-6%	2	2	2	2	3	3	4	4	2	3	3	4
Eosinophils	0-7%	4	3	1	1	3	0	0	1	0	0	0	2
Basophils	0-0.2%	0	0	0	0	0	0	0	0	0	0	0	0
Platelets	117-256	182	200	132	298	228	190	246	219	158	264	202	260
RBC (cee/mm^3)	6.0-10.4	8	7.6	7.1	8.7	6.8	7.5	7.5	8.4	6.4	8.4	7.8	7.4
Hematocrit (%)	27-43	35.5	35	33.9	38.4	33.2	31.8	36.7	39.6	30.3	37.7	37.3	34.4
Hemoglobin	10.1-16.1	11.9	11.5	11.1	12.8	10.4	10.2	11.8	12.8	9.8	12.4	12.1	10.6
MCV	37-49	44	46	48	44	49	43	49	47	48	45	48	47
MCH	13.7-18.2	14.9	15.1	15.5	14.7	15.3	13.6	15.8	15.3	15.4	14.8	15.5	14.4
MCHC	35.3-39.3	33.5	32.9	32.7	33.3	31.3	32.1	32.2	32.3	32.3	32.9	32.4	30.8
RDW-CV		26	27	27	29	30	27	26	29	27	25	29	34
Anisocytosis		Few	Few	Few	Few	Few	Few	Few	Few	Few	Few	Few	Few
Macrocyte		Few	Few	Few	Few	Few	Few	Few	Few	Few	Few	Few	Few
Microcyte		Few	Few	Few	Few	Few	Few	Few	Few	Few	Few	Few	Few
Poikilocytosis		Few	-	Few	Few	Few	Few	-	Few	-	-	Few	Few
Acanthocyte		Few	-	Few	Few	Few	Few	-	Few	-	-	Few	Few
Fibrinogen	100-400	600	1000	1200	600	700	500	700	300	900	400	300	1000
Blood chemistry													
Creatinine	0.4-2.2	1.7	1.9	1.7	1.8	1.7	2.3	1.5	2.3	1.8	1.7	1.9	1.7
Total protein	5.6-7.6	7.3	6.9	6.3	7.3	7.1	6.1	6.4	6.8	6.6	6.6	6.9	7.4
Albumin	2.6-4.1	3.5	3.3	3.4	3.4	3.2	3.3	3.2	3.5	3.3	3.5	3.4	3.2
AST (SGOT)	160-412	292	436	269	257	277	253	260	323	258	287	295	237

Hematology	Reference Range	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
WBC (cll/mm^3)	5.6-12.10	9	9.5	13.4	6.8	10.9	7.7	9	6.4	11.9	6.9	13.2	10.7	9.5
Neutrophils	52-70%	55	40	55	66	56	65	70	72	74	71	68	58	68
Band neutrophil	0-1%	0	0	0	0	0	0	0	0	0	0	0	0	0
Lymphocyte	21-42%	42	57	40	30	40	31	26	25	23	26	27	36	27
Monocyte	0-6%	3	3	3	4	4	4	4	3	3	3	3	3	3
Eosinophils	0-7%	0	0	2	0	0	0	0	0	0	0	2	3	2
Basophils	0-0.2%	0	0	0	0	0	0	0	0	0	0	0	0	0
Platelets	117-256	232	211	240	303	268	273	228	255	265	210	198	252	211
RBC (cee/mm^3)	6.0-10.4	8.5	10	8.3	7	6.6	6.5	7	6.4	6.6	7.5	7.7	8.3	6.8
Hematocrit (%)	27-43	35.8	43.6	39.5	30.9	27.8	30.9	30.2	27.9	30.3	29.7	33.6	36.1	31.3
Hemoglobin	10.1-16.1	11.8	15	12.6	10.4	9.4	10.6	10.3	9.5	10.3	10.4	11.5	11.7	10.6
MCV	37-49	42	44	47	44.4	42.4	47.2	43.5	43.7	46.3	39.5	43.5	44	46
MCH	13.7-18.2	13.9	15	15.1	14.9	14.4	16.2	14.8	14.9	15.7	13.8	14.9	14.2	15.6
MCHC	35.3-39.3	33	34.4	31.9	33.7	33.8	34.3	34.1	34.1	34	35	34.2	32.4	33.9
RDW-CV		26	24	29	25	24	24	23	24	24	25	24	28	26
Anisocytosis		Few	Few	Few	Few	Few	Few	Few	Few	Few	Few	Few	Few	Few
Macrocyte		Few	Few	Few	Few	Few	Few	Few	Few	Few	Few	Few	Few	Few
Microcyte		Few	Few	Few	Few	Few	Few	Few	Few	Few	Few	Few	Few	Few
Poikilocytosis		Few	-	Few	Few	Few	Few	Few	Few	Few	Few	Few	Few	Few
Acanthocyte		Few	-	Few	Few	Few	Few	Few	Few	Few	Few	Few	Few	Few
Fibrinogen	100-400	1000	800	600	200	300	200	100	300	300	200	200	500	200
Blood chemistry														
Creatinine	0.4-2.2	2	1.5	2.1	1.8	1.7	1.5	1.7	1.9	1.7	1.7	2.1	1.6	1.7
Total protein	5.6-7.6	6.8	6.9	7.2	7.4	6.7	7.6	7.3	7.4	6.5	6.5	7.4	6.9	6.7
Albumin	2.6-4.1	3.4	3.3	3.4	3.4	3.1	3.3	3.2	3.6	3.3	3.4	3.1	3.3	3.1
AST (SGOT)	160-412	306	306	234	274	302	310	261	256	244	352	251	283	268

Table 6: The Complete Blood Count (CBC) and serum chemistry profiles of experimental 25 grey horses.

Parameters	Groups			SEM	*P-value*	Reference Range
	Control	Mild EMN	Severe EMN			
Hematology						
WBC (cell/mm^3)	10.7	9.5	11.5	0.469	0.30	5.6-12.10
Neutrophils	5.43	5.34	6.82	0.311	0.08	5.2-7.0
Band neutrophils (Cell/mm3)	0.00	0.00	0.00	-	-	0-1%
Lymphocyte	4.85	3.83	4.14	0.381	0.56	2.1-4.2
Monocyte	0.36	0.25	0.34	0.020	0.08	0-6
Eosinophils	0.09	0.10	0.15	0.031	0.70	0-7%
Basophils	0.00	0.00	0.00	-	-	0-0.2%
Platelets	249^{b}	196^{a}	230^{ab}	8.228	0.04	117-256
RBC (cell/mm3)	7.55	7.68	7.47	0.176	0.90	6.0-10.4
Hematocrit (%)	33.7	34.2	34.4	0.800	0.94	27-43
Hemoglobin	11.1	11.4	11.3	0.255	0.91	10.1-16.1
MCV	44.8	44.9	46.1	0.480	0.48	37-49
MCH	14.8	14.9	15.1	0.129	0.54	13.7-18.2
MCHC	33.1	33.5	32.9	0.211	0.62	35.3-39.3
RDW-CV	26.6	26.2	26.6	0.507	0.95	-
Fibrinogen	420	633	567	63.32	0.39	100-400
Blood chemistry						
Creatinine	1.82	1.67	1.87	0.044	0.22	0.4-2.2
Total protein	7.05	6.65	6.96	0.081	0.15	5.6-7.6
Albumin	3.34	3.30	3.32	0.027	0.85	2.6-4.1
AST (SGOT)	280	289	284	8.533	0.91	160-412

[a b] Means in the same row with different superscripts were different (*P<0.05*) (One-way ANOVA)

4.3 Liquid Chromatography-Tandem Mass Spectrometry (LC–MS/MS)

4.3.1 Serum sample

This study revealed that the total component of 8,725 different proteins expression in all serum samples. From Venn diagram, this study found that, there were 8,668 proteins expression in all groups. There were 10 difference protein expressed overlapped between mild EMN and severe EMN. In addition, there were 37 different proteins expressed only in severe EMN (Figure 6). The element of the protein's expression is shown in Table 6.

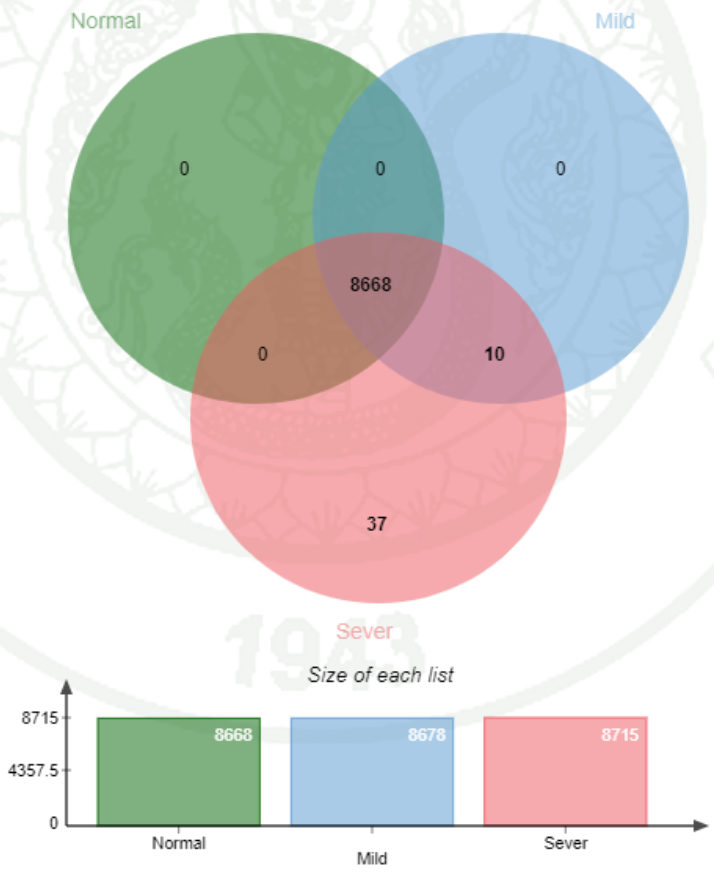

Figure 6: Venn diagram showing different serum proteins expression between normal, mild EMN and severe EMN group following the grey horses. Each set of diagrams indicating of proteins best hit that generated at each intersection by jvenn program (http://jvenn.toulouse.inra.fr/app/index.html).

Table 7: The element of the serum proteins expression

Protein	ID detail best hit	Peptide	ID Score
Element only in mild EMN group and severe group (10)			
A0A5F9CVG6	5'-3' exoribonuclease	GGRQSLHR	8.17
G1P492	5-hydroxytryptamine receptor 2B	VAMLDGSHK	5.91
H0VQS5	5-azacytidine induced 2	DNLKSK	19.42
L7N1F1	5-aminolevulinate synthase	LVSAQA	5.36
A3RL34	5-HT2b serotonin receptor	MAFSYRMSEQSK	2.81
G1SVT9	5'-nucleotidase domain containing 4	YVEKDAR	11.44
H0VA38	5'-nucleotidase domain containing 2	DMEKYILR	3.06
H0VEK1	5'-nucleotidase domain containing 1	MMPPDMLAKEYGTK	4.38
G1P4Z0	5'-nucleotidase	GDLIHVFNKHDGALK	3.59
A0A5F9CUR5	5'-nucleotidase ecto	LDNYSTK	5.13
Element only in severe EMN group (37)			
A0A286XVS2	14_3_3 domain-containing protein	YDEMVESMKK	9.01
H0V0X1	5'-3' exoribonuclease 1	RVANK	4.39
A0A286XPX3	5', 3'-nucleotidase, cytosolic	MESRPVR	9.56
G1T641	4HBT domain-containing protein	SKGTGWAAR	5.02
G1SDF8	40S_SA_C domain-containing protein	VRGTIFR	12.50
G1Q844	40S ribosomal protein S8	MGISRDDWHK	5.34
G1U0Z0	40S ribosomal protein S7	RILPKPTQK	6.30
G1TTA9	40S ribosomal protein S6	TSTSKSESSQK	4.93
A0A5K1UJS7	40S ribosomal protein S6	MATEVAADALGEEWK	6.19
A0A0A7RWC3	40S ribosomal protein S4	LVXTKGR	3.33
G1Q0J7	40S ribosomal protein S4	MAHGPKK	3.50
G1TKM8	40S ribosomal protein S3a	AAAIPHAR	3.31
G1TTE5	40S ribosomal protein S26	DISEASVFDAR	4.61
G1NZD3	40S ribosomal protein S12	TALIHDGLTR	17.03
H0WDH3	14_3_3 domain-containing protein	AMADGNEKK	3.21
G1SGI0	4-hydroxyphenylpyruvate dioxygenase	GQLLTPPK	10.48
A0A5F9DKM7	4-hydroxyphenylpyruvate dioxygenase	MTTYSDKGAK	2.93
H0UTE0	4-hydroxyphenylpyruvate dioxygenase	GNLTDLEPNGVASGM	9.78
L7N1J2	3HCDH_N domain-containing protein	FGELAMTR	6.34
A0A286XZI4	3-phosphoinositide dependent protein kinase 1	LGCEEMEGYEPLK	2.23
G1Q8M4	3-oxo-5-alpha-steroid 4-dehydrogenase	TLVYPFLMR	8.55
G1NSJ9	3-ketodihydrosphingosine reductase	MMLQAAGRR	3.85
A0A286XBL9	3hydroxymethyl 3methylglutaryl-CoA lyase like 1	VAQASFSALGS	2.75
G1PSX1	3hydroxyisobutyrylCoA hydrolase, mitochondrial	AMSEVVK	6.58
H0VGR7	3-hydroxybutyrate dehydrogenase 2	SMFLMTKAFLPK	5.51
A0A286XXH8	3-hydroxybutyrate dehydrogenase 1	VVNISSMLGRMANPAR	6.56

H0V7K3	3-hydroxy-3-methylglutaryl coenzyme A synthase	TDTWPK	7.58
G1PBI2	15-hydroxyprostaglandin dehydrogenase	AVAEALLHK	4.05
G1NU79	3'-phosphoadenosine 5'-phosphosulfate synthase 1	EGPGSLYK	6.38
G1PKA3	2Fe-2S ferredoxin-type domain-containing protein	SGQRIPVR	7.98
A0A286XHV6	26S proteasome non-ATPase regulatory subunit 2	ELDIMEPK	11.46
G1T897	2-methoxy-6-polyprenyl-1,4-benzoquinol methylase, mitochondrial	ATAQGHK	13.13
A0A5F9CS72	2-hydroxyacyl-CoA lyase 1	QPLLIVGK	9.02
G1NYM8	2-hydroxyacyl-CoA lyase 1	MKSNEAASK	4.36
Q70VZ7	2-acylglycerol O-acyltransferase 1	GIFQYNFGLIPYR	20.27
G1TYL5	2'-deoxynucleoside 5'-phosphate N-hydrolase 1	DDRALYGR	4.46
G1TLB8	2'-5'-oligoadenylate synthetase 3	AEVIAEIR	3.42

4.3.2 Feces sample

This study revealed that the total component of 8,725 different proteins expression in all serum samples. From Venn diagram, this study found that, there were 5,826 proteins expression in all groups. There were 1 different protein expressed only in normal group. One different protein expressed only in mild EMN group and five difference protein expressed overlapped between normal and mild EMN group. In addition, there were nine different proteins expressed only in severe EMN and 44 difference protein expressed overlapped between mild and severe EMN group. Moreover, 23 difference proteins expressed overlapped between severe EMN and normal group (Figure 7). The element of the protein's expression is shown in Table 7.

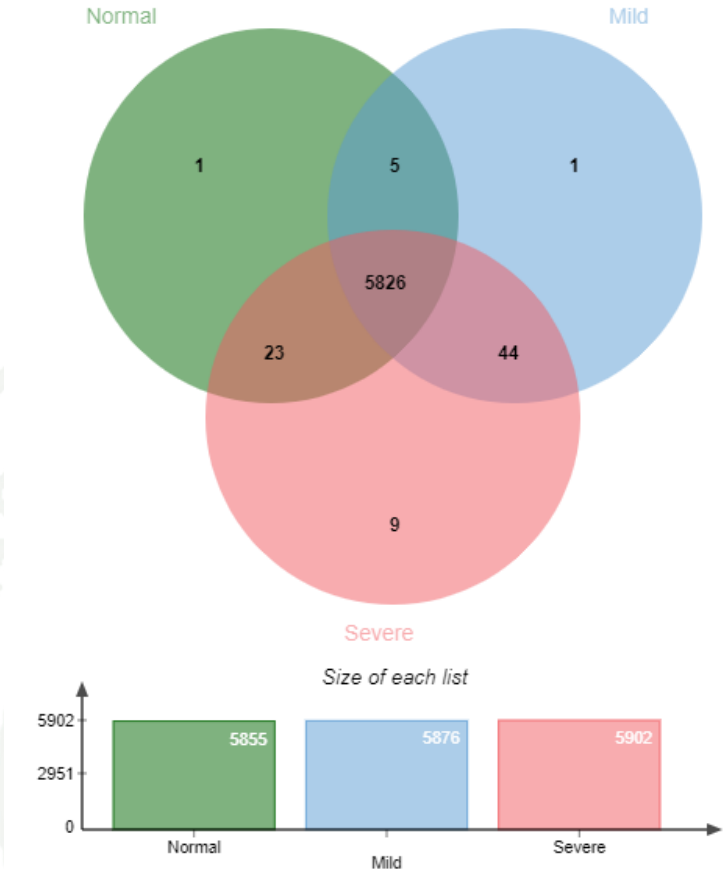

Figure 7: Venn diagram showing different feces proteins expression between normal, mild EMN and severe EMN group following the grey horses. Each set of diagrams indicating of proteins best hit that generated at each intersection by jvenn program (http://jvenn.toulouse.inra.fr/app/index.html).

Table 8: The element of the feces protein expression

Protein	ID detail best hit	Peptide	ID Score
	Element only in normal group (1)		
G1SU65	Par-3 family cell polarity regulator beta	NDNSPLQPFGTYSPQDKQK	8.09
	Element only in normal and mild EMN group (5)		
H0VJ02	Inositol polyphosphate-5-phosphatase D	SKDGSDDK	10.65
H0UU64	PMS1 homolog 2, mismatch repair system component	SQGLDSADPK	7.96
H0VXF5	Sortilin related VPS10 domain containing receptor 2	EDVGLVVTR	2.02
H0VSX6	Cilia and flagella associated protein 65	DMQGLGK	5.02
A0A286XS18	Slingshot protein phosphatase 2	MKVEK	6.47
	Element only in mild EMN group (1)		
G1PNC0	Ecotropic viral integration site 5 like	VREGQAVASAR	4.06
	Element only in mild EMN and severe EMN group (44)		
A0A286Y1U3	Pre-mRNA processing factor 40 homolog A	RSSLSPTMRPGTGAER	2.57

G1P7Y4	SHANK associated RH domain interactor	MDGPSSGDLMEK	11.03
A0A5F9DJA2	CSD_1 domain-containing protein	GADNEGAGEQGGPVR	11.87
A0A5F9DUD5	Zinc finger protein 451	DYSRCLQMMLDK	5.08
G1SKK4	Lysine demethylase 1B	ATETAEEDEDGGSEK	9.07
G1PHB6	FOS like 1, AP-1 transcription factor subunit	LELVLEAHRPICK	11.72
G1TGW9	Testis expressed 43	MASGKDTCPLFPK	13.52
H0W0S7	Tectonic family member 3	WSIISLLR	10.28
G1PRY9	CID domain-containing protein	RSTEQDVR	2.47
G1PKC8	Tyrosine-protein kinase	LSDPGMGLGALSR	12.60
A0A5F9CV15	Leucine rich repeats and immunoglobulin like domains 1	MAAPGGKEDSSR	4.51
A0A286XKA5	Interleukin 2 receptor subunit beta	SNQRPWNWTCPMFEVSR	4.83
G1PKD2	ATP binding cassette subfamily C member 6	SVMDCARVLVMDK	15.49
A0A5F9C7C6	Sortilin related VPS10 domain containing receptor 3	EEVKAPR	3.27
A0A286XQ72	Inositol 1,4,5-trisphosphate receptor type 1	IGTSPVK	9.50
H0V047	Regulatory factor X associated ankyrin containing protein	EPTRPAEELPTQPPSAPELR	4.01
G1SUL1	CD27 molecule	HCNSGLLIR	1.31
G1P2Y9	Terminal nucleotidyltransferase 5B	MMPENGVERSDR	7.22
G1P7E4	Histone deacetylase	IKLLLMR	6.40
H0VHD9	TBC1 domain family member 4	VHDGSQKPQAR	8.60
G1SQ11	Delta-1-pyrroline-5-carboxylate synthase	EASHSNR	1.57
G1SWH0	Semaphorin 3C	MGFRAICVLFGIFICSVSVK	9.99
H0VZS6	Catenin delta 2	MTEVHRSACGALR	9.16
H0UY41	Complement C5	EDFSTSGTTHFEIK	8.23
G1NSD4	Peptidyl arginine deiminase 1	ILIGSNFPKAGGR	9.08
A0A286XV09	Chromosome 1 open reading frame 226	SPSEAFPR	4.23
G1T6F3	Phosphorylase kinase catalytic subunit gamma 2	ATGNEFAVK	6.02
A0A5F9CQI5	MCF.2 cell line derived transforming sequence	MIMQGGFSVWIGHKK	7.18
W8CEN5	AMHY	QAEQWQPKQELVQHLSSK	6.49
G1TBF3	DENN domain containing 5A	VQCDEEELR	2.09
H0V1A4	Cullin 9	VRMLDDYEEITAGDK	4.43
G1PQ56	Homeobox A2	GSPGPAGALQPPEYPWMK	1.22
G1SSD4	F-box protein 25	NYFNILDK	3.15
G1PQ83	Karyopherin subunit beta 1	LSFSPAPIR	1.72
A0A286XGL0	Myosin heavy chain 1	KDIDDLELTLAK	9.23
A0A5F9CI41	Formin homology 2 domain containing 3	TISEFALEYR	9.80
G1PIQ1	Zinc finger protein 800	HMQMAHKITLSGTNSK	2.70
G1NXE3	ATP binding cassette subfamily B member 5	GTHAELMAK	1.15
Q6ZQW0	Indoleamine 2,3-dioxygenase 2	EAVPGIK	6.84
H0WC32	von Willebrand factor A domain containing 5B2	ANPVPGHLR	8.67
G1PWJ0	Extra spindle pole bodies like 1, separase	GAEALAEPRAAYSAR	9.84
H0V8A6	Drebrin like	LRSPFLQK	9.19
G1T4M4	Zinc finger protein 804B	IIHCGLSSQVSCVADR	6.58
H0W059	Rho guanine nucleotide exchange factor 16	AKTPTR	5.71
	Element only in severe EMN group (9)		
A0A5F9DQY0	KIAA0753	RMEEMEK	11.86
G1PJ91	Tetratricopeptide repeat domain 12	LLPSLLASGVLPIR	10.13
G1P0M5	CST telomere replication complex component 1	LRVIQPNLAGK	7.29

G1SNN8	Calcium release activated channel regulator 2A	EQVYQSRGTEDR	8.94
G1PMU6	Proline rich 35	AAAPAKPPAPPKGPPGTLAPGLLK	5.99
A0A5F9C6C9	Adipocyte plasma membrane associated protein	TRDDEPACGRPLGVR	5.18
A0A286XC38	Patatin like phospholipase domain containing 8	LSTSAPK	1.14
A0A286XVV3	Centromere protein H	INTESSVLMNTMK	3.76
A0A5F9CBX6	Eukaryotic elongation factor 2 kinase	QSDIGGGCPK	13.29
	Element only in severe EMN and normal group (23)		
G1SMS8	Pleckstrin homology domain containing S1	GQQKGAED	4.27
G1SPY0	Syntaxin binding protein 5	GVAARCSR	3.69
G1Q5L3	YY1 associated factor 2	SEKETTSK	5.05
G1PKS3	Large 60S subunit nuclear export GTPase 1	WNGGEMK	1.35
H0V885	Solute carrier family 25-member 14	GIFPGIILIFLR	25.32
H0W5B7	TNF receptor-associated factor	HVEDSVKPHLAMMCALVSR	8.81
H0VMI9	Scm polycomb group protein like 4	QHANTGPYLEGEK	8.75
G1SYS6	Acylphosphatase	LAGVGLLIALVSTLGSSVR	1.67
G1QCF5	DNA- (apurinic or apyrimidinic site) lyase	LRPSRAGSSK	13.43
G1PNJ3	X-ray repair cross complementing 4	KALVSGAGPADAYK	8.59
G1SNW1	Transmembrane serine protease 11B	NIWHLVGIVSWGDGCGK	7.65
G1PCH0	Serine dehydratase	DSMALSKVAGTSVYIK	4.07
G1NU43	Meiosis specific with OB-fold	LADPVEASRK	12.86
A0A5F9DLI7	Triacylglycerol lipase	VFNFCSTDTVVEK	13.99
A0A5F9D0A6	DUF3496 domain-containing protein	LDTASSK	3.83
A0A5F9D4W4	CDK5 regulatory subunit associated protein 3	YGGRPLEPMGEPGK	8.98
A0A5F9D8Q4	Centrosomal protein 95	TSFVEDPATPPGSVVPSAR	10.02
G1P167	Nuclear receptor coactivator 7	IQVPIEDMLPSKEEK	4.88
H0VGK9	Cell division control protein	EGKGFK	2.51
A0A5F9CJZ2	PPARG related coactivator 1	MGSACLLEPSKAMEPK	3.68
H0VHH6	ATP binding cassette subfamily B member 7	MQNHDNTKWDPK	1.66
H0UXN7	RAS guanyl releasing protein 3	MIEEFREVASQLGYEK	9.27
A0A5F9C7X7	Myotubularin related protein 12	EQDDVILQIHK	4.33

4.4 Functional annotation proteomics

4.4.1 Serum sample

It was discovered that there were total of the serum expressed proteins (3,619 annotated proteins out of 8,725 total proteins from the assessed proteomic data) were functionally assigned according to the KEGG database. The KEGG Orthology database were grouping that respond the highly similar the sequence group in main functional categories of cancer. The totally of the 6 main functional categories compose with metabolism, genetic information processing, environmental information processing, cellular processes, human diseases and organismal systems

were classified. As shown in Figure 8, the pie chart provides information about function of the main functional categories 3 groups of EMN (normal, mild EMN and severe EMN) compare each other. The main functional category was environmental information processing. In term of the proteins with metabolic function involved in the metabolism category were subcategorized, and the results are shown in Table 8. Moreover, the accounting of proteins involved in signal transduction (749 proteins), transport and catabolism (190 proteins), glycan biosynthesis and metabolism (100 proteins), digestive system (87 proteins), nervous system (34 proteins) and drug resistance: antineoplastic (2 proteins) were found to be higher in severe EMN group than in normal group and mild EMN group.

Table 9: Main functional–KEGG categories 3 groups of the experimental grey horses EMN of the serum sample

Main functional-KEGG categories	Normal grey horse	Mild EMN	Severe EMN
Unclassified proteins	5,066	5,075	5,099
Metabolism	786	786	789
Human Diseases	302	301	304
Cellular Processes	385	386	387
Organismal Systems	568	567	569
Genetic Information Processing	634	634	636
Environmental Information Processing	924	929	931

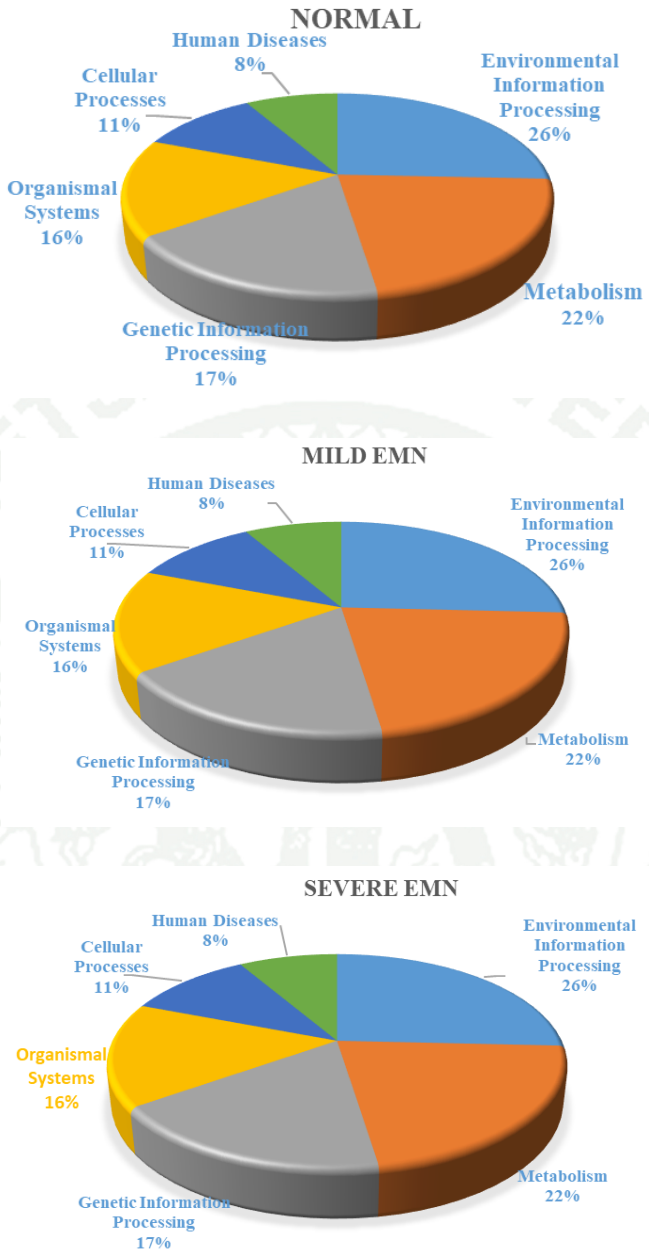

Figure 8: The pie chart provides proportion about main function of the main functional categories 3 groups of EMN (normal, mild EMN and severe EMN) compare each other. The main functional 6 categories composed with metabolism, environmental information processing, genetic information processing, organismal system, cellular processes and human diseases.

Table 10: Sub-functional KEGG categories 3 groups of the experimental grey horses EMN of the serum sample

Sub-functional KEGG categories	Normal grey horse	Mild EMN	Severe EMN
Unclassified proteins	5,066	5,075	5,099
Signal transduction	743	747	749
Translation	244	244	244
Carbohydrate metabolism	203	203	203
Folding, sorting and degradation	202	202	203
Transport and catabolism	188	189	190
Lipid metabolism	154	153	154
Signaling molecules and interaction	138	139	139
Immune system	113	113	113
Amino acid metabolism	112	111	112
Endocrine system	110	110	110
Cell growth and death	106	106	106
Sensory system	106	106	106
Replication and repair	102	102	102
Glycan biosynthesis and metabolism	99	99	100
Digestive system	87	87	87
Transcription	86	86	87
Infectious disease: viral	80	81	81
Nucleotide metabolism	73	73	73
Cancer: overview	71	70	71
Cellular community - eukaryotes	70	70	70
Neurodegenerative disease	68	67	68
Development and regeneration	67	66	67
Energy metabolism	62	63	63
Metabolism of cofactors and vitamins	51	51	51
Infectious disease: bacterial	44	44	44
Membrane transport	43	43	43
Nervous system	33	33	34
Environmental adaptation	29	29	29
Cell motility	21	21	21
Metabolism of other amino acids	16	17	17
Endocrine and metabolic disease	15	15	15
Cardiovascular disease	13	13	13
Metabolism of terpenoids and polyketides	10	10	10
Circulatory system	10	10	10
Aging	9	9	9
Xenobiotics biodegradation and metabolism	6	6	6
Infectious disease: parasitic	5	5	5
Excretory system	4	4	4
Cancer: specific types	2	2	2

Immune disease	2	2	2
Substance dependence	1	1	2
Drug resistance: antineoplastic	1	1	1

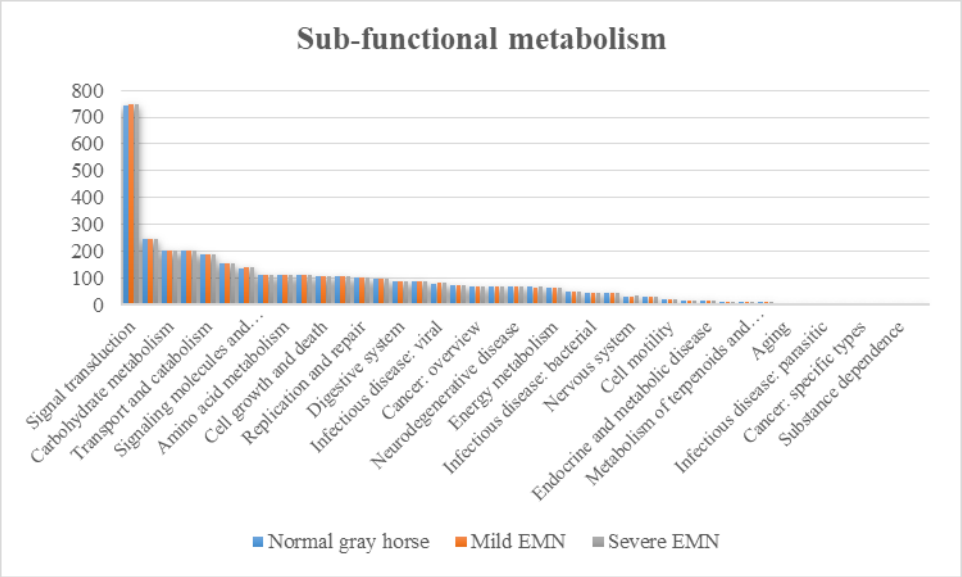

Figure 9: The bar chart provides proportion about sub-function of the KEGG categories 3 groups of serum EMN (normal, mild EMN and severe EMN) compare each other.

4.4.2 Feces sample

It was discovered that there were total of the feces expressed proteins (3,619 annotated proteins out of 8,725 total proteins from the assessed proteomic data) were functionally assigned according to the KEGG database. The KEGG Orthology database were grouping that respond the highly similar the sequence group in main functional categories of cancer. The totally of the 6 main functional categories compose with metabolism, genetic information processing, environmental information processing, cellular processes, human diseases and organismal systems were classified. As shown in Figure 10, the pie chart provides information about function of the main functional categories 3 groups of EMN (normal, mild EMN and severe EMN) compare each other. The main functional category was environmental

information processing. In term of the proteins with metabolic function involved in the metabolism category were subcategorized, and the results are shown in Table 11. Moreover, the accounting of proteins involved in glycan biosynthesis and metabolism, nucleotide metabolism, energy metabolism, metabolism of other amino acids, xenobiotics biodegradation and metabolism and metabolism of terpenoids and polyketides had the equal proteins all groups of experimental grey horses. While the categories of carbohydrate metabolism and lipid metabolism in normal group and severe group had outnumber of proteins than mild group. The sub-functional categories are shown in Table 12 and bar chart provides proportion about sub-function of the KEGG categories 3 groups of feces EMN (normal, mild EMN and severe EMN) compare each other are shown in Figure 11.

Table 11: Main functional –KEGG categories 3 groups of the experimental grey horses EMN of the feces sample

Main functional-KEGG categories	Normal grey horse	Mild EMN	Severe EMN
Metabolism	573	572	573
Human Diseases	189	189	189
Cellular Processes	255	255	257
Organismal Systems	81	82	82
Genetic Information Processing	450	450	454
Environmental Information Processing	602	612	614

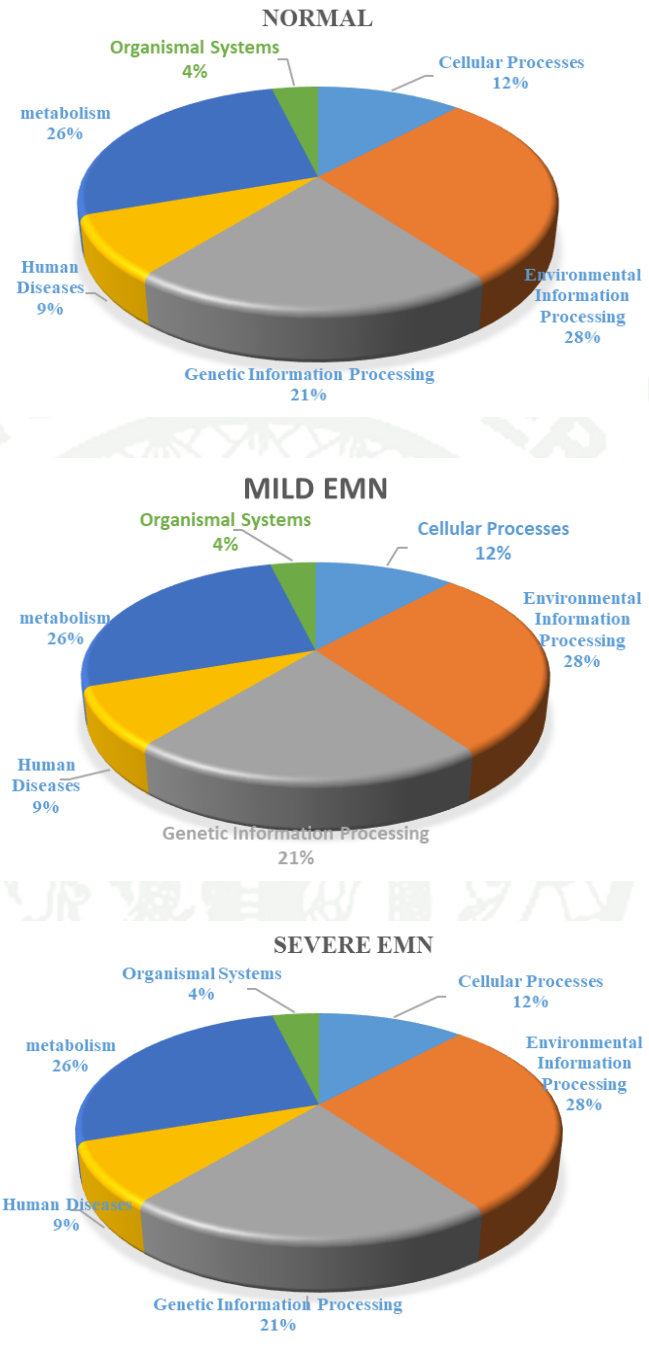

Figure 10: The pie chart provides proportion about main function of the main functional categories 3 groups of EMN (normal, mild EMN and severe EMN) compare each other. The main functional 6 categories composed with metabolism, environmental information processing, genetic information processing, organismal system, cellular processes and human diseases.

Table 12: Sub-functional KEGG categories 3 groups of the experimental grey horses EMN of the feces sample

Sub functional-KEGG categories	Normal grey horse	Mild EMN	Severe EMN
Unclassified proteins	5283	5304	5329
Lipid metabolism	107	106	107
Energy metabolism	38	38	38
Nucleotide metabolism	52	52	52
Amino acid metabolism	104	105	105
Carbohydrate metabolism	139	138	139
Metabolism of other amino acids	9	9	9
Glycan biosynthesis and metabolism	66	66	66
Metabolism of cofactors and vitamins	48	48	47
Metabolism of terpenoids and polyketides	4	4	4
Xenobiotics biodegradation and metabolism	6	6	6

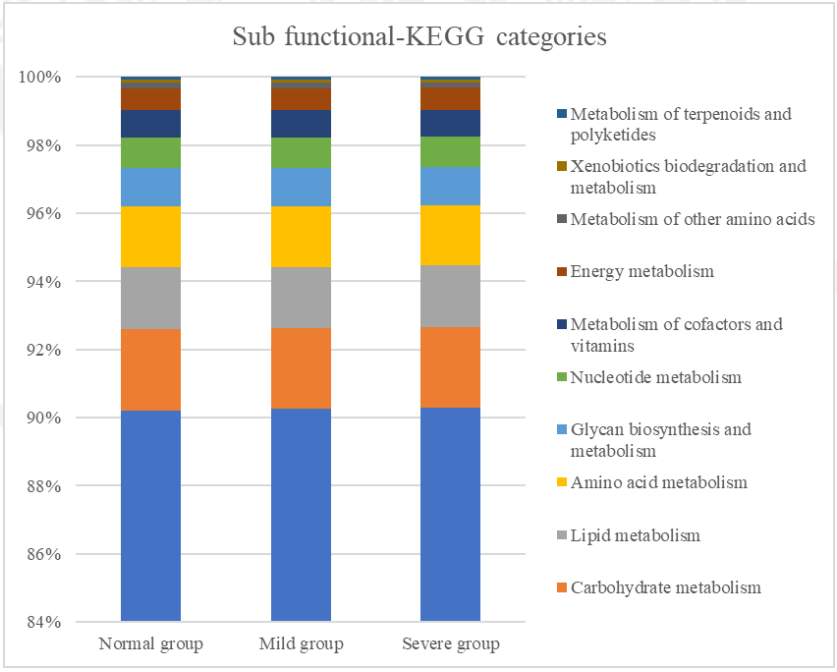

Figure 11: The bar chart provides proportion about sub-function of the KEGG categories 3 groups of feces EMN (normal, mild EMN and severe EMN) compare each other.

4.5 Analysis of differentially expressed proteins (DEPs) and functional annotation of grey horses

4.5.1 Serum sample

According to previous protein expression details both of serum sample proteomic analysis of EMN, all data were comprised in the differential expressed proteins or DEPs analysis with the Wilcoxon rank-sum test and FDR correction. Remarkably, 41 significant serum proteins were identified under an adjusted *P*-value <0.05. Based on study results, forty-one significant proteins between groups were found which involved in metabolism and non-metabolism at 10 and 31, respectively. Moreover, they were categories metabolism protein functions and non-metabolism along with KEGG database as shown in Table 12. According to the significant results for comparative protein expression levels (PELs), all groups are shown in Figure 12.

Table 13: List of 43 significant proteins and associated functions across EMN stages in serum sample

Protein ID	Protein function	PELs[a]		
		N	M	S
Metabolism				
A0A286XQH3	Lipin 2 (LPIN2)	19.08	20.44	20.10
A0A5F9C5G3	Serine hydroxymethyltransferase (SHMT1)	19.34	8.49	20.07
A0A5F9CSF7	Phosphoinositide phospholipase C (PLCH1)	3.28	13.59	14.48
G1PR13	Beta-carotene oxygenase 1 (BCO1)	16.94	18.34	18.00
G1Q6A9	Amine oxidase (LOC102438245)	18.34	19.09	19.09
G1T4J8	Elongation of very long chain fatty acids protein (ELOVL2)	6.15	16.18	15.04
H0V7K3	3-hydroxy-3-methylglutaryl coenzyme A synthase (HMGCS2)	19.20	20.00	20.04
H0VAB2	Aminoadipate-semialdehyde synthase (AASS)	0.00	15.27	15.91
H0VEW5	Phospholipase D family member (PLD3)	19.33	18.36	18.04
H0VT86	Sphingomyelin phosphodiesterase 3 (SMPD3)	0.00	16.27	14.77
Non-metabolism				
A0A286XCY2	Ubiquitin conjugating enzyme E2 K (UBE2K)	4.64	14.86	13.73
G1Q347	RRM domain-containing protein (LOC102432793)	16.23	17.23	17.09
G1U115	Proteasome 26S subunit, non-ATPase 6 (PSMD6)	17.27	19.07	18.79
H0VPF4	Tetratricopeptide repeat domain 37 (TTC37)	17.70	16.10	12.63
H0VTB1	DEAH-box helicase 16 (DHX16)	16.80	15.71	15.83
Q9BDT7	BRCA1 (Fragment)	16.73	17.77	17.90
A0A286XXU5	Ribosomal protein S6 kinase (RPS6KA4)	11.98	14.80	16.06
G1SJV2	Ras related GTP binding A (RRAGA)	0.00	16.25	15.88
G1SSU6	Contactin associated protein 1 (CNTNAP1)	18.04	18.78	19.19
G1U3S4	Phosphorylase b kinase regulatory subunit (PHKA1)	16.30	18.29	18.33
H0VGZ3	Rho-associated protein kinase (ROCK1)	0.00	18.55	17.69

H0VZ55	Vav guanine nucleotide exchange factor 2 (VAV2)	6.71	18.23	18.10
H0W6F7	Dishevelled segment polarity protein 2 (DVL2)	0.00	5.82	0.00
G1NWY2	Dedicator of cytokinesis 1 (DOCK1)	16.49	17.43	17.94
G1P8J9	Protein Mdm4 (MDM4)	14.81	15.93	16.69
G1QES4	Synaptopodin (SYNPO)	0.00	14.42	13.94
G1SHG6	Ubiquitin carboxyl-terminal hydrolase (USP2)	0.00	18.90	18.04
G1T956	ArfGAP with coiled-coil, ankyrin repeat and PH domains 1 (ACAP1)	18.29	19.61	19.34
G1TSN4	NBR1 autophagy cargo receptor (NBR1)	15.87	17.05	16.63
H0UTL4	Spartin (SPART)	19.06	20.14	19.83
A0A5F9DDG3	Uncharacterized protein	18.37	18.29	21.31
G1PQZ8	Uncharacterized protein (LOC102423280)	8.948	19.29	19.11
H0VGK9	Cell division control protein (CDC6)	18.25	19.45	19.51
A0A5F9D2V3	Olfactory receptor (OR3A1)	0.00	13.98	14.82
G1TW10	Mediator of RNA polymerase II transcription subunit 1 (MED1)	15.49	18.02	17.50
H0VDT5	Solute carrier family 17 member 8 (SLC17A8)	0.00	16.77	15.71
H0WBK8	Protein phosphatase 4 regulatory subunit 3C (PPP4R3C)	20.06	19.29	19.15
H0WCB7	Olfactory receptor family 1 subfamily I member 1 (OR1I1)	15.52	14.55	11.49
A0A286Y022	Hepsin (HPN)	13.13	14.67	16.06
A0A286Y239	Myosin IB (MYO1B)	17.18	17.98	17.87
G1SEQ3	Tyrosine-protein kinase receptor (Gross et al.)	0.00	20.00	19.52
G1SQI2	Peripherin (PRPH)	16.57	18.21	18.81
Q6V501	Lambda5 (IGLL1)	0.00	17.69	16.59

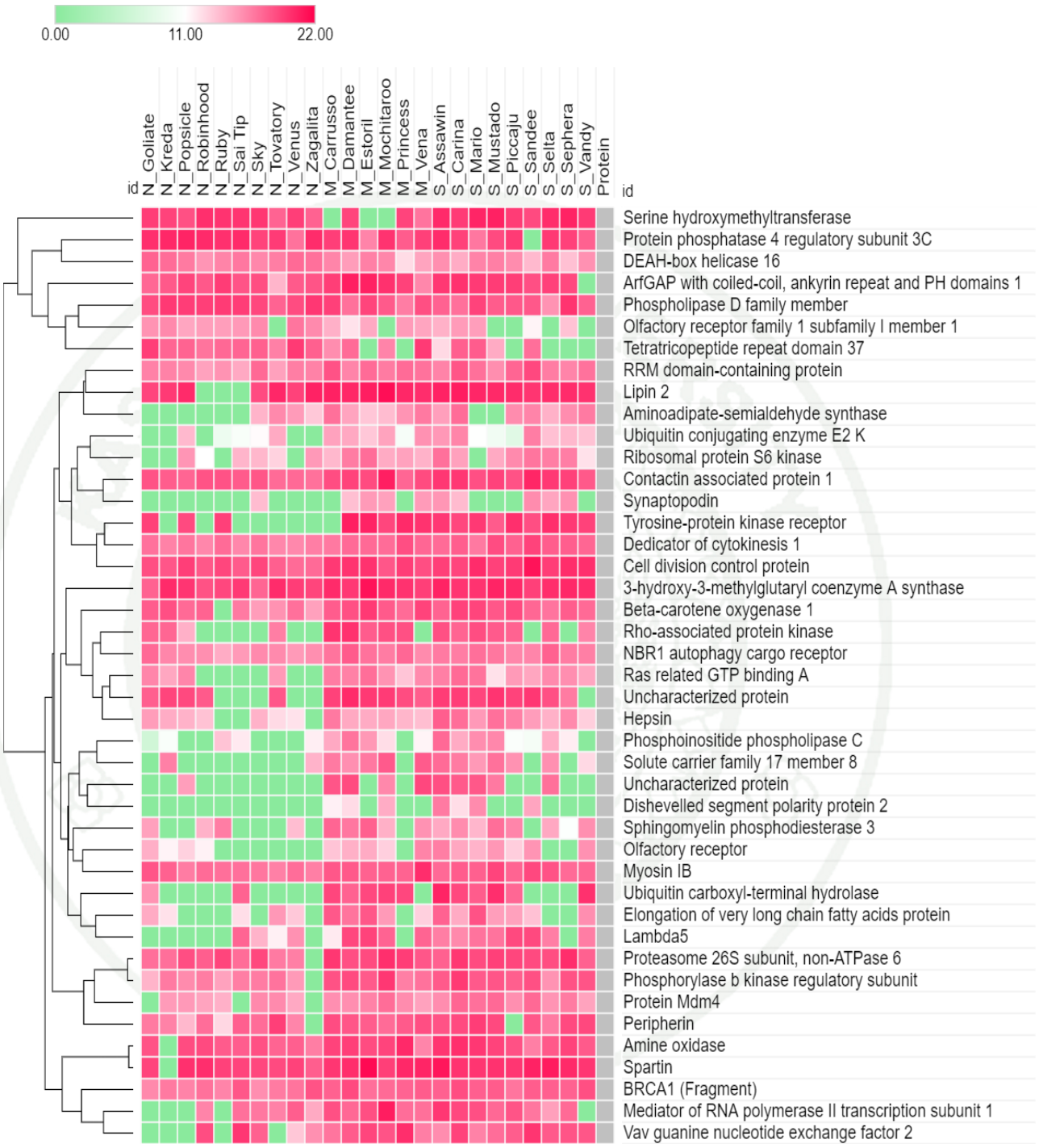

Figure 12: The comparative of protein expression level of the serum samples between normal grey horse group, mild EMN group and severe EMN group. The heatmap was generated using the CLUE program (https://clue.io/command). N, M, and S represent normal, mild and severe, respectively.

4.5.2 Feces sample

According to previous protein expression details both of feces sample proteomic analysis of EMN, all data were comprised in the differential expressed proteins or DEPs analysis with the Wilcoxon rank-sum test and FDR correction. Among these proteins, the expression of 109 significant proteins were categorized protein functions according to KEGG database as listed in Table 13. There were 28 proteins involved in metabolism and 81 proteins involved in non-metabolism categories i.e., cellular processes (15 proteins), environmental information processing (28 proteins), genetic information processing (26 proteins), organismal systems (5 proteins) and human diseases (7 proteins). Moreover, they were categories metabolism protein functions and non-metabolism along with KEGG database as shown in Table 13. According to the significant results for comparative protein expression levels (PELs), all groups are shown in Figure 13.

Table 14: List of 109 significant proteins and associated functions across EMN stages in feces sample

Protein ID	Protein function	PELs[a]		
		N	M	S
Metabolism (28)				
A0A286XHI2	COesterase domain-containing protein	0 (9/10)	14.5562	0 (7/9)
A0A5F9C6H5	Flavoprotein domain-containing protein (PPCDC)	0 (7/10)	14.9349	0 (6/9)
G1U159	Beta_elim_lyase domain-containing protein	0 (7/10)	16.1641	15.130
A0A5F9CKF0	Diacylglycerol kinase (DGKB)	0 (6/10)	14.9254	12.529
A0A5F9DLI7	Triacylglycerol lipase (PNLIP)	17.260	0 (6/6)	13.652
G1SMM6	Hcy-binding domain-containing protein (BHMT)	14.422	13.4364	14.681
G1Q112	Hydroxysteroid 11-beta dehydrogenase 1 like (HSD11B1L)	16.273	14.8005	0 (5/9)
G1T8U2	GDP-mannose 4,6-dehydratase (GMDS)	15.679	15.4775	14.698
A0A5F9CLC1	S-adenosylmethionine synthase (SFTPA1)	15.888	15.0714	14.217
A0A286XWT5	Urocanate hydratase 1 (UROC1)	16.028	15.9683	14.857
A0A286X7N5	Phospholipase A and acyltransferase 3 (PLAAT3)	15.922	16.1469	15.111
H0VHW2	Methylcrotonoyl-CoA carboxylase 1 (MCCC1)	16.912	16.6991	15.311
G1SFW8	Amine oxidase (LOC100353438)	15.449	13.4841	13.603
G1NYJ1	Glutathione S-transferase alpha 4 (GSTA4)	14.132	12.2057	11.853
H0VSK3	Serine palmitoyltransferase long chain base subunit 1 (SPTLC1)	16.273	0 (4/6)	15.132
A0A286Y388	SET and MYND domain containing 1 (SMYD1)	16.117	15.3967	14.960
A0A5F9D9E8	Molybdopterin molybdenumtransferase (GPHN)	16.323	7.86772	15.320
A0A286X826	AIRC domain-containing protein	15.446	14.9433	14.284
A0A5F9DQA4	Epoxide hydrolase (EPHX1)	15.645	14.3524	14.932

G1P5E9	Adenylate cyclase 9 (ADCY9)	16.003	15.2705	15.223
A0A286XZP0	SAC domain-containing protein	16.419	15.5267	15.532
G1PXP9	Phosphoglycerate kinase (PGK1)	16.223	14.7438	15.208
H0V979	N-acetylneuraminate pyruvate lyase (NPL)	18.318	0 (4/6)	16.319
G1U509	Hyaluronidase (LOC100337977)	16.794	15.2814	15.038
H0UW05	Cysteine dioxygenase (CDO1)	16.676	15.8742	15.488
H0WCW8	Argininosuccinate synthase 1 (ASS1)	17.175	15.9806	16.538
A0A5F9D3B6	Malic enzyme (ME2)	17.474	16.6210	15.409
O60774	Putative dimethylaniline monooxygenase [N-oxide-forming] 6 (FMO6P)	18.071	16.8172	16.113
Non-metabolism (81)				
G1PHB6	FOS like 1, AP-1 transcription factor subunit (FOSL1)	0(10/10)	8.73988	0 (5/9)
H0VZS6	Catenin delta 2 (CTNND2)	0(10/10)	15.8651	0 (5/9)
A0A286XNN2	Fibroblast growth factor receptor (FGFR2)	12.921	14.0723	14.464
G1P784	Transcription factor 7 (TCF7)	16.058	14.3422	13.646
A0A3Q8WH22	Peroxisome proliferative activated receptor gamma coactivator 1 (PGC1)	14.868	13.1301	13.576
H0VJZ3	Platelet-derived growth factor receptor alpha (PDGFRA)	15.751	13.4364	12.133
H0V1G0	Glutamate metabotropic receptor 1 (GRM1)	16.848	6.80747	13.901
H0VHD0	Collagen type I alpha 2 chain (COL1A2)	15.061	14.1917	14.316
G1P645	Glutamate ionotropic receptor NMDA type subunit 3A (GRIN3A)	15.658	13.9192	14.250
G1PLL1	SHC adaptor protein 4 (SHC4)	15.577	13.7627	14.167
H0VQA7	Interleukin 18 receptor accessory protein (IL18RAP)	15.740	15.1101	14.942
H0VF12	Caspase 8 (CASP8)	15.871	14.8093	15.078
H0VBX0	Dishevelled associated activator of morphogenesis 2 (DAAM2)	15.728	14.4307	14.834
H0WAY8	Cannabinoid receptor 1 (CNR1)	15.820	15.1585	14.948
A0A286Y5K6	Triadin (TRDN)	16.335	14.9018	15.184
H0V6P4	Glutamate metabotropic receptor 2 (GRM2)	16.604	15.1870	14.785
A0A5F9C4L3	Serotransferrin (Carrie et al.)	16.639	15.8092	15.465
A0A5F9D036	Murine retrovirus integration site 1 homolog (MRVI1)	16.880	15.3271	13.865
A0A286XSL3	MYC associated factor X (MAX)	16.921	16.0645	16.380
G1QDC2	GLUCAGON domain-containing protein	17.380	16.1440	15.249
H0V219	Ribosomal protein S6 kinase (RPS6KA1)	18.236	17.0992	17.295
A0A5F9CNK0	Cystic fibrosis transmembrane conductance regulator (CFTR)	19.024	18.3476	18.528
H0VCJ5	Mastermind like transcriptional coactivator 2 (MAML2)	0 (7/10)	8.15043	17.374
G1PAR3	SCY domain-containing protein	17.754	15.1699	13.936
G1PRZ7	Neogenin 1 (NEO1)	14.140	14.129	13.459
G1P513	Integrin subunit alpha 6 (ITGA6)	13.761	13.2868	11.482
G1P010	SOS Ras/Rho guanine nucleotide exchange factor 2 (SOS2)	15.235	15.5945	12.988
H0VHF9	Serine/threonine kinase 3 (STK3)	18.573	14.7247	13.189
A0A5F9CRH3	Structural maintenance of chromosomes 4 (SMC4)	0 (6/10)	17.5259	15.934
G1P0A6	Cathepsin K (CTSK)	15.001	13.9113	14.038
G1U707	WASP homolog associated with actin, golgi membranes and microtubules (WHAMM)	15.512	14.9549	14.464
H0VN72	Transcription factor (TFDP2)	15.354	14.4134	14.544
A0A286XMN5	Rho GTPase activating protein 5 (ARHGAP5)	16.164	15.6382	15.771

G1SFG8	Dedicator of cytokinesis 1 (DOCK1)	16.589	15.3978	15.593
G1PMR8	Transforming growth factor beta receptor associated protein 1 (TGFBRAP1)	16.547	15.7529	15.314
H0VD03	DnaJ heat shock protein family (Hsp40) member C6 (DNAJC6)	17.213	15.3083	15.365
A0A286XH11	Actinin alpha 4 (ACTN4)	17.687	16.1323	16.098
Q95MN1	p40-phox (NCF4)	21.242	21.5080	15.902
H0V0Y0	Charged multivesicular body protein 5 (CHMP5)	15.382	16.5139	15.557
A0A286XB07	Replication timing regulatory factor 1 (RIF1)	14.991	13.8350	13.327
G1PTV6	Zinc finger homeobox 3 (ZFHX3)	15.605	7.27744	14.061
A0A286XNG5	CD164 molecule (CD164)	19.842	19.5769	18.909
G1PDK5	LIM homeobox 5 (LHX5)	21.398	20.9894	16.970
G1PQ83	Karyopherin subunit beta 1 (KPNB1)	0(10/10)	6.99422	0 (5/9)
H0UU64	PMS1 homolog 2, mismatch repair system component (PMS2)	0 (8/10)	16.1875	0 (9/9)
G1SGR9	General transcription factor IIi (GTF2I)	13.913	6.02391	12.666
A0A286XGA6	Mitochondrial ribosomal protein L14 (MRPL14)	14.168	13.4557	11.649
G1SGI3	Leucyl-tRNA synthetase 2, mitochondrial (LARS2)	16.298	14.1246	15.969
H0W0V2	Replication factor C subunit 5 (RFC5)	15.844	14.6699	14.478
G1TX70	Ribosomal_L18e/L15P domain-containing protein	15.711	14.5448	14.496
G1SHR4	DNA polymerase delta 3, accessory subunit (POLD3)	15.921	15.2813	15.114
A0A286X796	Polyglutamine binding protein 1 (PQBP1)	16.460	15.1889	15.090
G1P4I4	Eukaryotic translation initiation factor 2 subunit beta (EIF2S2)	16.431	15.2732	15.352
H0UUT0	Isoleucyl-tRNA synthetase (IARS)	16.536	16.1518	15.583
G1PP17	Glutaminyl-tRNA synthetase (QARS)	17.316	15.8684	16.504
A0A286XF99	Fibrillarin (FBL)	17.926	17.0077	17.009
G1TZB5	Ribosomal_L2_C domain-containing protein	19.519	17.5432	17.586
G1PT46	Baculoviral IAP repeat containing 2 (BIRC2)	15.453	14.7971	16.628
H0VLS2	ElaC ribonuclease Z 1 (ELAC1)	14.606	14.6556	13.454
G1PF06	DNA polymerase iota (POLI)	14.781	13.6083	11.947
H0VRB6	Double-strand break repair protein (MRE11)	15.164	15.3019	14.910
H0VJ73	Cleavage and polyadenylation specific factor 1 (CPSF1)	16.295	15.3661	14.961
H0VI53	THO complex 5 (THOC5)	16.435	16.4691	15.783
H0VD64	UPF2 regulator of nonsense mediated mRNA decay (UPF2)	17.037	16.656	15.443
A0A5F9DTZ2	PWI domain-containing protein (SRRM1)	17.592	17.6671	15.845
G1SPJ7	UTP4 small subunit processome component (UTP4)	17.684	17.2912	16.765
A0A5F9DVJ8	X-ray repair cross complementing 1 (XRCC1)	18.662	14.9651	14.269
G1SDX2	OXA1L mitochondrial inner membrane protein (OXA1L)	18.652	18.3987	17.341
H0W1J3	RNA-binding protein 8A (RBM8A)	19.656	18.6914	15.461
A0A286Y4H6	Nuclear receptor subfamily 3 group C member 2 (NR3C2)	0 (9/10)	19.3616	0 (5/9)
A0A286X7C5	Collagen type XVI alpha 1 chain (COL16A1)	14.200	13.1893	13.172
A0A5F9DSG7	Collagen type XI alpha 2 chain (COL11A2)	14.394	13.5275	14.655
A0A5F9CML1	Collagen type V alpha 3 chain (COL5A3)	17.125	16.0657	15.981
A0A5F9CKR3	Fibroblast activation protein alpha (FAP)	18.773	16.0315	14.176
A0A5F9C4M4	IG domain-containing protein (PILRA)	0 (6/10)	15.2587	14.440
A0A286XFV7	Huntingtin (HTT)	16.223	15.5877	15.117

G1NT90	Myosin IA (MYO1A)	13.897	13.5326	12.289
G1PNJ0	OMA1 zinc metallopeptidase (OMA1)	19.023	18.8161	17.368
A0A286XEC3	Desmocollin 2 (DSC2)	21.034	20.7771	16.261
H0VXN6	TGc domain-containing protein (Tgm2)	0 (9/10)	15.3301	13.542
H0UWB8	Senataxin (SETX)	0 (9/10)	14.9092	0 (8/9)

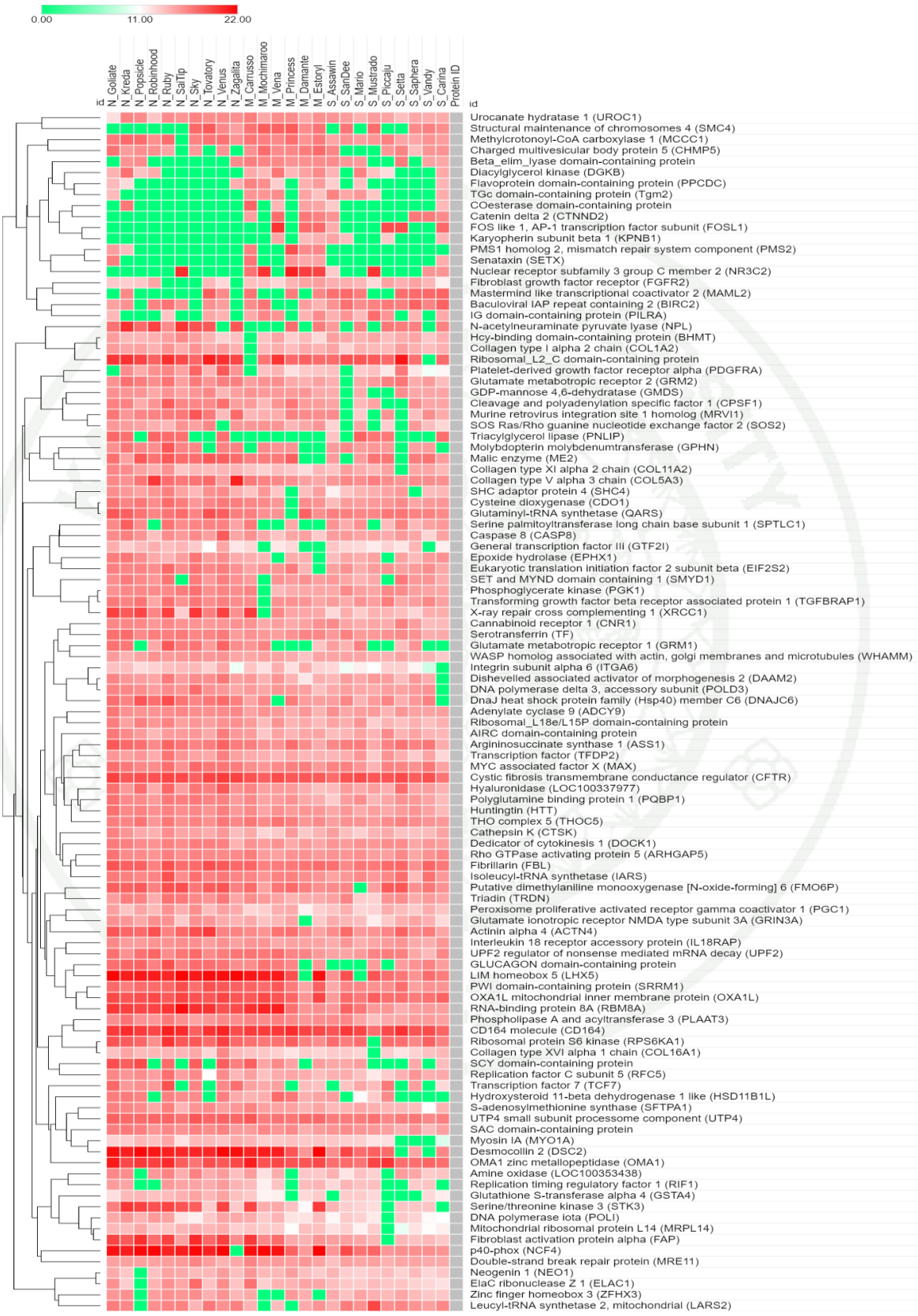

Figure 13: The comparative of protein expression level of the feces samples between normal grey horse group, mild EMN group and severe EMN group. The heatmap was generated using the CLUE program (https://clue.io/command). N, M, and S represent normal, mild and severe, respectively.

4.6 The relationship of candidate proteins and melanoma

4.6.1 The candidate proteins of serum sample

There were 3 candidate proteins showed they has been reported to be linked with melanoma tumor composed with BRCA1, ALK and DVL2. In addition, protein ROCK1 had showed highly expression especially in mild EMN group and severe EMN group but then it cannot be expressed in normal group. Moreover, protein PHKA1 had involved in progress of melanoma.

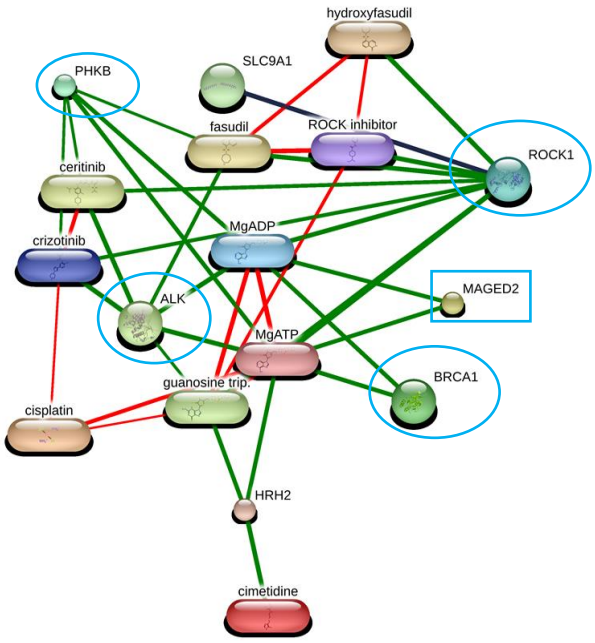

Figure 14: The interaction between Melanoma (MAGED2), ALK, BRCA1, ROCK1 and PHKB with cimetidine, cisplatin, ALK inhibitors (crizotinib) and ROCK inhibitor (fasudil) in Equus caballus that computational prediction between organism and interaction aggregated by Stitch program (www.stitch.embl.de) Version 5.0

4.6.2 The candidate proteins of feces sample

There were 6 candidate proteins had highly expression in mild EMN group and severe EMN group but cannot be found in normal group including diacylglycerol kinase (DGK, DAGK, DGKA), beta_elim_lyase domain-containing protein, mastermind like transcriptional coactivator 2 (MAML2), Structural maintenance of chromosomes 4 (SMC4), IG domain-containing protein and Transglutaminase 2 (TGM2). However, there were only 4 proteins had interaction with melanoma as shown in Figure 15.

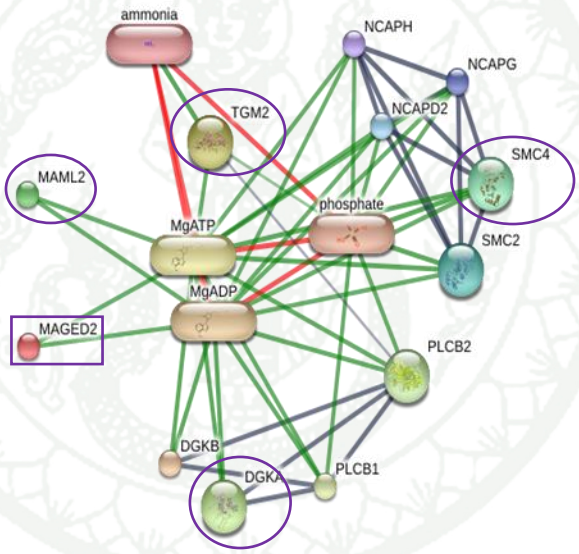

Figure 15: Contribution of DGK, MAML2, SMC4, TGM2 and MAGED2 in networks of protein-protein interaction and protein-chemotherapy interaction of *Equus caballus*. Abbreviations: DGKA; diacylglycerol kinase alpha, DGKB; diacylglycerol kinase beta, MAML2; mastermind like transcriptional coactivator 2, SMC4; structural maintenance of chromosomes 4, TGM2; transglutaminase 2, MAGED2; melanoma antigen family D2.

Interestingly, there were 6 candidate proteins had expressed only in mild stage composed with Flavoprotein domain-containing protein, FOS like 1; AP-1 transcription factor subunit (FOSL1), Catenin delta 2 (CTNND2), Karyopherin subunit beta 1 (KPNB1), PMS1 homolog 2, mismatch repair system component

(PMS2), Nuclear receptor subfamily 3 group C member 2 (NR3C2) and Senataxin (SETX). In addition, all of 6 candidate proteins had not interaction with melanoma as shown in Figure 16.

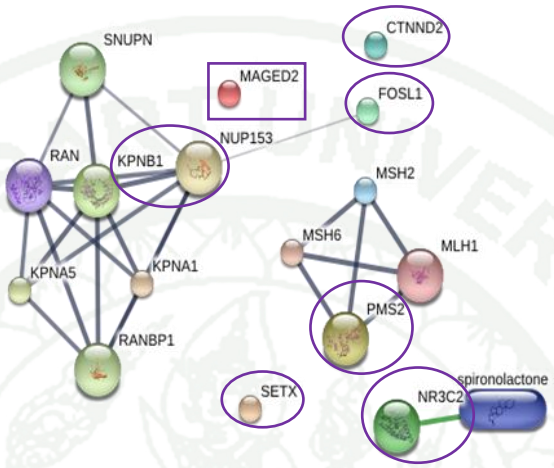

Figure 16: Contribution of FOSL1, CTNND2, KPNB1, PMS2, NR3C2, SETX and MAGED2 in networks of protein-protein interaction and protein-chemotherapy interaction of *Equus caballus*. Abbreviations: Flavoprotein domain-containing protein, FOSL1; FOS like 1; AP-1 transcription factor subunit, CTNND2; Catenin delta 2, KPNB1; Karyopherin subunit beta 1, PMS2; PMS1 homolog 2, mismatch repair system component, NR3C2; Nuclear receptor subfamily 3 group C member 2 and SETX; Senataxin.

CHAPTER V
DISCUSSION

5.1 Experimental grey horses

This study was performed in the grey coat color in different breeds in Lusitano, Thoroughbred and pony breed that has been known the greying is the importance predisposed. However, the common breed including Lusitano, Arabians, Lipizzaner and Percheron are the most that can be found its very often (Scott & Miller, 2003). Although, Thoroughbred and pony breed are not the predisposed but the grey coat color is the dominantly genetic (Rieder et al., 2000). This study found that Lusitano had the EMN condition about 53.33% (8/15) pony 33.33% (5/15) and Thoroughbred 13.33% (2/15) consistent with Reider et al. (2001) report that the breed predisposed especially Lusitano are grey color that they tend to be the opportunistic to EMN. However, it has been confirmed that the majority predisposed of EMN is the greying. EMN can be found in all breed caused of the color is majority of this incidence (Pielberg et al., 2008) associated with Sölkner et al. (2004) reported that melanoma prominence had relative with greying level and also as the result of negative pleiotropic effects of their grey mutation (Curik et al., 2013). In the same way, Pielberg et al. (2008) found that the horse with grey phenotype and the duplication in STX17 can be detected in all grey horses but cannot found in solid horses crossed breed and also consistent with the report of Seltenhammer et al. (2003) found that EMN highly common in greying but less common in the solid color horses. Likewise, this study found that the older grey horses (mild EMN 21.3±5.7 and severe EMN 24±3.6 years old) are more at risk to EMN which corresponds to the various report the common and highly prevalence is approximately 80% in the older grey horses (greater than 15 years old) (Moore et al., 2013; Rieder et al., 2000; B. A. Valentine, 1995). Moreover, EMN has been unaffected the old grey horse in different sex as the reported of Seltenhammer et al. (2003) and her colleague, it is not limited in gender and equine melanoma can be found both of male and female. Nevertheless, in the gender aspect cannot analyzed in this study cause of there are the mare 18/25 (73%), stallion 2/25 (8%) and gelding

5/25 (19%) in this population. Because the aim of this study did not survey at gender differences, gender was not a significant factor.

This study revealed that the primary location of EMN in mild group had occurred 100% in underneath of the tail with the feature of nodule 0.5 cm diameter (15/25=60%, 15/15=100%) which corresponds to Fleury et al. (2000) and his colleagues. In addition, the most common site was the underneath of the tail that can be found frequency occurred location that reported more than 79% of EMN had been showed the typical lesions present in the primary location including the below part of the tail and perineal area (vulva or penis or scrotum) also around the perianal region with one or several nodules in the vary sizes in the neighborhood of 0.5-2 cm diameter. Moreover, this study accounted for their spreading in per-anal region (8/25=32%, 8/15=53.3%), in place of vulva (5/25=20%, 5/15=33.3%) and others (2/25=8%, 2/15=13.3%). As a result, this study discovered that EMN occurred in approximately 60% (15/25) of grey horses, with mild EMN occurring in 24% (6/25) of grey horses, severe EMN occurring in 36% (9/25) of grey horses, and normal grey horses occurring in 40% (10/25). As for the specifics of the incident, this is the first report of EMN in Thailand.

There are no trustworthy hematological or biochemical markers available to encourage in diagnosis of equine melanoma which corresponds to the same work of Mählmann et al. (2015). However, the principle of this method is to determine the horse health status related with EMN and decided select the profile's blood chemistry in routine checking as creatinine, total protein, albumin and Aspartate aminotransferase (AST). Albumin is the most abundant protein in blood. It is synthesized in the liver and removed from circulation by the kidney, resulting in urine excretion. Albumin is frequently measured to detect liver or kidney damage, both of which can be a side effect of cancer or cancer treatment. Creatinine is a compound that the body produces and excretes in the urine. Because compounds that leave the body in the urine are processed by the kidney, creatinine can be used to assess kidney function. Some cancer treatments can harm the kidneys. AST is a liver enzyme that rearranges the amino acids that encodes a protein. It is emitted by damaged liver cells. Cancer may suffer from liver damage as a side effect of some

cancer treatments or as a result of cancer spreading to the horse liver. AST can also be abbreviated as SGOT (serum glutamic oxaloacetic transaminase).

This study showed that the experimental horses had the Lymphocytosis and fibrinogenesis and 1 grey horse had high level in AST. Lymphocytosis (increased numbers of lymphocytes) can be caused by exercise, excitement, or about cancers. The immune system possesses the most responsibility for managing the body's defenses. In addition, Antohe et al. (2018) found that the cutaneous melanoma stage I and stage III constantly associated with a chronic inflammatory infiltrate containing largely of lymphocytes. However, this study found that the experimental horses had the lymphocytosis both of normal group and EMN group. Plasma fibrinogen is commonly used as an imprecise optimistic marker of inflammatory conditions in equine practice (Tamzali et al., 2001). Besides, fibrinogenesis can be found all group (normal, mild EMN and severe EMN) of experimental grey horses. AST level can be elevated when various organs such as red blood cells, brain, kidneys, cardiac muscle, skeletal muscle and liver damage (Ślusarczyk et al., 2016). Hence, AST level was significantly higher in cases of acute liver or muscle damage. On the other hand, this study showed only 1 grey horse had high level in AST. It can be seen that this study had the consistent result with other researches. In the same way of human medicine, melanoma is not diagnosed using blood tests. However, lactate dehydrogenase (LDH) has been tested before treatment human malignant melanoma (Shanique et al., 2011). LDH is an enzyme involved in energy production that is released from damaged cells throughout the body, including the heart and liver. Cancer patients may have an elevated LDH as a result of cancer spreading to their liver or liver damage caused by certain cancer treatments. However, serum LDH concentration, albumin and platelets had been considered for the metastasis melanoma, which is a substance that occurs in greater quantities than normal in the presence of cancer. On the other hand, LDH concentration in the horse mean for the enzyme is found in a variety of tissues and must be separated into its various isoenzymes, each of which is associated with a different organ. Elevated LDH may indicate liver, muscle, or intestinal disease depending on the specific isoenzyme raised. In addition, horses

with colic may have significantly higher levels of LDH activity. Therefore, equine LDH concentration might not be only one marker capable of evaluating EMN.

Although the goal standard for diagnose equine melanoma, there were specific lesion and the position of tumors can be assessed it. In addition, the pointed of this study would like to identify the relationships of proteins expression in the blood and feces sample base on without invasive technique. It is noteworthy from the clinical examination in the experimental grey horses by inspection and palpation as the research in accordance with Desser et al. (1980) system. The results revealed that the melanotic tumor can be found the lesion in the underneath of the tail, perianal and perineum region in the same way with the reported of Fleury et al. (2000) and Rodríguez et al. (1997b). However, the difference idea in this study was to identify the mild EMN that had no reported yet. Besides, all the mild EMN group can be indicated the lump in the underneath of the tail area with the firm nodule 0.5 cm diameter. It is benefit enough to start the treatment or early detection of EMN.

5.2 Serum proteome

According in the serum sample, this study revealed the potential proteins in all groups amount 44 proteins that associate with the cancer pathway. Interestingly, there were 4 proteins involved in lipid biosynthesis composed with LPIN2, PLCH1, ELOVL2, and HMGCS2. The metabolic progression in lipid metabolism provides in energy loading needed, cell membrane configuration and play the role of signaling molecules (Brohée et al., 2015). Therefore, lipid metabolism vital for mechanisms cellular in regulating appearance of the cancer cell. As the result, the overview in protein intensity is highly assessment in mild EMN and severe EMN. In addition, LPIN2 protein action as phosphatidate phosphatase (Metcalfe et al.) enzyme that necessary in glycerol, lipid biosynthesis and regulated lipid metabolism (Reue, 2009). PLCH1 is the enzyme associated with phospholipid component and expressed in pigmented layer of the retina and it is related with tyrosine kinase (Kadamur & Ross, 2013). Moreover, ELOVL2 associated with the fatty acid synthesis and correlated with cutaneous melanoma that high potential for prognostic factors in human melanoma (Dai et al., 2019). HMGCS2 is a catalyze enzyme that associated with reaction in ketogenesis (Grabacka et al., 2016). Moreover, there were 3

candidate proteins showed they has been reported to be linked with melanoma tumor composed with BRCA1, ALK and DVL2.

Despite the fact that they were the most common diseases, a link involving melanoma and BRCA1/2 mutations has already been discovered. BRCA1/2 abnormalities are known to be linked to endometrial cancer (Ginsburg et al., 2010; Petrucelli et al., 2016) . However. *BRCA1/2* mutations can be predisposed to melanoma as the reported of many researchers. The BRCA1 protein is a 220 kDa in the biology function is regulation of transcription and regulatory proteins (Rosen et al., 2003). The BRCA 1 mutation was associated with a greater risk of cancer, including melanoma (Wu, 2015). In addition, the various researcher reported that BRCA carrier had been mindfulness for cutaneous and ocular melanoma (Gumaste, 2015; Moran et al., 2012). Moreover, BRCA mutation carriers had been an increased incidence of ocular melanoma (Iscovich et al., 2002). Additionally, the molecular function of BRCA1 is DNA and zinc ion binding also ubiquitin-protein transferase activity that it is essential for the process of recombination restoration in DNA lesion including maintain genome stability (Kim et al., 2019). Therefore, BRCA1 mutation resulted the rising of cutaneous melanoma were found (Cruz et al., 2011; Lorenzo & Hemminki, 2004).

Remarkably, this study showed the expression of protein ALK tyrosine kinase receptor (anaplastic lymphoma kinase, ALK) in mild EMN group and severe EMN group but this cannot be found in the normal grey horses' group. This protein is indeed a tyrosine kinase for neuronal receptors that is found in both the central and peripheral nervous systems and plays as the differentiation of the nervous system (Motegi et al., 2004). ALK tyrosine kinase receptor composed with the lipid membranes binding, a transmembrane portion, and a cytoplasmic tyrosine kinase part (Cao & Nambudiri, 2017). In addition, ALK is also play another crucial role as the ATP coupling and tyrosine kinase activation in signaling pathway receptor proteins. Moreover, ALK also modulates hematopoietic progenitor cell differentiation, proliferation, and survival, as well as dendritic cell survival. Additionally, In human melanoma tumors, ALK stimulates kinase activity such as mitogen-activated protein kinase (MAPK) signaling that work as regulator various cellular functions such as

differentiation, the cells cycle, tumor growth, also apoptosis (Bergami et al., 2008). Furthermore, ALK has been associated in the pathogenesis of various cutaneous neoplasm including melanoma (Cao & Nambudiri, 2017). Another reason, ALK can be modulation of Rho family GTPases (ROCK1 and ROCK2) that induce actin cytoskeleton reorganization, cell migration, including invasion (Colomba et al., 2008). Busam et al. (2014) have been identified the protein ALK expression in melanoma lesion by IHC technique from mild to late stage. Correspondingly, the protein ALK is the therapeutic targets also used as the biomarker for the melanoma in human (Wiesner et al., 2015). Therefore, ALK expression discovered is very important for identified the treatment occasions of melanoma in human for example Inhibitors of ALK (TAE-684, crizotinib and ceritinib). Similarly, the protein ALK was associated for poorly prognostic condition and has been confirmed of the ALK mutations by next generation sequencing of melanoma (Xia et al., 2014). Even though ALK had not been reported in equine melanoma industry before but it could be possibly established as the potential protein for furthered diagnosing or treating EMN.

Besides, this study found that protein DVL2 cannot found in normal grey horses' group but it was found highly intensity in mild EMN stage and severe EMN stage. It is 95 kDa cytoplasmic protein and also the developmental protein in molecular function. DVL2 had been associated with melanoma via biological process as the canonical Wnt transcription factor is important in cell proliferation regulation. (Michniewicz & Czyz, 2020). DVL2 is involved in signal transduction and neuroectodermal cell migration, precursor cell of melanocyte, homeostasis maintaining of melanocytes between epidermis and keratinocytes (Larue & Delmas, 2006) also activated of MITF, a crucial role in melanoma progression (Vance & Goding, 2004). In addition, in non-Canonical Wnt signaling aspect, DVL activity involved in Rho-associated kinase (Z Chalmers et al., 2017) to actin cytoskeletal elements reconfiguration structures also motility of cells (Ackers & Malgor, 2018). Moreover, DVL2 had the positive regulation of protein tyrosine kinase activity that developed many tumors including melanoma (Easty et al., 1997).

Interestingly, protein ROCK1 had showed the expression in mild EMN group and severe EMN group but it cannot be found in the normal grey horses' group. Although, it is not directly involved with melanoma but it is associating with ALK and DVL2 that had an effect on activated by binding to RhoA-GTP. It was found highly intensity when the grey horse began the small nodule as the mild EMN stage also in severe EMN stage. Likewise, there was poorly research about equine neoplasia and ROCK1, also it had not been showed in EMN but there were many reasons cooperated with human melanoma.

ROCK is a serine/threonine kinase with a molecular weight of 160 KDa that has been the main focus of the signal transduction pathway. ROCK1 and ROCK2 are two isoenzymes that are found in a variety of vasculature. ever since early embryonic development to adulthood (Julian & Olson, 2014). ROCK1 is found in abundance in the pleural space, hepatic, lymphoid tissue, renal, reproductive organs, spleen, and granulocytes. It is expressed in the brain at low levels, principally in nerve cells whereas, ROCK2 is most abundant in the central nervous system, muscular, and cardiovascular system. (Hartmann et al., 2015). However, ROCK1 and ROCK2 are difference aspects such as subcellular localizations, upstream regulators as well as functions in specialized cells and biochemical mechanisms (Coleman et al., 2001). In addition, it has a peptide bond kinase activity, a spiral region in the central portion, a protein kinase region, and a carboxy-terminal cysteine-rich domain. that leads to multiple cell modifications (Chan and Olson, 2016). ROCK is activated, which results in the transcriptional activation of protein targets that control actin filaments configuration and transcriptional regulation. ROCK1 is the protein kinase which play importance role of regulator of cell polarity and the fibrils cytoskeleton. Therefore, it encourages cellular morphology, cell mobility, and cell adhesion. In addition, Chin et al. (2015) reported that ROCK1 controls a variety of cellular mechanisms, which include cell migration, proliferation, as well as cell permeability consequently encouraged angiogenesis activation as of accomplishment critical vascular endothelial growth factor (VEGF) regulator. Moreover, Through activation of LIM kinase, ROCK1 promotes actomyosin contractility by phosphorylating the myosin heavy chains and strengthening thin filaments (the protein that controlling the

formation of invadopodia, a unique cancer structure responsible for destroying extracellular matrix) (Julian & Olson, 2014). Furthermore, in the cancer appearance, ROCK1 regulates cell infiltration, cell proliferation, and extracellular matrix modification (Kaczorowski et al., 2020) including the formation of integrin receptors and cytoskeletal fibers, which monitor and control tissue contractures. On the other hand, ROCK inhibitor can be reduction melanoma expansion, invasion, and metastasis formation, as well as other cancer adhesions such as breast, pancreatic, hepatic (Mikuriya et al. , 2015) and ovarian (Jeong et al. , 2012) . However, Kaczorowski et al. (2020) and his colleagues had the crucial reported that the expression of ROCK1 and ROCK2 proteins have not even been identified in interventional melanoma specimens, they do play a regulatory role in immune function, particularly in tumor-infiltrating lymphocytes (TILs). In the management of severe melanoma, Smit et al. (2014) and his colleagues, found that ROCK1 has been identified as a target in conjunction with current BRAF mutated melanoma therapeutic approaches, as well as a potential drug target for BRAF mutant melanoma. Furthermore, when combined with BRAF or ERK anticancer drug therapies, ROCK1 silencing greater melanoma cell elimination that kind of information of ROCK1 is the great exploration of BRAF mutant melanoma therapies. Moreover, in cultured cells, a ROCK inhibitor enhanced melanoma apoptosis when BRAF or ERK were inhibited. Furthermore, Kaczorowski and his colleagues 2020, reviewed that ROCK1 and ROCK2 expression in TILs were as a substitute down-regulated in progressive and advancing tumors. Huang et al. (2017) and his co-workers reported the result of glucocorticoids (GCs) on metastasis melanoma in mice that GCs considerably enhanced melanoma cell adhesion, migratory, incursion, and lung metastatic spread in cultured cells. In addition, GCs had not been transformation of the Rho proteins signaling, however, the expression was considerably greater, elevated Rho-associated kinase 1/2 (ROCK1/2) activity as well as ROCK1/2 stability via the glucocorticoid receptor, while decreasing the expression of tissue antagonists of matrix metalloproteinase-2. Although, in EMN had been poorly detail about ROCK1 but Frampton et al. (2007) and his colleagues, had been illustrated equine herpesvirus type 1 (EHV-1) enters the equine dermis via two mechanisms: endocytosis or direct fusion disparate and serine/threonine

activation. ROCK1, a Rho kinase, is required for infection. Moreover, Frampton et al. (2010) and his colleagues had further experimental conducted and found that the inhibitor ROCK1 inhibition with the therapeutic agent Y-27632 could prevent EHV-1 from transferring towards the nucleoplasm.

As a result, the database of candidate proteins and predicted interactions between chemical and the proteins analyzed by Stitch program, it is shown in Figure 12 that there was indirect interaction of ALK, BRCA1, DVL2 and ROCK1 with melanoma cell via $Mg2^{+}$ ATP. Magnesium compound plays a role of cofactor with various catalyst enzymes in the body structure, exertion as the energy generation process from ATP and dilating effected the veins and arteries leading to responsible for these protein differentiations (Fig 12).

It was demonstrated that, the cellular originate melanocytes are the specialized dendritic cells that are derived from neural stem cells melanoblasts and principally found in the skin's basal layer and the outer root sheath of hair follicles then produce melanin. Consequently, there are the transcription factor, transmembrane receptors, extracellular ligands, intracellular signaling and numerous of proteins systematized of these processes of EMN. All of phenomena, while the protein posttranslational had been occurred could be induced excessed oxidative phosphorylation which caused cell differentiated or gene mutation and tissue damage. Therefore, it is suggested that when tissue differentiated or gene mutation occurred from melanoma cell induced impairment cell release and also DNA repaired leading ALK, BRCA1, DVL2 and ROCK1 into the blood circulation.

5.3 Feces proteome

Focusing on the protein expression on the 6 candidate proteins had shown highly expression in EMN groups both of mild and severe but cannot found in normal group. As the result. In metabolism category were diacylglycerol kinase (DGK, DAGK, DGKA) and beta_elim_lyase domain-containing protein while mastermind like transcriptional coactivator 2 (MAML2), Structural maintenance of chromosomes 4 (SMC4), IG domain-containing protein and Transglutaminase 2

(TGM2) were in non-metabolism category. Interestingly, DGK is a protease which increasing and motivating diacylglycerol (DAG) as for phosphatidic acid (PA) using ATP as the source of phosphate (You et al., 2014) so the crucial function of DGK is the regulated lipid signaling of lipid metabolic pathway (Eichmann & Lass, 2015). It is the cancer progression, invasion, migration and support survival of cancer cell including lymphoma, hepatoma and melanoma (Ayuso et al., 2014). In addition, DGK had been showed in melanoma cell lines from humans but not in non - malignant melanocytes; and NF-kappaB regulator, which inhibits transcription factor melanoma apoptotic cell death (Yanagisawa et al., 2007). Moreover, the DGK in α isozyme develops cancer cell proliferation and encourages the nonresponsive immune as T-cell (Takao et al., 2021). Accordingly, TGM2 is an enzyme of the transglutaminase (TG) family of enzymes that stimulate protein post-translational modification. Besides, TMG2 mediated cross-link of extracellular matrix (ECM) proteins, TGM2 is a protein that can catalyze the hydrolysis nucleic acid guanine triphosphate (GTP) for maintaining ECM stability. In addition, TGM2 performed a number of activities, including regulating tumorigenesis and acting as a receptor-mediated endocytic and also stimulated cellular adhesive capacity of fibroblasts (Gross et al., 2003). Hence, TGM2 is related to inflammation and modulated the roles in various tumors (Agnihotri et al., 2013; Coussens & Werb, 2002) for example breast cancer (Ai et al., 2008), cervical cancer (Caffarel et al., 2013), ovarian cancer (Singer et al., 2006), pancreatic cancer (Mehta, 2009), colorectal cancer (Miyoshi et al., 2010), hepatocellular carcinoma(Sun et al., 2008), lung cancer (Esposito et al., 2003), prostate cancer (Lewis et al., 2005) and melanoma (Xu et al., 2006). Moreover, TGM2 regulated many biological comportments of tumor cells as differentiation, invasion, adhesion, apoptosis, migration, proliferation, survival, growth, interaction with microenvironment metastasis, angiogenesis and chemoresistance through these mechanisms including pro-crosslink, protein binding, signal transduction and transformation via microvesicles (Huang et al., 2015). This study reveals that the intensity of TGM2 in mild and severe stages had level up to 13-15-fold (median value) and 7-11-fold (average value) higher than the normal stage obviously. In patients human had showed the sturdy TG2 expression in melanoma samples, in the same way, metastasis melanoma cell lines had also

expressed TG2 level up to 24-fold during progressive stages of melanoma (Huang et al., 2015). The MAML2 acts as transcription coactivator activity which associated with Notch signaling pathway, transcriptional positive regulatory oversight by RNA polymerase II and capable developed a multiprotein complex (Wu et al., 2002). The MAML2 in oncogenic role disturbance normal cell cycle, differentiation and developmental of tumor (Noda et al., 2013). Aspect in melanoma, the Notch signaling had related with melanoblast including maintain undeveloped stage, appropriate location controlling and prevention of differentiated of melanocytes (Moriyama et al., 2006) so Signaling via the Notch pathway can activated in skin melanoma (Zhang et al., 2015). Another protein, the SMC4 is protein complex in family of the chromosomal ATPases that plays role regulated chromosome assembly and chromosome seclusion (Feng et al., 2014). In additions, The SMC4 had been showed up-regulated in various malignancies, including lung cancer (Bidkhori et al., 2013), breast cancer (Kulawiec et al., 2008), liver cancer (Zhou et al., 2012) and especially colorectal cancer that showed protein SMC4 expression highly in comparison to normal vasculature (Feng et al., 2014) also it is associated with tumor proliferation also tumor cell development (Zhang et al., 2016). The SMC4 and equine melanoma had not been reported yet however it may affect chromosomal stability via p53 pathway in breast cancer (Wirtenberger et al., 2006) also may chromosomal rearrangements from increase double-stranded DNA disruptions leads to mutation and mismatches (Kulawiec et al., 2008). The associated between these proteins and melanoma is shown in Fig 13.

As the result, these proteins were expressed only in mild stage composed with Flavoprotein domain-containing protein, FOS like 1 (FOSL1), Catenin delta 2 (CTNND2), Karyopherin subunit beta 1 (KPNB1), PMS1 homolog 2, mismatch repair system component (PMS2), Nuclear receptor subfamily 3 group C member 2 (NR3C2) and Senataxin (SETX). These could be suggested the tumors had occurred but not yet metastasis. The associated between these proteins and melanoma is shown in Fig 14.

Accordingly, Flavoproteins are proteins that contain a riboflavin nucleic acid derivative and play an important role in mitochondrial respiration, fatty acid

debasement, and oxidation regulatory oversight (Peng et al., 2019). In addition, the aspect of molecular function is catalytic activity such as radical expulsion that contributes to photosynthetic activity, DNA repair and oxidative stress (Dijkman et al., 2013). Therefore, the uncharacteristic expression of flavoproteins could lead to skin conditions undergo degenerative changes, or neurological system and peripheral neuropathy, and how it influences cancer cell proliferation and motility such as breast cancer, gastric, colorectal, and lung cancers (Medina et al., 2013). Excitingly, FOSL1 is one of several in the transcription factor family AP-1 and have been considered as regulators of cell proliferation, differentiation, and transformation. Besides, the FOSL1 is the predominant initial AP-1 participant stimulated by malignant tumors especially melanoma. Moreover, the FOSL1 were expressed highly on melanocytes cell, afterward it can be developed and transformed to tumors growth (Maurus et al., 2017). The CTNND2 play crucial role in biological process as Neuronal advancement, specifically the structure and preservation of synaptic connections and synaptogenesis, is also implicated in Wnt signaling pathways regulatory as amplified in esophageal carcinoma (Brown et al., 2011). These features in Wnt signaling restrict expression of genes and attenuate Ras superfamily Rho GTPases in extracellular matrix rearrangement which consistent in the study of Tesena et al. (2021) and her colleagues reported a Rho-binding domain leads to multiple cell modifications in equine melanoma that interact with lipid modification as well. It probably acts on beta-catenin turnover in neuron (Bareiss et al., 2010) as well as the prostate cancer has arisen from overexpression of beta-catenin that increased its mediated signaling (Lu et al., 2016). By regulating adhesion molecules, it plays a role in nerve cells adhesion as well as tissue integrity and organogenesis (Lu et al., 2016). The PMS2 is an enzyme coding by gene also involved mismatch repair as well as latent endonuclease action that introduces marks into a discontinuous DNA component (van Oers et al., 2010). Diseases related to the PMS2 mutagenesis Colorectal cancer, hereditary nonpolyposis, type 4, and Mismatch Repair Cancer Syndrome are a few examples. Mutations in the PMS2 promoter region are significantly associated with high tumor mutational burden (TMB), particularly in skin cancers as melanoma. (ZR Chalmers et al., 2017). The SETX is the protein involved in lysosomal degradation and autophagy. It is act as the helicase

activity, identical protein binding and transcription termination site sequence-specific DNA binding (Kim et al., 1999). Besides, SETX is supposed to be expressed in the early stage through formation of autophagosome (Richard et al., 2020). Diseases involved SETX mutation usually associated with the nervous system including The first is in-coordination with oculomotor apraxia type 2 (AOA2) (Moreira et al., 2004) and the second is ALS4 (Chen et al., 2004). The KPNB1 stands the nuclear transport receptors that responsive to the importins and exportins, the pores located between nucleus and cytoplasm. Besides, proteins importins and exportins be dependent on the specific to NLS (nuclear localization signal) and NES (nuclear export signal), respectively (Chook & Suel, 2011). The KPNB1 act the crucial roles as mitotic and chromosomal integrity that had been influenced in relation to cancerous cells biology (Cagatay & Chook, 2018). Dysregulation of the expression of nuclear transporter, KPNB1 has been used as the tool for the prognostic disease under investigation cancer including breast cancer, brain cancer, gastric cancer, prostate cancer, ovarian cancer, bladder cancer, liver cancer, lung cancer, oesophageal cancer and melanoma (Stelma et al., 2016). The NR3C2 is the receptor as the nuclear receptor also act as DNA-binding transcription and zinc ion binding. The NR3C2 expression can be found in various tissue including the kidney, adipose tissue, colon, central nervous system, heart, and sweat glands are all affected. The mutation NR3C2 can caused hypertension, chronic heart failure.

5.4 Lipid metabolism and EMN

Accordingly, this study both of serum and fecal proteome found that most of protein were associated with lipid metabolism.

Totally 41 significant proteins in serum EMN, there were 10 proteins in metabolic category, whereas 31 proteins involved in non-metabolism category. Focusing on metabolic category of serum proteome, there were 7 out of 10 proteins involved in lipid metabolism including lipin 2 (LPIN2), phosphoinositide phospholipase C (PLCH1), beta-carotene oxygenase 1 (BCO1), elongation of very long-chain fatty acid proteins (ELOVL2), 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMGCS2), phospholipase D family member (PLD3) and sphingomyelin

phosphodiesterase 3 (SMPD3). In addition, uniquely proteins expressed involved in lipid metabolism were highly expressed in both mild and severe stages except the expression of PLD3 had high intensity in normal stage.

Consequently, among these proteins in fecal EMN, the expression of 109 significant proteins were categorized protein functions according to KEGG database. There were 28 proteins involved in metabolism and 81 proteins involved in non-metabolism categories. Particularly in metabolic category of fecal proteome, there were 10 out of 28 proteins involved in lipid metabolism together with COesterase domain-containing protein, diacylglycerol kinase (DGKB), triacylglycerol lipase (PNLIP), hydroxysteroid 11-beta dehydrogenase 1 like (HSD11B1L), phospholipase A and acyltransferase 3 (PLAAT3), methylcrotonoyl-CoA carboxylase 1 (MCCC1), serine palmitoyltransferase long chain base subunit 1 (SPTLC1), epoxide hydrolase (EPHX1), SAC domain-containing protein and phosphoglycerate kinase (PGK1). In addition, the expression of proteins DGKB that involved in lipid metabolism had obviously high intensity in mild and severe stages. Moreover, the expression of proteins COesterase domain-containing protein involved in lipid metabolism had high intensity only in mild stage. Furthermore, the most proportion of expression protein in metabolism category in fecal proteome still were lipid metabolism as the result in serum proteome. However, in fecal metabolism had various function proteins expression such as carbohydrate metabolism, nucleotide metabolism, amino acid metabolism, cofactor and vitamin metabolism, and glycan biosynthesis and metabolism but there were less proportion than lipid metabolism.

Therefore, this study revealed that the expression protein involved in lipid metabolism plays the crucial role in EMN with the high percentage than other function in metabolism category both of serum and fecal proteomes. Lipid metabolism is composed of two major pathways: fatty acid biosynthesis (anabolism) and fatty acid breakdown into acetyl CoA via beta-oxidation (catabolism) (Lubert, 1995). There are several pathways to synthesize lipids from the lipid membrane, triacylglycerols, and cholesterol. Membrane lipid biosynthesis produced two types of membrane lipids: sphingolipids and glycerophopholipids (Messias et al., 2018). The precursor of triglyceride biosynthesis is phosphatidic acid, phosphatidylinositol acid

phosphorylation translates this just to diacylglyceride and then to triacylglyceride by acyltransferase (Athenstaedt & Daum, 1999). Another example is fatty acid biosynthesis, where acetyl-CoA serves as a precursor to malonyl-CoA, which is then assembled to form palmitate and palmitic acid. The isoprenoid pathway converts Acetyl-CoA is transformed into acetoacetyl-CoA, which is then converted into cholesterol. Furthermore, before entering the cell, triglyceride decomposes into fatty acid and glycerol via lipid catabolism. In the glycolysis pathway, glycerol is converted to glyceraldehyde-3-phosphate, that is then oxidized to generate energy. Following that, long chain fatty acids in acyl-CoA format will cross the mitochondria membrane. Fatty acid catabolism begins in the cytoplasm as acyl-CoA, then moves to the mitochondrial membrane to begin the process of beta oxidation. The primary products of the beta oxidation pathway are acetyl-CoA, NADH, and FADH (Wood et al., 2014).

As known well, EMN developed from precursor cells melanocytes found at the skin's dermal-epidermal junction. The early stage of EMN growth starting with the tumor cell had been interaction with keratinocyte surrounding microenvironmental area (Kim et al., 2017). Afterward, EMN progression by invasive of mostly populated adipocytes through subcutaneous layer. Melanoma cells were direct contact with adipocytes tissue and become metastasize to others organs both of local area and metastasis tumor microenvironments. In addition, they suggested that the tumor cell microenvironmental were released the lipid which can be resulting EMN growth, proliferative and invasion (Zhang et al., 2018).

As a result, this study discovered that the majority of Proteins involved in lipid metabolism are found across both serum and fecal experiments, which is consistent with other studies on lipid metabolic reprogramming in melanoma. Instead, these findings revealed an increasing progression of the tumor microenvironment surrounding it, as well as the role of fatty acid metabolism inside this aggressiveness of melanoma cells. Moreover, the expression of these proteins is linked to significant lipogenic synthesis has been related to tumor cell invasion and a poor prognosis. Furthermore, modifications in the lipid metabolic network can support cell invasion and metastasis in melanoma cells (Pellerin et al., 2020).

5.5 Conclusions

This study found that the occurrence of EMN in the grey horses was approximately 60% with mild EMN and severe EMN stage in the study population. Blood parameters did not difference between the studied groups. Moreover, highly level of ALK, BRCA1, DVL2 and ROCK1 could be used as the candidate serum proteins expression of EMN to differentiate between control, mild and severe EMN. Additionally, in the feces protein expression showed FOSL1 that can be only found in mild stage besides the high level of DGKA, MAML2, SMC4 and TGM2 could be used as the candidate feces proteins expression of EMN. Furthermore, the candidate serum and feces protein expression may have potential for early diagnosis and prediction of EMN in grey horses which can reduce the prevalence of EMN control the disease and also provide the higher profits for horse industry.

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