Journal of Animal and Veterinary Advances 12 (2): 146-152, 2013
ISSN: 1680-5593

An Investigation of the Elemental Composition of Horse Hair Affected by Equine Metabolic Syndrome (EMS) Using SEM EDX and ICP-OES

[1]Krzysztof Marycz, [2]Nezir Yaar Toker, [1]Joanna Czogaa, [3]I. Michalak, [4]J. Nicpon and [1]Jakub Grzesiak
[1]Electron Microscopy Laboratory, Department of Animal Hygiene,
Faculty of Biology and Animal Science, University of Environmental and Life Sciences,
Koiuchowska 5b, 50-375 Wrocaw, Poland
[2]Department of Biochemistry, Faculty of Veterinary Medicine, Istanbul University,
34320 Avcilar, Istanbul, Turkey
[3]Institute of Inorganic Chemistry and Mineral Fertilizers, University of Technology,
ul. Smoluchowskiego 25, 50-372 Wrocaw, Poland
[4]Department of Surgery, Faculty of Veterinary Medicine,
University of Environmental and Life Sciences, pl. Grunwaldzki 51, 50-366 Wroclaw, Poland

Abstract: Equine metabolic syndrome is a metabolic disorder which is characterized by obesity, insulin resistance and laminitis. At present, its therapy and especially, diagnostic process has not been fully elaborated. The study aimed to examine the elemental composition of hair collected from horses affected by equine metabolic syndrome in order to introduce this kind of analysis as an ancillary diagnostic tool in the course of this disease. Seven horses with diagnosed EMS were chosen as research group. Seven healthy horses served as a control group. Measurements were conducted by means of two independent methods: Scanning Electron Microscopy combined with roentgen microanalysis and Inductively Coupled Plasma-Optical Emission Spectrometry to amplify the accuracy of the results. The data obtained revealed differences between the elemental composition of hair collected from horses with equine metabolic syndrome when compared to healthy controls as far as Ca, Zn, S, Pb and Cr contents are concerned. Moreover, it was shown that the mineral composition of the hair of the examined animals was similar to the elemental composition of human hair from individuals suffering from type II diabetes which further confirmed the similarity of this condition in people and horses. In summary, multi-elemental hair analysis in the course of equine metabolic syndrome could be considered as a valuable, noninvasive and convenient diagnostic method complementary to clinical examination and blood testing.

Key words: Summer eczema, horses, ultrastructure, hair, blood testing

INTRODUCTION

The obesity phenomenon both in human and in veterinary medicine has recently become increasingly popular as a research subject (Persky and Eccleston, 2010; Tsenkova *et al*., 2011; Yao-Borengasser *et al*., 2011). In the case of humans, it is considered as a disease of civilization and is connected with the reduction of physical activity and inappropriate mode of nutrition. As far as horses are concerned, the main reasons for excessive body weight are usually a high energy and high glycemic index diet but also a specific genetic background Dugdale *et al*., 2010). Equine disease states connected with obesity, i.e., increased serum insulin level and laminitis were first introduced to the veterinary medicine in 2002 and are called Equine Metabolic Syndrome (EMS), Obesity Depended Laminitis (ODL) or Peripheral Cushing's syndrome. EMS is considered to be similar with type II diabetes in humans and cats (Frank *et al*., 2010; Johnson, 2002). By definition, obesity dependent laminitis is consistent with the clinical or subclinical active or chronic form of laminitis which is still the most difficult in terms of treatment and prognosis (Geor, 2010). Not infrequently it is equivalent with premature sport activity ending or even with euthanasia of the animal.

The diagnostic process in the course of EMS is not easy and has not been fully worked out yet. It usually includes specific dynamic metabolic tests such as an oral

Corresponding Author: Krzysztof Marycz, Electron Microscopy Laboratory, Department of Animal Hygiene, Faculty of Biology and Animal Science, University of Environmental and Life Sciences, Koiuchowska 5b, 50-375 Wrocaw, Poland

and intravenous glucose tolerance test and the Glucose Insulin Combine Test (CGIT) performed to assess insulin sensitivity (Frank *et al*., 2006, 2010; Durham *et al*., 2009; Frank *et al*., 2010). The aforementioned procedures are occasionally practically demanding and often may lead to life threatening hypoglycemia. In order to simplify the EMS diagnostic process and make it more clinically convenient further ancillary tests are needed. Trace element analysis of hair has been described as an additional source of information on homeostasis of the organism. The mineral composition of given hair is much more constant in comparison with the elemental content in the internal organs or body fluids. Moreover, hair may be considered for retrospective purposes when blood and urine are no longer available. Hair coat chemical composition to some extent may mirror the metabolic status of the organism. Changes in hair elemental content have been noted in the course of different metabolic disturbances including human type II diabetes (Falkiewicz *et al*., 2000; Afridi *et al*., 2010; Kolachi *et al*., 2010; Lakshmi Priya and Geetha, 2011). Both deficiencies and excess of chosen elements may be connected with functional and morphological anomalies of different tissues. However, it should be underlined that the use of multi-elemental hair analysis test should be treated as a ancillary test and should always be considered together with the patient's history and physical examination (Dlugaszek *et al*., 2008; Hong *et al*., 2009; Madejon *et al*., 2009). In the course of this study researchers decided to analyze the given spectrum of micro and macro elements (Ca, Zn, Mg, Fe, S, K, Cr and Pb) because correlation between those particular minerals hair contents and diabetes type II were indicated in human beings (Skalnaya and Demidov, 2007). There is diversity of diagnostic methods to analyze elemental composition: Inductively Coupled Plasma Emission Spectrometry (ICP-ES), Scaning X-ray Electron Microscope (SEM-EDX), Instrumental Neutron Activation Analysis (INAA) and Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES). SEM EDX at present is a generally recognized cognitive tool in many fields of science including medicine and biology. Scanning electron microscopy combined with Energy Dispersive Spectroscopy enables imaging and archiving of ion distribution on the surface of the tested organic matter. Minimal interference with the physical and chemical structure of the tested material allows for repeatability of tests which is of great importance in medical diagnostic. In the research the researchers present potential and practical possibilities of the use of this research method, in the course of EMS diagnostic process. ICP-OES method enables a relatively fast combined determination of many elements in biological materials with maintenance of good analytical parameters (accuracy, precision, limit of detection and quantification). This is a direct method preceded by biomass mineralization (indirect analysis). SEM EDX and ICP OES relevance in biomaterials examination confirmed by researchers previous study encouraged to revise the aptitude of this combine analytical technique with regards to hairs. Both mentioned methods are at present commonly use in nearly every filled of science, medicine and technology. The aim of the following study was to investigate the association between hair elemental content and Equine Metabolic Syndrome (EMS) with the application of two independent methods: scanning electron microscopy combined with roentgen microanalysis and Inductively Coupled Plasma-Optical Emission Spectrometry.

MATERIALS AND METHODS

Horses: Fourteen horses of different breeds (draft horses, warm blood and ponies), both sexes, aged from 9-15 years were included in this research. None of the mares was pregnant. Horses chosen for this study came from different parts of Poland and they were owned by private persons. Body weight measurements were precisely defined using mobile electronic platform scale (Equine Bosh mobile scale). Body weight ranged from 312-780 kg. All horses underwent clinical examination, the Glucose Insulin Combine Test (GCIT), an assessment of serum insulin level and radiological investigation of the hooves. On the basis of the aforementioned procedures, the animals were divided into two groups: equine metabolic syndrome horses research group (n = 7) and healthy horses control group (n = 7). From both control and experimental animals mane hair was collected. All experimental protocols were approved by Local Ethical Committee at Wroclaw University of Environmental Science.

Clinical examination: Clinical examination was performed by two well trained veterinary surgeons. Special emphasis was put on the presence of body fat tissue accumulations in locations characteristic for EMS. Animal gait and hoof capsule were examined (palpation, visual examination and x-ray control) in order to identify subclinical or clinical signs of laminitis.

Collection of peripheral blood: From the examined population of horses (14 individuals), samples of peripheral blood were collected into EDTA tubes and coagulation tubes by jugular venipuncture. Blood insulin level was determined using the Equine Elisa Test (BioVendor).

Glucose Insulin Combine Test (GCIT): Before performing GCIT, all horses were starved overnight with only limited access to Timothy hay (2 kg). The procedure took place in surroundings familiar to the animals in the presence of the horse owner in order to try to reduce the level of stress stimuli to a minimum. First, the blood was collected in order to measure the baseline insulin and glucose level. Then, 150 mg kg^{-1} body weight (BW) 50% dextrose solution was injected intravenously, immediately followed by IV 0.10 U kg^{-1} BW regular insulin bolus (Humulin R, Elili). Next, blood glucose concentrations were measured at 1, 5, 15, 25, 35, 45, 60, 75, 90, 105, 120, 135 and 150 min postinfusion by means of glucometer (Glucosens TD 42-07).

Hair elemental analysis

SEM-EDX: Twenty hairs from mane were pull out from each horse (from 7 control and 7 research horses) using sterile gloves. A standardized method was developed for collecting samples from the same anatomical areas. There was no contact with the ground or any other surface which might provide the contamination. Hair was washed and degreased in demineralised water with detergent then rinsed tree times and dried. After preparation, the samples were analyzed with an Evo LS 15 Scanning Electron Microscope combined with a Bruker 129 eV microroentgenographic detector (SEM-EDX) in order to determine elemental composition. Hair was removed and the peribulbar part of every hair was analyzed for its elemental content: Ca, Zn, Mg, K, S, Fe, Pb and Cr. The analysis was performed at five different points of every hair under 20 kV voltage.

ICP-OES: Hair samples from fourteen horses (seven from the research group and seven from the control group) were examined by means of ICP-OES to evaluate their elemental composition. The samples of hair (0.25 g) were digested with 5 mL of concentrated 69% HNO_3 supra-pure grade from Merck (Darmstadt, Germany) in Teflon vessels with the use of a Milestone Start D microwave oven. After above mineralization the samples were diluted to 50 g and analyzed with a Varian VISTA MPX ICP-OES (Victoria, Australia) Inductively Coupled Plasma-Optical Emission Spectrometer with a pneumatic nebulizer in the Chemical Laboratory of Multielemental Analyses at Wrocaw University of Technology which is accredited by ILAC-MRA and the Polish Centre for Accreditation (No. AB 696) in accordance with EN-ISO 17025.

Statistical analysis: The results were elaborated statistically using the student t-test (Statistica 9.0) and were considered to be significantly different when $p<0.05$. Additionally, the Mann-Whitney test was performed with a significant level when $p<0.01$.

RESULTS

Clinical examination and blood test: All horses included in the study were obese and part of them exhibited regional adiposity observed close to the tailhead, above the eyes in the form of cresty neck and occasionally as bumps along the sides of the horse. Some of the horses from the research group exhibited hoof deformation and difficulties in walking as the result of past laminitis. During palpation none of the horses from both research and control group did exhibit the signs of hoof pain. For all horses (research and the control group) baseline glucose levels were within reference ranges. Resting serum insulin concentrations ranged from 39.2-172 mU mL^{-1} in the research horses while in the control group they were within referral ranges (<20 mU mL^{-1}). During GICT in horses from research group the glucose level did not return to baseline level within 45 min while glucose baseline level in horses from control group where within normal ranges between 20-30 min from insulin injection. On the basis of this test all research horses were considered to be affected with equine metabolic syndrome while horses from control group exhibiting negative GICT result which means they were healthy.

SEM-EDX: The content of Ca, Zn, Mg, K, Pb, Fe, S and Cr in hair was presented as a mean value calculated from 20 individual hairs for each horse (from 7 control and 7 research horses) in the case of SEM-EDX technique. Elemental analysis was conducted of the peribulbar regions of mane hair from healthy horses and from horses affected with Equine Metabolic Syndrome (EMS). The presence of the following elements Ca, Zn, Mg, K, Pb, Fe, S and Cr was examined. In horses with EMS compared to healthy animals, significantly elevated Pb ($p<0.01$) hair content and decreased levels of Ca ($p<0.01$), Zn ($p<0.02$), Cr ($p<0.04$), Fe ($p<0.09$), S ($p<0.08$) was observed. Statistical analysis of the results showed significant differences in Ca, Zn, Pb, Cr and S hair content between healthy and EMS horses ($p<0.05$) whereas no significant difference in K, Mg and Fe level was detected (Table 1).

ICP-OES: The mineral content of the examined hair, collected from healthy and EMS horses, revealed the presence of Ca, Zn, Mg, K, Pb, Fe, S and Cr. In the healthy horse group compared to horses with metabolic syndrome, significantly ($p<0.01$) elevated Ca and Cr levels were detected. Table 2 presents the mineral composition

Table 1: The mineral composition of the hair (wt./%) of healthy horses and horses with EMS

	(Mean±SD)	
Elements	Healthy horses	EMS horses
Ca	0.55±0.13	0.09±0.044
Zn	0.37±0.24	0.10±0.030
Mg	0.56±0.19	0.38±0.100
Fe	0.12±0.06	0.08±0.008
S	6.07±1.10	6.70±0.500
K	0.02±0.03	0.28±0.560
Cr	0.08±0.01	0.07±0.010
Pb	3.13±0.56	5.93±1.810

Table 2: The mineral composition of hair (mg kg^{-1}) of healthy horses and horses with EMS

	(Mean±SD)	
Elements	Healthy horses	EMS horses
Ca	1468±789	1004±537
Zn	128±22.2	96.6±43.3
Mg	314±116	273±149
Fe	414±409	200±196
S	30440±1459	30587±1640
K	772±627	1465±2037
Cr	8.13±6.32	1.52±1.89
Pb	22.2±10.7	34.4±29.0

SD: Standard Deviation; EMS: Equine Metabolic Syndrome

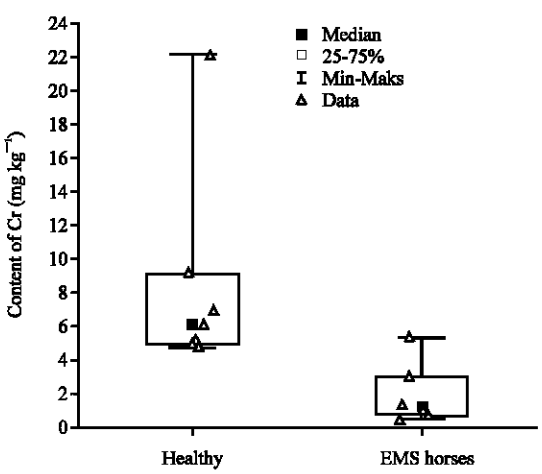

Fig. 1: The content of Cr in EMS and healthy horses (mg kg^{-1})

of hair (mg kg^{-1}) of healthy and EMS horses. The content of Ca was 46.2% higher, Zn 32.5% higher, Mg 15.0% higher and Fe 107% higher in the control group than in the experimental group. Higher differences were observed in the case of Pb and K. Healthy horse hair contained 35.5% less Pb and 47.2% less K than hair of horses with EMS. A statistically significant difference ($p = 0.0124$; Mann-Whitney test) was noticed for the content of Cr in hair healthy horses contained about 5 times more Cr than horses with EMS. The detailed results for Cr are presented in Fig. 1.

DISCUSSION

Obesity is a medical condition in which excess body fat is accumulated to the extent that it may have an adverse effect on health. It increases the likelihood of various metabolic diseases, both in humans and animals including type II diabetes. Being overweight has also become a more apparent problem in the field of veterinary medicine (Nguyen *et al*., 2011; Reaven, 2011; Johnson *et al*., 2009). There are intrinsic and extrinsic factors which are considered to be responsible for the development of obesity in horses. Cold blooded breads and ponies are especially prone to gaining weight easily (Carter *et al*., 2010). It arises partly from their genetic predisposition, but most often it is caused by improper feeding strategies. A relatively newly described disease which interrelates with pathological obesity in horses is Equine Metabolic Syndrome. The clinical picture of EMS usually includes regional adiposity, lameness and/or evidence of earlier laminitis such as divergent growth rings on the hooves (Frank *et al*., 2010). Fat tissue accumulation is mainly observed in the neck region and tail base area. EMS in every day practice is very often related to significant diagnostic and therapeutic difficulties. It may result from the lack of well elaborated diagnostic algorithms and the unpractical and relatively complicated nature of existent ones. Examination of hair, both in human and veterinary medicine is recognized as an ancillary diagnostic tool (Campbell, 1985; Seidel *et al*., 2001). There are some advantages to this method such as non invasiveness and speed. However, some researchers criticize it for insufficient accuracy (Manson and Zlotkin, 1985). In this study, hair as an easily obtainable, biologically and chemically stable material were examined in order to establish their potential as a valuable diagnostic tool in the course of EMS. Hair follicles are metabolically active tissues that require nutrients to support both structural and functional activities. For this reason, every metabolic disorder can influence the morphological and particularly chemical properties of hair as hair follicle products. Equine skin carries several types of hair temporary hair that comprises the majority of the coat and the permanent hair of the mane, tail, feathers and eyelashes. Permanent hair seems to be the most relevant subject for the examination (Galbraith, 1998; Dunnett and Lees, 2004). Accuracy of hair mineral examination was amplified by employment of two different and independent techniques Scanning Electron Microscope combined with microroentgenographic detector (SEM-EDX) and Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES). Analysis included both micro and macroelements as they are equally essential for the homeostasis of the organism. As was indicated, metabolic disorders such as diabetes result in inappropriate elemental composition of the body (Skalnaya and Demidov, 2007). Suboptimal zinc levels induce graying of hair, prolonged wound healing, parakeratosis, susceptibility to infections and developmental orthopedic diseases in young horses. As far as human medicine is concerned, the correlation between zinc content of tissues and metabolic disorders in general was noticed (Skalnaya and Demidov, 2007). Low zinc content indicated in the hair of EMS horses in comparison with healthy horses may also be mirrored in bad quality of hoof horn and predisposition to laminitis. Moreover in type II diabetes in humans it has been described that Zn plays an important role in insulin metabolism and its conformational integrity (Chaumer, 1998). Because type II diabetes and EMS are considered to be parallel disorders, zinc level reduction may also lead in horses to improper insulin secretion.

Some researchers have suggested that magnesium and calcium play a role in glucose homeostasis and insulin action (Ma *et al*., 2006; Wu *et al*., 2009). Moreover, significantly lower hair Ca and Mg levels were observed in the case of humans suffering from diabetes and obesity when compared to healthy controls. This last observation was also confirmed in the present study, as far as the magnesium and calcium content in hair of EMS horses is concerned. Taking into consideration the aforementioned findings it seems to be reasonable to pay particular attention to the dietary Ca and Mg supplementation in horses suffering from metabolic syndrome. With regards to toxic elements high lead concentrations in blood were noticed in obese individuals with type II diabetes. Similarly, significantly higher hair lead content was spotted in obese, diabetes women (Skalnaya and Demidov, 2007). These observations and the fact that horses with EMS also exhibited higher lead hair contents may suggest the essential role of this toxic element in pathogenesis of type II diabetes both in humans and horses. Insulin is known to increase potassium cellular uptake (DeFronzo, 1988). On the other hand, a regulatory role for K in insulin metabolism has been postulated (Tzamaloukas and Avasthi, 1987) which may correspond with insulin resistance. Sulphur is also considered to be the main macro element responsible for proper formation of skin appendages, such as hooves. It may explain the bad quality of hoof walls in horses with EMS. Chromium is an essential mineral that appears to have a beneficial role in the regulation of insulin action and its effects on carbohydrate, protein and lipid metabolism. Studies show that people with type II diabetes have lower levels of chromium in their blood than healthy people. Interestingly, low hair chromium content was confirmed in the case of horses affected with equine metabolic syndrome compared to control animals. Because of this finding and because it is known that chromium absorption from the gastrointestinal tract is a difficult and complicated process, further research seems to be necessary to find the best bio available form of this element for horses as an effective supplement in the treatment of EMS (Anderson, 2000). Hair elemental analysis conducted on horses with equine metabolic syndrome revealed a lot of similarities with hair mineral contents of human with type II diabetes. Measurements were performed by means of two independent methods and showed comparable results which confirmed their usefulness and accuracy.

CONCLUSION

Finally, the data obtained suggested that multi-element hair analysis is a valuable diagnostic tool in the prevention and treatment of overweight, obesity and metabolic syndrome in horses. Moreover, it seems to be reasonable to supplement the diet of animals with EMS with chosen elements such as Zn, Mg, Ca and Cr. Despite in the course of this study research animal group was not very numerous, the obtain result seems to be very promising as far as simplification of EMS diagnostic protocol is concerned. For this reason in researchers opinion farther investigation of hair elemental contents in horses with metabolic disorders are indicated.

ACKNOWLEDGEMENT

Research financed from grant KBN No. N 73498 budget. The study was conducted within the research project No. NN 308266138 financed by the National Science Center Poland.

REFERENCES

Afridi, H.I., T.G. Kazi, N. Kazi, Sirajuddin and G.A. Kandhro *et al*., 2010. Chromium and manganese levels in biological samples of Pakistani myocardial infarction patients at different stages as related to controls. Biol. Trace Elem. Res., 142: 259-273.

Anderson, R.A., 2000. Chromium in the prevention and control of diabetes. Diabetes Metab., 26: 22-27.

Campbell, J.D., 1985. Hair analysis: A diagnostic tool for measuring mineral status in humans. J. Orthomol. Psychiatry, 14: 276-280.

Carter, R.A., L.J. McCutcheon, E. Valle, E.N. Meilahn and R.J. Geor, 2010. Effects of exercise training on adiposity, insulin sensitivity and plasma hormone and lipid concentrations in overweight or obese, insulin-resistant horses. Am. J. Vet. Res., 71: 314-321.

Chaumer, A.B., 1998. Zinc, insulin and diabetes. J. Am. Coll. Nutr., 17: 109-115.

DeFronzo, R.A., 1988. Obesity is associated with impaired insulin-mediated potassium uptake. Metabolism, 37: 105-108.

Dlugaszek, M., M. Szopa, J. Rzeszotarski and P. Karbowiak, 2008. Magnesium, calcium and trace elements distribution in serum, erythrocytes and hair of patients with chronic renal failure. Magnes. Res., 21: 109-117.

Dugdale, A.H., G.C. Curtis, P. Cripps, P.A. Harris and C.M. Argo, 2010. Effect of dietary restriction on body condition, composition and welfare of overweight and obese pony mares. Equine Vet. J., 42: 600-610.

Dunnett, M. and P. Lees, 2004. Hair analysis as a novel investigative tool for the detection of historical drug use/misuse in the horse: A pilot study. Equine Vet. J., 36: 113-117.

Durham, A.E., K.J. Hughes, H.J. Cottle, D.I. Rendle and R.C. Boston, 2009. Type 2 diabetes mellitus with pancreatic beta cell dysfunction in 3 horses confirmed with minimal model analysis. Equine Vet. J., 41: 924-929.

Falkiewicz, B., E. Dabrowska, J. Lukasiak, D. Cajzer and I. Jablonska-Kaszewska, 2000. Zinc deficiency and normal contents of magnesium and calcium in metabolic X syndrome patients as assessed by the analysis of hair element concentrations. Biofactors, 11: 139-141.

Frank, N., R.J. Geor, S.R. Bailey, A.E. Durham and P.J. Johnson, 2010. Equine metabolic syndrome. J. Vet. Internal Med., 24: 467-475.

Frank, N., S.B. Elliott, L.E. Brandt and D.H. Keisler, 2006. Physical characteristics, blood hormone concentrations and plasma lipid concentrations in obese horses with insulin resistance. J. Am. Vet. Med. Assoc., 228: 1383-1390.

Galbraith, H., 1998. Nutritional and hormonal regulation of hair follicle growth and development. Proc. Nutr. Soc., 57: 195-205.

Geor, R.J., 2010. Current concepts on the pathophysiology of pasture-associated laminitis. Vet. Clin. North Am. Equine Practice, 26: 265-276.

Hong, S.R., S.M. Lee, N.R. Lim, H.W. Chung and H.S. Ahn, 2009. Association between hair mineral and age, BMI and nutrient intakes among Korean female adults. Nutr. Res. Pract., 3: 212-219.

Johnson, P.J., 2002. The equine metabolic syndrome peripheral Cushing's syndrome. Vet. Clin. North Am. Equine Pract., 18: 271-293.

Johnson, P.J., C.E. Wiedmeyer, N.T. Messer and V.K. Ganjam, 2009. Medical implications of obesity in horses-lessons for human obesity. J. Diabetes Sci. Technol., 3: 163-174.

Kolachi, N.F., T.G. Kazi, H.I. Afridi, N. Kazi and S. Khan *et al*., 2010. Status of toxic metals in biological samples of diabetic mothers and their neonates. Biol. Trace Elem. Res., 143: 196-212.

Lakshmi Priya, M.D. and A. Geetha, 2011. Level of trace elements (copper, zinc, magnesium and selenium) and toxic elements (lead and mercury) in the hair and nail of children with autism. Biol. Trace Elem. Res., 142: 148-158.

Ma, B., A.B. Lawson, A.D. Liese, R.A. Bell and E.J. Mayer-Davis, 2006. Dairy, magnesium and calcium intake in relation to insulin sensitivity: Approches to modeling a dose-dependent association. Am. J. Epidemiol., 164: 449-458.

Madejon, P., M.T. Dominguez and J.M. Murillo, 2009. Evaluation of pastures for horses grazing on soils polluted by trace elements. Ecotoxicology, 18: 417-428.

Manson, P. and S. Zlotkin, 1985. Hair analysis-a critical review. Can. Med. Ass. J., 133: 186-188.

Nguyen, N.T., X.M. Nguyen, J. Lane and P. Wang, 2011. Relationship between obesity and diabetes in a US adult population: Findings from the National Health and Nutrition Examination Survey, 1999-2006. Obes. Surg., 21: 351-355.

Persky, S. and C.P. Eccleston, 2011. Impact of genetic causal information on medical students clinical encounters with an obese virtual patient: Health promotion and social stigma. Ann. Behav. Med., 41: 363-372.

Reaven, G.M., 2011. The metabolic syndrome: Time to get off the merry-go-round? J. Internal Med., 269: 127-136.

Seidel, S., R. Kreutzer, D. Smith, S. McNeel and D. Gilliss, 2001. Assessment of commercial laboratories performing hair mineral analysis. J. Am. Med. Assoc., 285: 67-72.

Skalnaya, M.G. and V.A. Demidov, 2007. Hair trace element contents in women with obesity and type 2 diabetes. J. Trace Elem. Med. Biol., 21: 59-61.

Tsenkova, V.K., D. Carr, D.A. Schoeller and C.D. Ryff, 2011. Perceived weight discrimination amplifies the link between central adiposity and nondiabetic glycemic control (HbA(1c)). Ann. Behav. Med., 41: 243-251.

Tzamaloukas, A.H. and P.S. Avasthi, 1987. Serum potassium concentration in hyperglycemia of diabetes mellitus with long-term dialysis. Western J. Med., 146: 571-575.

Wu, T., W.C. Willett and E. Giovannucci, 2009. Plasma C-Peptide is inversely associated with calcium intake in women and with plasma 25-hydroxy vitamin D in men. J. Nutr., 139: 547-554.

Yao-Borengasser, A., V. Varma, R.H. Coker, G. Ranganathan, B. Phanavanh, N. Rasouli and P.A. Kern, 2011. Adipose triglyceride lipase expression in human adipose tissue and muscle. Role in insulin resistance and response to traning and pioglitazone. Metabolism, 60: 1012-1020.