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Expression of Leptin, NGF and Adiponectin in Metabolic Syndrome

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Adipose tissue secretes a variety of adipokines involved in the regulation of energy metabolism and insulin resistance. Metabolic syndrome corresponds to a clinical condition in which white adipose tissue is characterized by an increased production and secretion of inflammatory molecules which may have local effects on adipose tissue physiology but also systemic effects on other organs. The aim of this study was to assess the expression of leptin, NGF and adiponectin in women with metabolic syndrome compared to healthy controls. Plasma leptin, NGF and adiponectin levels were measured by the ELISA method. Leptin and NGF immunohistochemical expression was analyzed in subcutaneous adipose tissue. The results indicated that in women with metabolic syndrome waist circumference, body mass index, HOMA index, glucose, total cholesterol and triglyceride levels were significantly increased in parallel with over-expressed plasma levels of leptin and NGF and decreased adiponectin. The immunohistochemical expression of leptin and NGF was very strong. In conclusion, this is the first study demonstrating a complex of immunochemical and immunohistochemical expression of the key adipokines including leptin, NGF and adiponectin in women with metabolic syndrome. Locally-produced pro-inflammatory adipokines probably contribute to the ethiopathogenic mechanisms of metabolic syndrome.

Key words: Metabolic syndrome, adipose tissue, leptin, NGF, adiponectin.

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Metabolic syndrome (MetSyn) is a complex of metabolic disorders, which is based on obesity, dyslipidemia, insulin resistance, compensatory hyperinsulinemia. Areas of active investigation focus on the molecular basis of metabolic inflammation and potential pathogenic roles in insulin resistance, diabetes, and cardiovascular disease. Increasing evidence suggests that chronic, low-grade inflammation may be common in the pathogenesis of MetSyn (YANG *et al*. 2013). Playing a central role in the development of MetSyn and in its clinical consequences is obesity. Recent studies have shown that obesity induces chronic local inflammation in adipose tissue. Adipose tissue is an active immune and endocrine organ that secretes various humoral factors (adipokines). Its production of proinflammatory cytokines in obesity likely contributes to low-level systemic inflammation that is seen in metabolic syndrome-associated chronic pathologies (NISHIMURA *et al*. 2009). Alteration of white adipose tissue mass in obesity affects the production of most adipose secreted factors and the activation of some pro-inflammatory signalling pathways, resulting in the induction of several biological markers of inflammation (HOTAMISLIGIL et al. 1993). A body of evidence suggests the presence of an overall, low-grade inflammation in obesity, with altered levels of several circulating factors such as an increase in the plasma levels of C-reactive protein, tumor necrosis factor-α, interleukin-6, transforming growth factor-beta and other biological markers of

inflammation (NISHIMURA *et al.* 2009; HOTAMISLIGIL *et al.* 1993; FRIED *et al*. 1998; BASTARD *et al*. 2002; SAMAD *et al*. 1996, 1997;SARTIPY & LOSKUTOFF 2003). Other adipose-specific molecules that are involved in the control of energy metabolism, also regulate immune responses. For example leptin, in addition to its key role in food intake and energy expenditure, also regulates immune processes (BASTARD *et al*. 2006). Leptin levels have been shown to be directly associated with BMI and MetSyn (CHALDAKOV *et al*. 2001). Adiponectin is another adipokine associated with obesity and MetSyn, its circulating levels being decreased (GONZÁLEZ *et al*. 2012). There is a strong positive correlation between adiponectinemia and insulin sensitivity. Moreover, adiponectin may modulate the TNF-α-induced inflammatory response by reducing TNF-α secretion of macrophages, thus fulfilling anti-inflammatory and anti-atherogenic effects (BASTARD *et al*. 2006). Neurotrophins are also involved in the development and progression of inflammatory and immune diseases. Because obesity is related to low grade systemic inflammation, in a study evaluating the plasma NGF levels in obese women with MetSyn, NGF was found to be upregulated (BULLÓ *et al*. 2007). On the contrary, other reports indicated that hyponeurotrophinemia was present in a group of patients with MetSyn and cardiovascular disease (CHALDAKOV *et al*. 2001; CHALDAKOV *et al*. 2004).

In general, the involvement of adipokines in MetSyn is not elucidated. The aim of our work was to elucidate this question by studying the expression of selected adipokines including leptin, NGF and adiponectin, in women with MetSyn.

Material and Methods

60 healthy controls (at the age 30-45 years, before menopause) were included in the study. They were patients in the Clinic of Endocrinology, Medical University, Plovdiv. The protocol was approved by the Ethics Committee of the Medical University, Plovdiv. All subjects gave an informed consent. The waist circumference (WC), body mass index (BMI), plasma glucose level (RA 1000 Technicon, USA), plasma insulin, HOMA – index (MEIA, ABBOTT, USA, AxSYM), lipids profile (Optima KONE) were measured for each subject. BMI was calculated as weight/(height(m))^2; kg/m^2. Waist circumference was measured midway between the lower rib margin and the iliac crest. Plasma leptin, NGF and adiponectin levels were measured by commertial ELISA assay performed according to the manufacturer's instructions (Human leptin ELISA, Human adiponectin (HEK) ELISA, Human NGF ELISA, BioVendor Laboratory, Medicin, Inc., Czech Republic). The data were statistically processed by SPSS 16.0 (Windows) and expressed as$X \pm SD$

Immunohistochemistry. Immunohistochemical demonstration of leptin and NGF the ABC immunostaining kit (Santa Cruz, USA). The material was fixed in Bouin's solution and embended in parrafin. Five-micrometer-thick sections were cut, mounted on glass slides and air-dried for 24 h at 37^{o}C. Dewaxed sections were processed using the following incubation steps: (1) hydrogen peroxide 0.3% in metanol for 30 min to block endogenous peroxidase, (2) normal goat serum 1:75 for 20 min to reduce nonspecific background staining, (3) primary antibody (polyclonal rabbit anti-mouse leptin antibody diluted 1:200 or polyclonal rabbit anti-mouse NGF antibody diluted 1:100 in PBS, overnight at 4^{o}C, (4) biotinylated secondary antibody – goat anti-rabbit IgG 1:200 for 30 min, (5) ABC complex for 1 h, (6) histochemical visualization of the peroxidase using diaminobenzidine (0.0125% DAB solution containing 0.0025%hydrogen peroxide), (7) sections were counterstained with Harris hematoxylin, rinsed with distilled water, dehydrated and mounted. The specificityof the immune reaction was confirmed by omission of the primary antibody.

MetSyn –metabolic syndrome
NGF – nerve growth factor
BMI – body mass index
TNFα – tumor necrosis factor alpha
WC – waist circumference
HOMA-index – Homeostasis Model Assessment index
ABC method – avidin-biotin peroxidase method
NCEP – National Cholesterol Education Program
ATP III – Adult Treatment Panel III

Results

Metabolic syndrome diagnosis was given using the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) (2002), which was updated by the American Heart Association and the National Heart Lung and Blood Institute in 2005 (GRUNDY *et al*. 2005). According to NCEP ATP III metabolic syndrome is present if three or more of the following five criteria are met: hyperglycemia/insulin resistance, visceral obesity, atherogenic dyslipidemia and hypertension. For this purpose we measured the waist circumference, body mass index, glucose, insulin, total cholesterol and triglyceride plasma levels for each subject. Our study demonstrated

that detection rates of MetSyn components such as hyperglycemia, dyslipidemia, insulinemia and insulin resistance and obesity were significantly higher. Women with MetSyn had significantly higher BMI, WC, glucose, insulin, total cholesterol and triglyceride levels than the healthy controls. The clinical chemical indexes are demonstrated in Table 1.

BMI of the women with MetSyn was 35.43±3.18 kg/m^2, healthy controls – 23.43±5.11 kg/m^2 (P<0.05). Insulin resistance was present in the women as estimated by the HOMA index. HOMA index in MetSyn differed significantly from the values in the control group. HOMA-index of the women with MetSyn was 4.15±1.10, healthy controls – 1.15±0.80 (P<0.05).

The immunochemical results showed increased levels of leptin and NGF and decreased adiponectin levels in blood samples of women with MetSyn in comparison with healthy women. Plasma levels of leptin of the objects with MetSyn were over-expressed (P<0.01). The registered plasma leptin level in MetSyn women was 35.33±9.74 ng/ml, healthy controls – 8.63 ± 1.35 ng/ml. (Fig. 1) Leptin expression was related to lipid profile, insulinemia and BMI. Leptin showed positive correlation with BMI (r = 0.70). We also found positive correlation between leptin and HOMA index (r = 0.30).

Plasma NGF levels were also significantly increased in the group of MetSyn compared to the controls (P<0.01). MetSyn plasma NGF levels were 69.2±2.1 μg/ml; healthy controls – 42.13±5.52 μg/ml. In the subjects with MetSyn plasma NGF was correlated with body mass index (BMI) (r = 0.63) and waist circumference (r = 0.56).

Adiponectin levels were inversely associated with Met Syn. Adiponectin levels of MetSyn women were significantly decreased in comparison with the healthy controls (10.34±4.47 μg/ml; respectively 23.02 ± 2.54 μg/ml; P<0.01) (Fig. 2). Leptin: adiponectin ratio was significantly higher in MetSyn women (P<0.001) (Fig. 3).

Biopsies of subcutaneous adipose tissue were taken and immunohistochemical reactions were performed in order to obtain a detailed assessment of the expression of adipokines at their place of origin. The immunohistochemical expressions of the investigated adipokines corresponded to the immunochemical data. The immunohistochemical reactions of leptin and NGF were positive in all the adipocytes of adipose tissue samples of both MetSyn women and control healthy women (Figs 4, 5). Leptin immunohistochemical expression in women with MetSyn was stronger than that of healthy women (Fig. 4a,b). In women with MetSyn, NGF expression was also stronger as compared to that of healthy women (Fig. 5a,b). Using semiquantitative analysis, the expression of both reactions is demonstrated in Table 2 .

Table 1

Some clinical chemical indexes. Total cholesterol, triglycerides and glucose are significantly higher in MetSyn than controls

Indexes	Groups	
	Controls	MetSyn
Total cholesterol mmol/l-1	4.1±0.9	6.3±0.28 P<0.001
Triglycerides mmol/l-1	1.5±0.3	2.1±0.73 P<0.05
Glucose mmol/l-1	4.46±1.53	6.91±1.04 P<0.05

Discussion

This is the first study that evaluates a complex of plasma levels of some adipokines (leptin, NGF and adiponectin) and immunohistochemical expression of leptin and NGF in a group of women with MetSyn.

Our study demonstrated that detection rates of the MetSyn components such as hyperglycemia, dyslipidemia, insulinemia and insulin resistance and obesity were significantly higher. Subjects with MetSyn showed significantly higher levels of leptin and NGF. At the same time we found an inverse correlation between adiponectinemia and MetSyn. These data coexists with the results

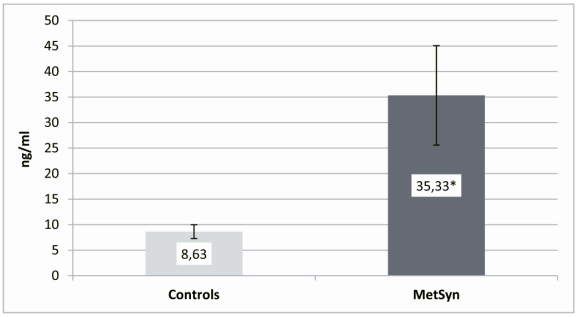

Fig. 1. Plasma levels of leptin of the objects with MetSyn and healthy controls. Plasma leptin levels of the MetSyn objects were over-expressed (P<0.01).

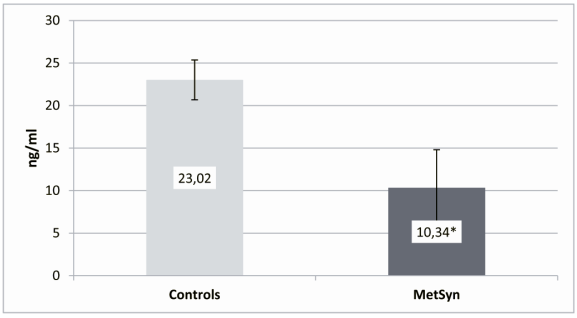

Fig. 2. Adiponectin levels of the objects with MetSyn and healthy controls. Adiponectin levels of MetSyn women were significantly decreased in comparison with the healthy controls (P<0.01).

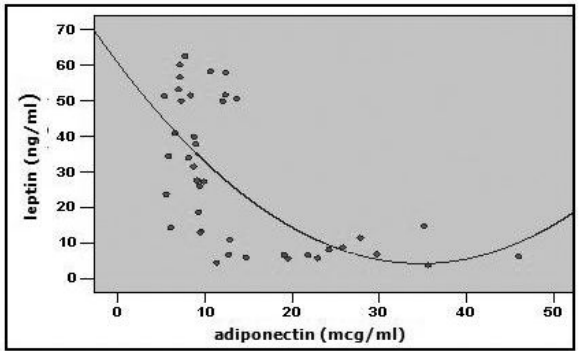

Fig. 3. Leptin: adiponectin ratio. Leptin: adiponectin ratio was significantly higher in MetSyn women (P<0.001).

Table 2

Semiquantitative analysis of immunohistochemical expression of leptin and NGF. MetSyn immunohistochemical expressions of leptin and NGF are stronger (+++) than that of controls (+)

Indexes	Groups	
	Controls	MetSyn
Leptin	+	+++
NGF	+	+++

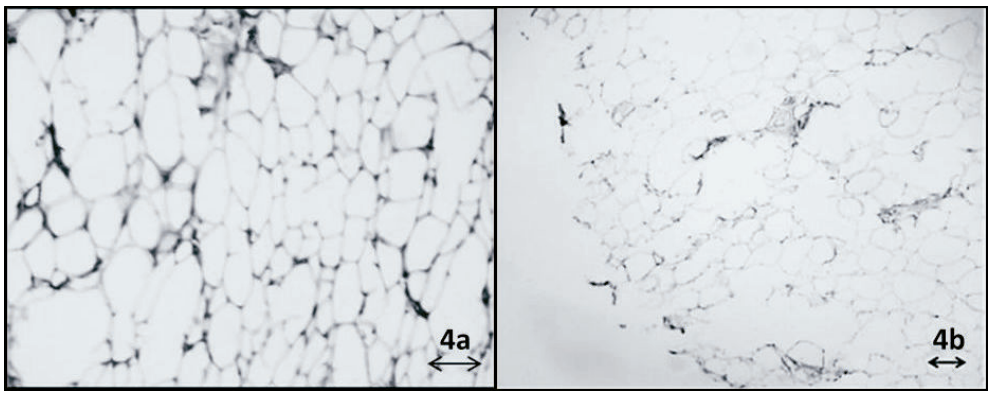

Fig. 4. Immunohistochemical expression of leptin. a) MetSyn. b) Controls. Magn. X 200. (Bar = 10 μm). Immunohistochemical reaction of leptin is positivated in all the adipocytes of adipose tissue samples of both MetSyn women and control healthy women. The leptin immunohistochemical reaction in women with MetSyn is stronger than that of healthy women.

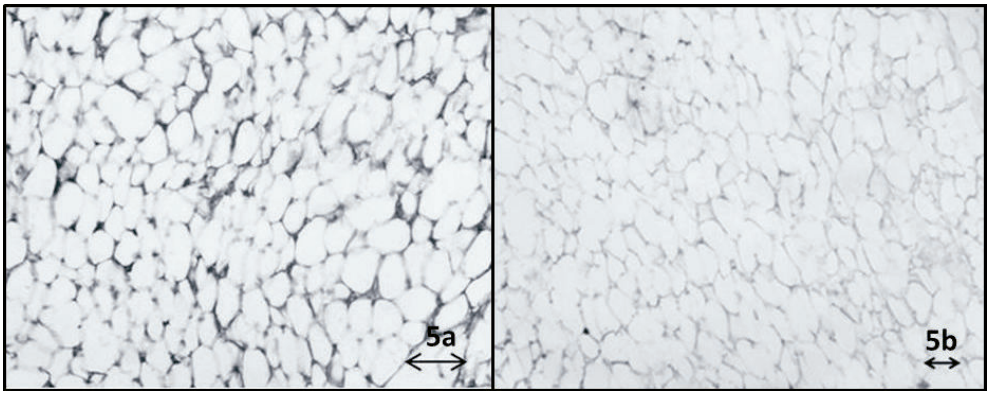

Fig. 5. Immunohistochemical expression of NGF. a) MetSyn. b) Controls. Magn. X 200. (Bar = 10 μm). Immunohistochemical reaction of NGF is positivated in all the adipocytes of adipose tissue samples of both MetSyn women and control healthy women. The NGF immunohistochemical reaction in women with MetSyn is stronger than that of healthy women.

concerning obese humans where serum immunoreactive-leptin concentrations are increased (CONSIDINE 1996). It is known that in obesity leptin circulating levels and adipose tissue leptin mRNA expression are overexpressed in association with BMI and fat mass (CONSIDINE *et al*. 1995; VIDAL *et al*. 1996). Our findings correlate also with the results of a study on the inflammatory markers of metabolic syndrome among adolescents (GONZÁLES *et al*. 2012) where circulating adiponectin levels were decreased and increased leptin levels were directly associated with MetSyn and BMI. ZHANG *et al*. (2012) also reprted increased serum level of leptin and decreased serum adiponectin level in MetSyn.

We found increased plasma levels of NGF in the MetSyn women as compared to the healthy women. In another study plasma NGF was also found upregulated in obesity and MetSyn (BULLÓ *et al*. 2007). We suggest that NGF is a part of the etiopathological mechanisms of developing MetSyn . Recent years have witnessed NGF's role not only as a stimulator of nerve growth and survival, but also as an inducer of trophic effects on immune cells, endothelial cells and pancreatic beta cells (YANEV *et al*. 2013). NGF metabotrophicnactions of glucose, lipids, energy, and cardiovascular homeostasis have been reported (CHALDAKOV 2011). Chaldakov and coworkers have hypothesized that NGF production could be implicated in the pathogenesis of obesity and related comorbidities reporting hyponeurotrophinemia present in a group of patients with MetSyn and cardiovascular disease CHALDAKOV *et al.* 2001; CHALDAKOV *et al*. 2004).

In this work we have demonstrated that not only leptin and NGF plasma levels are upregulated but also immunohistochemical expression of both proteins is stronger in subjects with MetSyn. This is the first study documenting immuno- histochemical expression of leptin and NGF in subcutaneous adipose tissue of women with MetSyn. Similarly to our results, (BULLÓ *et al.* 2007) found higher levels of NGF mRNA in subcutaneous adipose tissue tissue from a small subgroup of morbidly obese patients using another method of investigation. There are also other previous reports of NGF gene expression in human adipose tissue in a small group of morbidly obese volunteers with a stronger signal in omental than in subcutaneous fat (PEERAULLY et al. 2004).

Our results contribute to the idea that the main adipose tissue product, leptin, participates not only in the regulation of energy balance and food intake and informs the brain about the amount of body fat (KHAN *et al*. 2012) but is also both a metabolic and neuroendocrine hormone. At the same time both adipokines, leptin and NGF, are involved in inflammation. Several studies have implicated neurotrophins in the pathogenesis of various inflammatory diseases (DICOU *et al*. 1993; BONINI *et al*. 1996). In 2006 a neurotrophic hypothesis of the etiopathogenesis of MetSyn was put forward. This hypothesis considered neurotrophins as a key factor in MetSyn development (HRISTOVA & ALOE 2006). Chronic inflammatory and/or psychoemotional distress provoke a series of neuroimmuno- endocrine interactions such as increased tissue and plasma levels of proinflammatory cytokines and neurotrophins (HRISTOVA 2013). Recent studies have shown that obesity, playing a central role in the development of metabolic syndrome and in its clinical consequences, induces chronic local inflammation in adipose tissue. Inflammation is now considered by some authors to be an important feature of both obesity and MetSyn, neurotrophins being involved in these pathologies (CHALDAKOV *et al*. 2004). Whether leptin and NGF may contribute to inflammation and the metabolic derangements associated with body weight gain remains to be elucidated.

Conclusion

This study demonstrates for the first time a complex of immunochemical and immunohistochemical expression of the key adipokines leptin, NGF and adiponectin in women with metabolic syndrome. Locally-produced pro-inflammatory adipokines probably contribute to the etiopathogenic mechanisms of MetSyn implicated in obesity, insulin resistance, cardiovascular disorders and hypertension. Leptin, adiponectin and NGF could be used as biomarkers to predict MetSyn and its risks among adolescents as well as the new targets for new therapies.

References

BASTARD J.P., MAACHI M., LAGATHU C., KIM M.J., CARON M., VIDAL H., CAPEAU J., FEVE B. 2006. Recent advances in the relationship between obesity, inflammation, and insulin resistance. Eur. Cytokine Netw. **17**: 4-12.

BASTARD J.P., MAACHI M., TRAN VAN NHIEU J., JARDEL C., BRUCKERT E., GRIMALDI A., ROBERT J.J., CAPEAU J., HAINQUE B. 2002. Adipose tissue IL-6 content correlates with resistance to insulin activation of glucose uptake both *in vivo* and *in vitro*, J. Clin. Endocrinol. Metab. **87**: 2084-2089.

BONINI S.E., LAMBIASE A., BONINI S.T., ANGELUCCI F., MAGRINI L., MANNI L., ALOE L. 1996. Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma. PNAS **93**: 10955-10960.

BULLÓ M., PEERAULLY M.R., TRAYHURN P., FOLCH J., SALAS-SALVADÓ J. 2007. Circulating nerve growth factor levels in relation to obesity and the metabolic syndrome in women. Eur. J. Endocrinol. **157**: 303-310.

CHALDAKOV G.N., FIORE M., STANKULOV I.S., HRISTOVA M., ANTONELLI A., MANNI L., GHENEV P.I., ANGELUCCI F., ALOE I. 2001. NGF, BDNF, Leptin, and mast cells in human coronary atherosclerosis and metabolic syndrome. Arch. Physiol. Biochem. **109**: 357-360.

CHALDAKOV G.N., FIORE M., STANKULOV I.S., MANNI L., HRISTOVA M.G., ANTONELLI A., GHENEV P.I., ALOE L. 2004. Neurotrophin presence in human coronary atherosclerosis and metabolic syndrome: a role for NGF and BDNF in cardiovascular disease? Prog. Brain. Res. **146**: 279-289.

CHALDAKOV G.N. 2011. The metabotrophic NGF and BDNF: an emerging concept. Arc. Ital. Biol. **149**: 257-263.

CONSIDINE R.V., SINHA M.K., HEIMAN M.L., KRIAUCIUNAS A., STEPHENS T.W., NYCE M.R., OHANNESIAN J.P., MARCO C.C., MCKEE L.J., BAUER T.L., CARO J.F. 1995. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N. Engl. J. Med. **334**: 292-295.

DICOU E., MASSON C., JABBOUR W., NERRIERE V. 1993. Increased frequency of NGF in sera of rheumatoid arthritis

and systemic lupus erythematosus patients. Neuroreport. **5**: 321-324.

FRIED S.K., BUNKIN D.A., GREENBERG A. 1998. Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. J. Clin. Endocrinol. Metab. **83**: 847-850.

GONZÁLEZ M., DEL MAR BIBILONI M., PONS A., LLOMPART I., TUR J.A. 2012. Inflammatory markers and metabolic syndrome among adolescents. Eur. J. Clin. Nutr. **66**: 1141-1145.

GRUNDY S.M., CLEEMAN J.I., DANIELS S.R., DONATO K.A., ECKEL R.H., FRANKLIN B.A., GORDON D.J., KRAUSS R.M., SAVAGE P.J., SMITH S.C. et al. 2005. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung and Blood Institute scientific statement. Circulation **112**: 2735-2752.

HOTAMISLIGIL G.S., SHARGILL N.S., SPIEGELMAN B.M. 1993. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science. **259**: 87-89.

HRISTOVA M., ALOE L. 2006. Metabolic syndrome – neurotrophic hypothesis. Med. Hypotheses **66**: 545-549.

HRISTOVA M.G. 2013. Metabolic syndrome – From the neurotrophic hypothesis to a theory. Med. Hypotheses **81**: 627-634.

KHAN S.M., HAMNVIK O.P., BRINKOETTER M., MANTZOROS C.S. 2012. Leptin as a modulator of neuroendocrine function in humans, Yonsei Med. J. **53**: 671-679.

NISHIMURA S., MANABE I., NAGAI R. 2009. Adipose tissue inflammation in obesity and metabolic syndrome. Discov. Med. **8**: 55-60.

PEERAULLY M.R., JENKINS J.R., TRAYHURN P. 2004. NGF gene expression and secretion in white adipose tissue: regulation in 3T3-L1 adipocytes by hormones and inflammatory cytokines. American Journal of Physiology. Endocrinol. Metab. **287**: E331-E339.

SAMAD F., YAMAMOTO K., LOSKUTOFF D.J. 1996. Distribution and regulation of plasminogen activator inhibitor-1 in murine adipose tissue in vivo. Induction by tumor necrosis factor-alpha and lipopolysaccharide. J. Clin. Invest. **97**: 37-46.

SAMAD F., YAMAMOTO K., PANDEY M., LOSKUTOFF D.J. 1997. Elevated expression of transforming growth factor-beta in adipose tissue from obese mice. Mol. Med. **3**: 37-48.

SARTIPY P., LOSKUTOFF D.J. 2003. Monocyte chemoattractant protein 1 in obesity and insulin resistance. Proc. Natl. Acad. Sci. USA **100**: 7265-7270.

VIDAL H., AUBOEUF D., DE VOS P., STAELS B., RIOU J.P., AUWERX J., LAVILLE M. 1996. The expression of ob gene is not acutely regulated by insulin and fasting in human abdominal subcutaneous adipose tissue. J. Clin. Invest. **98**: 251-255.

ZHANG X.J., LI M., GAO S., WANG Y.H., LIU S.J. 2012. Relationship between metabolic syndrome and adipokines on diabetes among high-risk populations. Zhonghua Liu Xing Bing Xue Za Zhi **33**: 418-422.

YANEV S., ALOE L., FIORE M., CHALDAKOV G. 2013. Neurotrophic and metabotrophic potential of nerve growth factor and brain-derived neurotrophic factor: Linking cardiometabolic and neuropsychiatric diseases. World J. Pharmacol. **2**: 92-99.

YANG T., CHU C.H., HSIEH P.C., HSU C.H., CHOU Y.C., YANG S.H., BAI C.H., YOU S.L., HWANG L.C, CHUNG T.C., SUN C.A. 2013. C-reactive protein concentration as a significant correlate for metabolic syndrome: a Chinese population-based study. Endocrine **43**: 351-359.