Vitamin D as a Regulator of Adipocyte Differentiation
Effects *in vivo* and *in vitro*

ALIN CONSTANTIN PINZARIU[1], TEODOR OBOROCEANU[1]*, FLORIN ZUGUN ELOAE[1], IOANA HRISTOV[1], VICTOR VLAD COSTAN[2], LUMINITA LABUSCA[3], PETRU CIANGA[1], LILIANA VERESTIUC[4], BIANCA HANGANU[5], DRAGOS VALENTIN CRAUCIUC[5], IRINA SMARANDA MANOILESCU[5], EUSEBIU VIOREL SINDILAR[6], VERONICA MOCANU[1]

[1]Grigore T. Popa University of Medicine and Pharmacy, Departments of Morphofunctional Sciences I and II, 16 Universitatii Str., 700115, Iasi, Romania

[2]Grigore T. Popa University of Medicine and Pharmacy, Department of Oral and Maxillofacial Surgery, 16 Universitatii Str., 700115, Iasi, Romania

[3]National Institute of Research and Development for Technical Physics 47 Mangeron Blvd., 700050, Iasi, Romania

[4]Grigore T. Popa University of Medicine Pharmacy, Faculty of Medical Bioengineering, 9-13 Kogalniceanu Str.,700454, Iasi, Romania.

[5] Institute of Legal Medicine, 4 Buna Vestire Str., 700455, Iasi, Romania

[6]Ion Ionescu de la Brad University of Agricultural and Veterinary Medicine Iasi, 3 Mihail Sadoveanu Alley, 700490, Iasi, Romania

The age-associated adiposity and the effect of long-term vitamin D was studied in vitamin D deficient rats. In in vivo experiments, the influence of a 9 months of vitamin D treatment (weekly oral gavage with 0.125 mg vitamin D3 (5000 IU)/100g body weight) on the adipocyte precursors from the omental adipose tissue was examined. In in vitro experiment, rat adipose-derived mesenchymal stromal/stem cells (ASCs) were induced to differentiate into adipocytes in the presence or absence of 25(OH)D3 (0.25, 25, and 2500 nmol/L). ASCs derived from vitamin D-treated animals showed an increase adipogenic potential as compared to vitamin D-deficient rats. The addition of 25(OH)D3 inhibits the adipocyte differentiation and lipid deposition in a dose dependent manner.

Keywords: vitamin D, sarcopenia, adipocyte precursors, adipose-derived mesenchymal stromal/stem cells

A characteristic feature of aged organisms is the progressive and generalized loss of muscle mass and function (sarcopenia), which is a major cause of frailty and morbidity among the elderly [1]. The weakness and loss of muscle mass are related with changes in muscle morphology, demonstrating a preferential atrophy of type II muscle fibers [2]. This reduction in muscle mass with aging may result from a general decline in protein synthesis, an enhancement of overall protein degradation rates or a failure of miogenic potential. Moreover, the loss of myofibres is associated with inflammatory cell and adipocyte infiltration [3, 4]. The fat inûltration may correspond to aberrant transdifferentiation of myogenic precursor cells into adipocytes resulting in the formation of fat within the intermuscular space [2, 5].

Prolonged deficiency of vitamin D is associated with severe muscle weakness and loss of muscle mass and strength [6]. In 15-month-old rats, 9 months of vitamin D depletion induced skeletal muscle atrophy [2]. Vitamin D deficiency contribute not only to skeletal muscle atrophy [2] but also to increased adiposity [7, 8].

In vitro studies showed that the addition of 1,25-dihydroxy vitamin D3 (1,25(OH)2D3) decreases skeletal muscle cells proliferation and enhances myogenic differentiation [9-11]. Regarding adipogenic trans-differentiation of muscle satellite cells, previous studies reported that high physiological and supraphysiological doses of 1,25(OH)2D3 inhibited adipogenic trans-differentiation [12].

The age-associated adiposity acts synergistically with sarcopenia, worsening disability through 'sarcopenic obesity'. Increased lipid accumulation in many tissues is associated with the appearance of insulin resistance [13, 14] and decreased muscle protein synthesis.

We used vitamin D deficiency and aged induced sarcopenia model to study the effect of prolonged treatment with high physiological doses of vitamin D on adipose tissue, muscle regeneration and adipogenic transdifferentiation. As we have previously shown, long-term administration of vitamin D (weekly oral gavage with 0.125 mg vitamin D3 (5000 IU)/100g body weight for 9 months) resulted in reduced fat accumulation in old male Wistar rats used as a model of vitamin D deficiency and aged induced sarcopenia [15].

The present study considered the *in vivo* effect of prolonged high dose vitamin D3 treatment on the potential for differentiation of adipocytes precursors derived from vitamin D-treated and vitamin D deficient rats and the *in vitro* effect of different concentrations of 25 hydroxy vitamin D3 (25(OH)D3) on adipocyte differention in primary culture.

Experimental part

Animal experiments

Twenty young (N=10, 3 months old, 128±13g) and old (N=10, 9 months old, 290±24 g) male Wistar rats were purchased from the Cantacuzino Institute, Bucharest, Romania. The animals were housed in polypropylene cages and maintained on a 12 h light/12 h dark cycle under ultraviolet B-free incandescent light to minimize endogenous vitamin D production. All rats were allowed free access to laboratory food pellets (Cantacuzino Institute, Bucharest, Romania)[15].

After 1 week of acclimation, the young and adult animals were randomly divided in control and treated vitamin D groups. The animals were kept under similar conditions except that the vitamin D groups received weekly via oral gavage vitamin D (Vigantol oil, KGaA, Darmstadt, Germany,

* email: teodor.oboroceanu@yahoo.com; Phone: 0770554483

0.5 mg vitamin D3/ml), in dose of 0.125 mg vitamin D3 (5000 IU)/100g body weight and vitamin D deficient groups received palm oil [15].

The experiment respected the instructions of the general guidelines for the care and use of laboratory animals, recommended by the Council of European Communities [16]. All experimental procedures were approved by the Laboratory Animal Care Committee of the Grigore T. Popa University of Medicine and Pharmacy Iasi, Romania.

Preparation of adipose-derived mesenchymal stromal/stem cells (ASCs) and cell culture

Animals were euthanized with isoflurane and omental adipose depots were excised. Tissues were minced and incubated in 1 mg/mL collagenase in Dulbecco's modified Eagle's medium (DMEM)/F12 for 1 hr at 37°C with 5% CO_2. After inactivation of the digest with an equal volume of DMEM/10% fetal bovine serum (FBS), samples were passed through a 70 mm cell strainer. For cell culture, samples were centrifuged at 600 g for 10 min, and the resulting ASCs were resuspended in complete media (DMEM/10% FBS/1% penicillin/streptomycin) and plated for imaging or differentiation [17].

Cell differentiation and treatment

ASCs were used for adipogenic differentiation. After reaching 80-90% conûuence, cells were transferred to six-well plates and stimulated to differentiate into adipocytes either between adipogenic induction media (AIM), containing 0.1µM dexamethasone, 1% ITS (Insulin Transferrin Selenium), 200 µM indomethacin, 0.5 µM IBMX (3-isobutyl-1-methylxanthine), 10% fetal bovine serum, and penicillin/streptomycin (all from Sigma Aldrich, Co., St. Louis, MO), or adipogenic maintenance medium (10% ITS, 10% fetal bovine serum, and penicillin/streptomycin) every 3 days for 3 weeks, until obtaining an adipogenic phenotype as previously described [17]. During differentiation, cells were treated with either PBS or 25-hydroxy vitamin D3 (25(OH)D3) (Sigma-Aldrich, Co., St. Louis, MO), at three different concentrations (0.25, 25, and 2500 nmol/L). Non-induced cells were used as a control.

Oil Red O staining

Oil red O (ORO) staining was used to assess adipocyte differentiation as an indicator of intracellular lipid accumulation. On day 21, the culture medium was removed from tissue culture wells and the cells were rinsed with phosphate buffered saline (PBS) once, followed by ûxation using 3.7% formaldehyde in PBS for at least 1 hour. The ûxative was then aspirated and the cells were washed with 60% isopropanol before being allowed to dry completely. The cells were stained for 10 min at room temperature with a diluted solution of ORO prepared from a 0.5% (w/v) ORO dissolved in isopropanol. Photo-micrographs were taken using a Carl Zeiss microscope (Axio Observer Z1, Jena Germany) and imaging software. To quantify lipid deposition, ORO was eluted with 1 mL 100% isopropanol for 10 min and absorbance measured at 490 nm [17].

Nuclear and cytoskeleton fluorescent dye

Nuclear fluorescent dye was performed using 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). The stained adipocytes were visualized under a fluorescent microscope for verification of purified intact adipocytes using the laser with wavelength of 405 [17].

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using the Student's t test and Bonferroni's multiple comparison test (Statistical Software Package SPSS®, version 13, SPSS Inc., Chicago, IL, USA). Unpaired Student's t-tests were performed to determine whether there were significant differences between groups ($p<0.05$).

Results and discussions

In vivo experiments. Differentiation of adipose-derived mesenchymal stromal/stem cells (ASCs) isolated from vitamin D-treated or vitamin D-deficient rats

We treated young and old vitamin D deficient male Wistar rats with 0.125 mg vitamin D3 (5000 IU)/100g body weight or palm oil for 9 months [15]. The ASCs from omental adipose tissue were isolated, proliferated and adipogenic differentiated. As shown in figure 1A, long-term

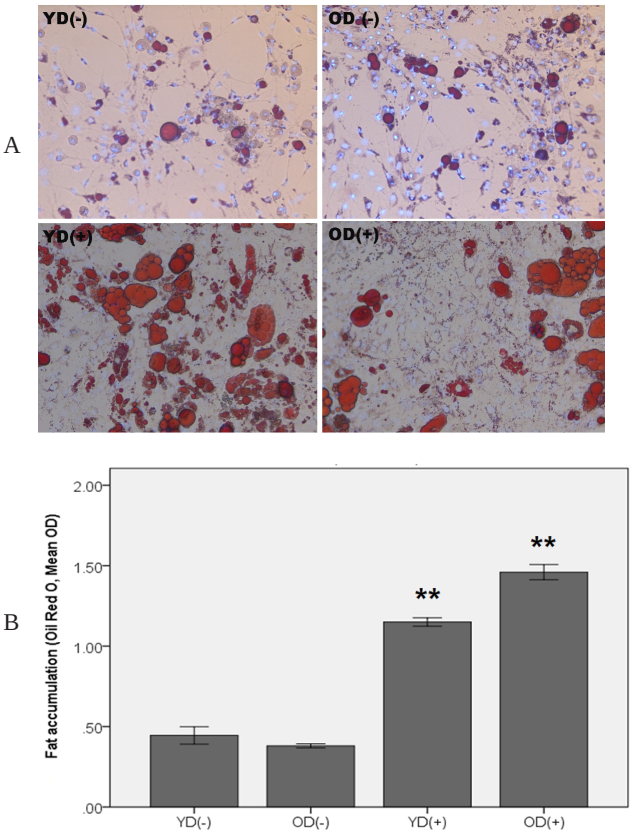

Fig. 1. Differentiation of ASCs isolated from vitamin D-deficient or vitamin D-treated rats. (A) At week 3, photomicrographs taken using a light microscope (magniûcation x10) shows higher amount of fat droplets in adipocytes derived from vitamin D-treated rats as compared to those derived from vitamin D-deficient rats. (B) Increased fat accumulation (ORO, mean OD) in adipocytes derived from long-term vitamin D-treated as compared to vitamin D-deficient rat (* $p<0.05$, ** $p<0.01$). Abbreviation: ASCs, adipose-derived mesenchymal stromal/stem cells (ASCs); 25(OH)D3, 25 hydroxy vitamin D3, Oil Red O: ORO

treatment with vitamin D3 increased the adipogenic potential demonstrated by increasing proliferation and adipogenic differentiation (higher amount of fat droplets) of ASCs derived from vitamin D-treated rats as compared to ASCs derived from vitamin D-deficient rats. After 21 days, mature adipocytes were subjected to Oil Red O (ORO) staining and quantitative analysis was conducted. ORO absorbance at 490 nm by spectrophotometry showed a significant increase ($p<0.01$) in lipid deposition in cell

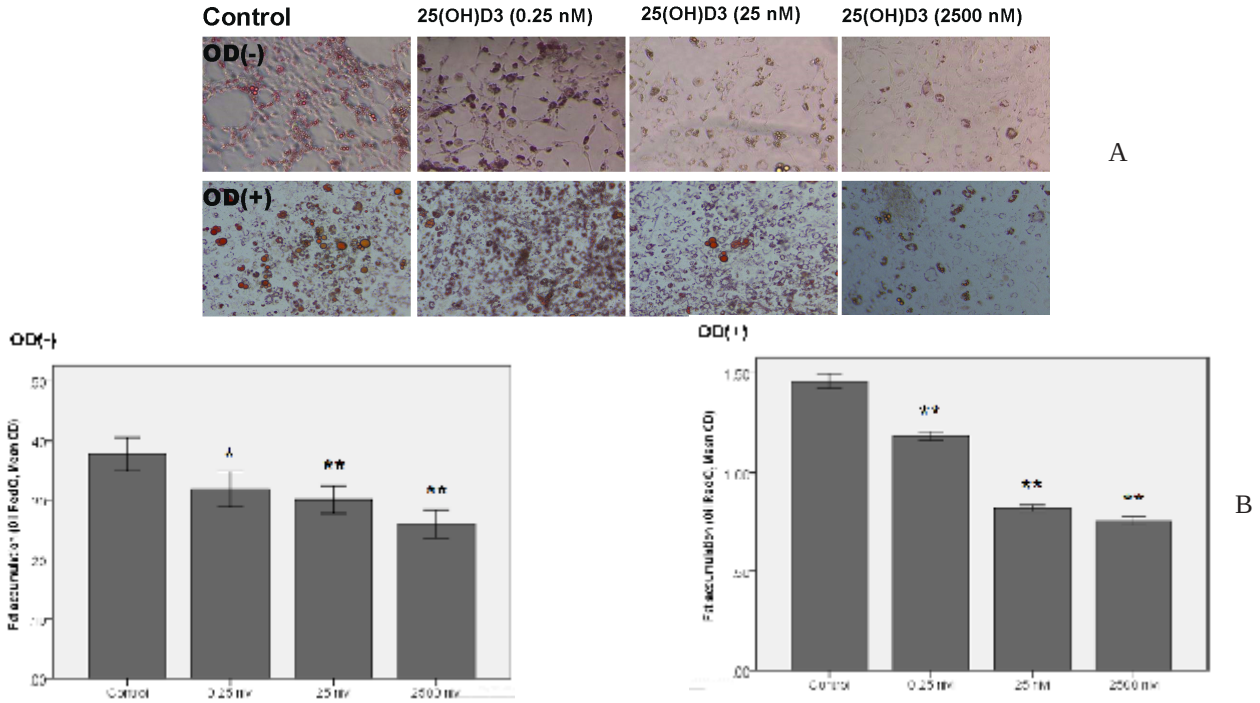

Fig. 2. 25(OH)D3 inhibits adipogenesis in vitro: (A) ASCs derived from old Wistar male rats were committed to differentiate into adipocytes and treated for 3 weeks with either 25(OH)D3 (0.25, 25, and 2500 nM) or PBS. At week 3, photomicrographs taken using a light microscope (magniûcation x10) shows lower amount of fat droplets in 25(OH)D3-treated cells as compared to PBS-treated cells (control). (B): The strong inhibitory effect dose-dependent of 25(OH)D3 on fat accumulation (ORO, mean OD) in differentiating adipocytes as compared to PBS (* p <0.05, ** p <0.01). Abbreviation: ASCs, adipose-derived mesenchymal stromal/stem cells (ASCs); 25(OH)D3, 25 hydroxy vitamin D3, Oil Red O: ORO

derived from vitamin D-treated as compared to vitamin D-deficient rats (fig. 1B).

In vitro experiment. The effect of 25(OH)D3 on differention of ASCs

We used ASCs isolated from omental adipose tissue of old male Wistar rats with vitamin D deficiency (9 months) treated or not with vitamin D. The 80-90% confluent ASCs were treated with adipogenic induction medium containing PBS (control) or 25(OH)D3 dissolved in DMSO at progressive concentrations: 0.25, 25, and 2500 nmol/L.

As shown in figure 2A, the proportion of cells having easily identiûable fat droplets (adipocytes) was inversely related to the 25(OH)D3 concentration in the media. To assess lipid deposition, ORO was eluted and quantiûed by measuring the absorbance at 490 nm by spectrophotometry (fig. 2B). All three concentrations of 25(OH)D3 induced a signiûcant decrease in lipid deposition in cell derived from old vitamin deficient vitamin D ($p < 0.05$, $p < 0.01$ and $p < 0.01$, repectively) and old vitamin D-treated rats ($p < 0.001$, $p < 0.001$ and $p < 0.001$, repectively) as compared to PBS-treated cells.

At old age, fat is redistributed outside fat depots, accumulating in bone marrow, muscle, liver, and other ectopic sites [18]. Muscle fat infiltration is associated with deterioration of muscle strength and functionality. As in humans, mice and rats have fat redistribution and ectopic fat deposition with aging [19]. Little is known about the impact of aging on fat tissue and regulation of adipocyte proliferation and differentiation.

White adipose tissue (WAT) depots are composed of a heterogeneous mixture of cell populations including terminally differentiated lipid-filled adipocytes and a stromal vascular fraction (SVF) that contains blood lineage cells, endothelial cells, immune cells, other uncharacterized stromal cells and adipocyte precursor cells [17]. As mature adipocytes are terminally-differentiated postmitotic cells, differentiation of adipocyte precursors is required for hyperplastic WAT growth. The differentiation of adipocyte precursors could be affected by vitamin D deficiency and age [20-22].

Several studies have tried to identify the origin of the stromal/stem cell population (adipocyte precursors) within adipose tissue *in situ*. Howevver, it is difficult to identify native adipose-derived stromal/stem cells (ASCs) [23]. Isolated and cultured ASCs are a non-uniform preparation consisting of several subsets of stem and precursor cells. In general, ASCs are described as immature cells within the adipose tissue; they are a rare and quiescent population (or populations) within the perivascular niche. However, native ASCs could not be clearly separated from the whole heterogeneous mixture as they share membrane antigens with other cells found in the SVF [24].

Stromal cells and preadipocytes express functional vitamin D receptors [7, 25]. However, 1,25(OH)2D3 has been reported to act both as an adipogenic agonist and antagonist in a variety of systems. Several possible explanations may account for the discrepant adipogenic effects by 1,25(OH)2D3 reported in the literature. One is that the tissue site of origin for each of the preadipocyte cell models influences the response to 1,25(OH)2D3 [26, 27].

In our study, the inclusion of 25(OH)D3 to an adipogenic differentiation cocktail significantly inhibited adipocyte differentiation at the concentrations of 25 and 2500 nmol/L. The previous studies also reported an inhibitory effects of 1,25(OH)2D3 on adipocyte fat storage in mature 3T3-L1 by higher concentrations (1, 10 and 100 nmol/L) of 1, 25 (OH)2D3 but not by lower concentrations (0.1 and 0.01 nmol/L) [7, 27, 28].

The present study demonstrates increased adipogenic potential of cells derived from vitamin D-treated rats (0.125 mg vitamin D3 (5000 IU)/100g body weight for 9 months) as compared to vitamin D-deficient rats. Once the ASCs

are removed from the rat and grown *in vitro*, cells derived from the vitamin D-treated rats have an increased capacity for both division and fat accretion. The increased adipogenic potential could be related to increased number of ASCs and increase their competence to proliferate and differentiate [29]. In a previous study, Domingues-Faria et al. reported that in old rats vitamin D-depleted for 9 months the plasma 25(OH)D3 was reduced by 74% as compared to controls. Furthermore, vitamin D deficiency induced skeletal muscle atrophy and reduced the myogenic potential [2]. Regarding the adiposity, this is the first study to report the increased adipogenic potential in long-term vitamin D treated old rats.

In a previous paper, we have reported the prolonged high dose vitamin D treatment resulted in dynamic remodeling of adipose tissue, consisting in decreased number of omental adipose cells and smaller, polygonal-shaped adipocytes. These histological findings support the observation that prolonged vitamin D treatment increased the adipogenic potential and decreased the fat stores [15].

Taken together, all these changes suggest that vitamin D plays an important role as a regulator of adiposity and could maintain the cellularity and metabolic characteristics of adipose tissue.

Conclusions

The present data demonstrated that long-term treatment with high doses of vitamin D may have favorable effects on adipocyte proliferation and differentiation, by selection of potent preadipocytes, decreased lipid accumulation and by improving metabolic functions.

References

1.ROSENBERG, I.H., J Nutr, **127**, no. 5 Suppl, 1997, p. 990S.
2.DOMINGUES-FARIA, C., CHANET, A., SALLES, J., BERRY, A., GIRAUDET, C., et al., Nutr Metab (Lond), **11**, no. 1, 2014, p. 47.
3.RELAIX, F. and ZAMMIT, P.S., Development, **139**, no. 16, 2012, p. 2845.
4.PARKER, M.H., Front Genet, **6**, no., 2015, p. 59.
5.PINZARIU, A., SINDILAR, A., HALIGA, R., CHELARU, L. and MOCANU, V., Rev Med Chir Soc Med Nat Iasi, **118**, no. 3, 2014, p. 699.
6.HOLICK, M.F., N Engl J Med, **357**, no. 3, 2007, p. 266.
7.JI, S., DOUMIT, M.E. and HILL, R.A., PLoS One, **10**, no. 6, 2015, p. e0126142.
8.KOB, R., FELLNER, C., BERTSCH, T., WITTMANN, A., MISHURA, D., et al., J Cachexia Sarcopenia Muscle, **6**, no. 2, 2015, p. 181.
9.GARCIA, L.A., KING, K.K., FERRINI, M.G., NORRIS, K.C. and ARTAZA, J.N., Endocrinology, **152**, no. 8, 2011, p. 2976.
10.WAGATSUMA, A. and SAKUMA, K., Biomed Res Int, **2014**, no., 2014, p. 121254.
11.BRAGA, M., SIMMONS, Z., NORRIS, K.C., FERRINI, M.G. and ARTAZA, J.N., Endocr Connect, **6**, no. 3, 2017, p. 139.
12.RYAN, K.J., DANIEL, Z.C., CRAGGS, L.J., PARR, T. and BRAMELD, J.M., J Endocrinol, **217**, no. 1, 2013, p. 45.
13.TARDIF, N., SALLES, J., GUILLET, C., TORDJMAN, J., REGGIO, S., et al., Aging Cell, **13**, no. 6, 2014, p. 1001.
14.SLAWIK, M. and VIDAL-PUIG, A.J., Genes Nutr, **2**, no. 1, 2007, p. 41.
15.PINZARIU, A.C., PASCA, S.A.., SINDILAR, A., DROCHIOI, C., BALAN, M.,OBOROCEANU, T., NICULESCU, S., CRAUCIUC, D.V., CRAUCIUC, E.G., LUCA, A., BUTCOVAN, D., SINDILAR, E.V., MOCANU, V.., Rev. Chim. (Bucharest), **68**, no. 9, 2017, p. 2139
16.GENERAL GUIDELINES FOR THE CARE AND USE OF LABORATORY ANIMALS RECOMMENDED BY THE COUNCIL OF EUROPEAN COMMUNITIES, Official Journal of European Communities Brussels, no. 1-18, 1986.
17.CHURCH, C.D., BERRY, R. and RODEHEFFER, M.S., Methods Enzymol, **537**, no., 2014, p. 31.
18.BUTCOVAN, D., OBOROCEANU, T., CIMPEANU, C., MIRONESCU , A., HALIGA, R.E., et al., Rev. Chim..(Bucharest), **68**, no. 4, 2017, p. 886.
19.TCHKONIA, T., MORBECK, D.E., VON ZGLINICKI, T., VAN DEURSEN, J., LUSTGARTEN, J., et al., Aging Cell, **9**, no. 5, 2010, p. 667.
20.PEREZ, L.M., DE LUCAS, B., LUNYAK, V.V. and GALVEZ, B.G., Mol Genet Metab Rep, **12**, no., 2017, p. 51.
21.DROCHIOI, C., COSTAN, V. V., ZAHARIA, M., BOISTEANU, O., SANDU, I.G., et al., Rev. Chim (Bucharest), **66**, no. 9, 2015, p. 1302
22.DUFRANE, D., Cell Transplant, **26**, no. 9, 2017, p. 1496.
23.BOURIN, P., BUNNELL, B.A., CASTEILLA, L., DOMINICI, M., KATZ, A.J., et al., Cytotherapy, **15**, no. 6, 2013, p. 641.
24.BAER, P.C., World J Stem Cells, **6**, no. 3, 2014, p. 256.
25.HALIGA, R., ZUGUN-ELOAE, F., OBOROCEANU, T., PINZARIU, A. and MOCANU, V., Rev Med Chir Soc Med Nat Iasi, **120**, no. 2, 2016, p. 404.
26.MUTT, S.J., HYPPONEN, E., SAARNIO, J., JARVELIN, M.R. and HERZIG, K.H., Front Physiol, **5**, no., 2014, p. 228.
27.KELLY, K.A. and GIMBLE, J.M., Endocrinology, **139**, no. 5, 1998, p. 2622.
28.LEE, H., BAE, S. and YOON, Y., Int J Mol Med, **30**, no. 5, 2012, p. 1219.
29.HAUSMAN, G.J. and DODSON, M.V., J Genomics, **1**, no., 2013, p. 56.

Manuscript received: 18.09. 2017