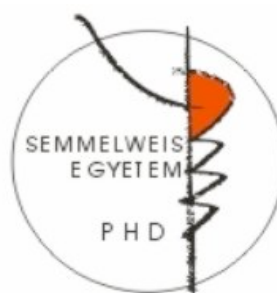


# ALTERATIONS OF ARTERIOLAR REACTIVITY IN A RAT POLYCYSTIC OVARY SYNDROME MODEL - EFFECTS OF PARALLEL VITAMIN D<sub>3</sub> ADMINISTRATION

PhD thesis

**Levente Sára MD**

Basic Medicine Doctoral School  
Semmelweis University Budapest



Supervisor: Szabolcs Várbiro MD, PhD

Official Reviewers:

Attila Szabó MD, PhD, Dsc

Sándor Alföldi MD, PhD

Head of the Final Examination Committee:

János Rigó Jr. MD, PhD, Dsc

Members of the Final Examination Committee:

Ádám László MD, PhD

Péter Studinger MD, PhD

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## **BACKGROUND**

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disease and the most frequent disorder in women of reproductive age. Nowadays, PCOS is in the focus of research because of its increasing prevalence. PCOS affects approximately 4-10% of fertile women around the world, but some researchers estimate the overall rate to be between 4-25%. The aetiology of this complex and heterogenous disorder is still uncertain. Environment factors such as physical inactivity, malnutrition and obesity have crucial role in development of the disorder. The most common features of PCOS, called the “Rotterdam criteria”, are menstrual disorders (amenorrhoea) such as oligo- or anovulatory menstrual cycles; polycystic, large ovaries as detected by ultrasound; and clinical or laboratory signs of excess androgen. It often associated with insulin resistance (IR) obesity, acne, hirsutism, cardiovascular disorders (CVD). There is a wide variability in phenotypes; symptoms and severity vary among affected patients. PCOS also alters cardiovascular function through various mechanisms. The mechanisms underlying this increased risk and its possible therapeutic approaches are still under investigation. IR and obesity were proposed to have a central role in the development of CVDs. Although there is a higher incidence of cardiovascular risk factors among PCOS patients, the existence of a significantly higher CVD incidence compared to control patients is debated. In research yet little is known about cardiovascular alterations of PCOS. In the background of PCOS circle vicious of IR and hyperandrogenism can increase the risk of CVDs.

***Vascular alterations in PCOS.*** In PCOS, increased arterial stiffness and pulse wave velocity were demonstrated by ultrasound assessments. The mechanism of these alterations is unclear, but endothelial dysfunction and altered collagen metabolism of the vessel walls may be involved. This is similar to the patterns seen in IR and metabolic syndrome. Human studies have shown both macro- and microvascular dysfunction measured by ultrasound in PCOS, as indicated by the reduction in acetylcholine(ACh)-dependent vasodilation. This impaired response to ACh is similar to that observed in non-insulin-dependent diabetes mellitus. It is proposed to be related to metabolic alterations, especially insulin resistance in PCOS. It was showed that those women who have a decreased internal carotid artery pulsatility index in PCOS, have a

higher cardiovascular risk as well. Women with PCOS have a high prevalence of IR, metabolic syndrome, early-onset atherosclerosis, and they might develop hypertension during their reproductive period. These abnormalities are partly explained by the pharmacological and biomechanical remodelling of resistance arteries. The intima-media thickness of the carotid arteries was confirmed to be greater in PCOS than in age- and BMI-matched controls. Similarly, coronary artery calcification was found to be greater in PCOS patients. An endothelial dysfunction is the first step in the formation of atherosclerosis. The exact molecular mechanisms of the vascular alterations in PCOS are still unclear. It was hypothesised that the accumulation of reactive oxygen species, toxic oxygen-derived products and nitrogen-derived products might contribute to an uncontrolled lipid peroxidation of the cell membranes. The stimulation of NADPH oxidase and the uncoupling of mitochondrial oxidative phosphorylation can impair NO synthesis. These might explain the cardiovascular disorders often seen in PCOS. Hyperinsulinemia and IR often associates with PCOS. The independent role of insulin in human vessel relaxation has been demonstrated. Insulin induced dilation on efferent arterioles in kidney was reported to be independent of EDRF/NO and prostaglandins, however the mechanism of insulin induced direct vascular effect remained unclear. Insulin was suggested to affect IP<sub>3</sub> release of Ca<sup>2+</sup> by a cGMP-dependent mechanism that would contribute to its vasodilatory effect. Insulin may also act through NO and Na<sup>+</sup>-K<sup>+</sup> ATP-ase in human muscle arterioles.

***Possible role of vitamin D in PCOS.*** Vitamin D is a secosteroid hormone that differs from other steroid hormones and can be obtained mainly from de novo synthesis in the skin. The accompanying metabolic alterations and obesity in PCOS might increase the risk of vitamin D deficiency. A recent study showed that vitamin D<sub>3</sub> deficiency occurred in approximately 72% of PCOS women. The level of vitamin D<sub>3</sub> was significantly lower in metabolic syndrome, and supplementation during infancy was observed to protect against T1DM. Vitamin D deficiency is strongly determined by the degree of obesity, and genetic variants of vitamin D<sub>3</sub> receptors can enhance the risk of deficiency. A rat vitamin D deficiency model was reported in which altered vascular endothelial and smooth muscle function and elevated blood pressure were observed. In accordance, increased arterial stiffness and endothelial dysfunction were demonstrated to be associated with vitamin D insufficiency in humans. Several researchers suggest that vitamin D deficiency may contribute to elevated blood pressure and that it has a crucial

role in the development of hypertension. Direct effect of vitamin D<sub>3</sub> on resistance vessels was demonstrated earlier by researchers. However, vitamin D<sub>3</sub> supplementation was assigned as an adjuvant therapy in PCOS, its effectiveness and possible role on metabolic symptoms need to be further characterised. However, even though several studies have confirmed the relationship between IR and vitamin D deficiency, the role of vitamin D<sub>3</sub> in glucose metabolism is still under debate.

## **AIMS**

The aim of our study was to induce PCOS by a 70-day-DHT treatment in a modified animal model for examining early metabolic and functional changes - the earliest detectable lesions. We tried to determine the early effects of DHT treatment on carbohydrate metabolism. We examined the potentially beneficial effects of vitamin D<sub>3</sub>. To identify early mechanical and pharmacological alterations in a morphologically stable skeletal muscle resistance vessel such as the gracilis arteriole in rat in PCOS. We also investigated the effect of low-dose vitamin D<sub>3</sub> treatment on the vascular biomechanical adaptation and the pharmacological responsiveness of the gracilis resistance arterioles. We aimed to clarify the effects of DHT on insulin-dependent vasodilatation on resistance arterioles to determine associations of the possible modulatory role of protective doses of vitamin D<sub>3</sub>.

## **METHODS**

**Animals.** Thirty adolescent (21- to 28-day-old) female Wistar rats that weighed 100-140 g when the study began were used. Twenty of the animals received subcutaneous pellets containing 7.5 mg DHT implanted under the back skin under anaesthesia (Nembutal 45 mg/kg i.p. ) in sterile conditions (DHT-treated female groups), which induces polycystic ovary syndrome. Ten animals underwent sham operations. Ten DHT-treated animals received weekly doses of 120 ng /100 g 1,25 (OH)<sub>2</sub> D<sub>3</sub> vitamin (DHT+D<sub>3</sub> group) subcutaneously. A vehicle was given to the remaining 20 animals. The investigation conforms to the Principles of Laboratory Animal Care NIH publication No. 85-23, revised 1985) as well as the Euroconform Hungarian Law on Animal Care (XXVIII/1998), and the study protocol was accepted by the institutional Animal Care Commission (IRB approval: 22.1/2960/003/2009).

**Oral Glucose Tolerance Test (OGTT).** After eight weeks of treatment, an oral glucose tolerance test was performed under short ether narcosis. Blood glucose and plasma insulin were measured following overnight fasting and 120 minutes after an oral glucose load of 0.3g / 100 g body weight given through a gauge.

**In vivo blood pressure measurement.** After ten weeks of DHT treatment blood pressure was measured directly through the carotid artery in anaesthetised rats (Nembutal 45 mg/kg i.m.) with a Statham transducer connected to a Cardiosys CO-104 system (Experimetria, Budapest, Hungary).

**Biomechanics of a Musculocutaneous Arteriole (Pressure Arteriography).** After opening the iliofemoral region, the arteriole, which had an in vivo diameter of approximately 150  $\mu\text{m}$  and is the blood supply for the gracilis muscle, was removed and placed into a vessel chamber filled with normal Krebs-Ringer (nKR) solution. It was cannulated at both ends with plastic microcannulas and extended to its in vivo length. Both cannulas were connected to pressure-servo pumps, and the arterioles were pressurised under a no-flow condition to 50 mmHg intraluminal pressure. The outer and the inner diameters of the arterioles were measured by video-microscopic pressure microangiometry. In this setup, the glass-bottomed tissue bath was positioned in the light path of an inverse Leica microscope. A magnified picture of the vessel was formed with the aid of a video camera (Leica DFC320) and Leica QWin software. The digitalised pictures were saved, and off-line measurements of the inner and outer diameter were made using Leica QWin image analysis software. The gracilis arterioles were allowed to equilibrate for 30 minutes at 50 mmHg intraluminal pressure in an oxygenised nKR solution. Following incubation, the pressure was decreased to 10 mmHg and then increased to 100 mmHg in 10-mmHg pressure steps. The steady-state diameter was measured. The diameter was also measured during norepinephrine contraction after a 10-minute incubation period with  $10^{-6}\text{M}$  NE. After recording the NE-diameter (pre-contraction) at 50 mmHg, dose-diameter curves were recorded in the presence of 30, 60, 120, 240 and 600 mU/ml of insulin, leaving an equilibration period of 8-10 minutes for each dose, which allowed a stable diameter to be reached and measured. After rinsing, NE was added to the organ bath in the same dose as previously described. After a 10-minute incubation the pressure-diameter curve was repeated as described above. After recording the NE-curve, at  $P=50$  mmHg,  $10^{-6}$  M ACh was added to the organ bath. Following a 20-minute equilibration period, the outer and inner

diameters were measured. Next,  $10^{-5}$  M L-NAME was administered, and the diameters were again measured after reaching equilibrium (25-30 minutes at P=50 mmHg). Finally, the passive diameter was measured in a  $\text{Ca}^{2+}$ -free Krebs solution. Arteriolar segments were incubated for 20 minutes, and the steady-state, fully relaxed diameter was then measured as described above. The effect of insulin + NE was compared to the NE-induced contraction without insulin and also was compared to full relaxation. We used a micrometre etalon (Wild) for the calibrations.

**Histology.** The ovaries were formaldehyde fixed, stained with hematoxylin-eosine and histologically examined for controlling PCOS morphology. The morphometry was measured using the Panoramic Viewer software (3DHISTECH Ltd., Budapest, Hungary).

**Statistical analysis.** For statistical analysis, repeated-measures ANOVA was used. In vitro parameters were plotted as a function of intraluminal pressure, and the curves were analysed by paired comparisons of the treatment groups. A one-way ANOVA was applied for the discrete parameters (e.g., blood pressure) and two way ANOVA for analyzing glucose and insulin curves of OGTT. As a post hoc test, Tukey's test was used.  $P < 0.05$  was uniformly accepted as a significant difference.

## RESULTS

**Physiological parameters.** The mean arterial pressure was  $122 \pm 3$  mmHg,  $123 \pm 6$  mmHg and  $123 \pm 4$  mmHg for the controls, the DHT-group and the DHT+vitamin D<sub>3</sub>-treated group, respectively (non-significant). The body weights at the end of the experiment were  $298 \pm 8$ ,  $354 \pm 16$  and  $353 \pm 9$  g for the same groups, respectively. The DHT-treated animals had significantly higher body weights than the controls ( $p < 0.01$ ), but there was no difference between the two DHT-treated groups.

**Ovarian morphology.** We detected a polycystic morphology in the DHT-treated groups and normal ovaries in the controls by histological analysis. Follicle diameters were significantly smaller in DHT-treated ovaries compared to control ( $1609 \pm 617$  and  $2334 \pm 451$  pixels at 40x magnification,  $p < 0.05$ ). Follicles of DHT+D<sub>3</sub> ovaries weren't significantly different from the other two groups ( $2054 \pm 442$ ).

**Glucose metabolism.** The blood glucose levels and the fasting insulin levels were not significantly different among the experimental groups. However, significant differences were found among the groups for the 120 min postload insulin values ( $0.71 \pm 0.14$ ,  $1.42 \pm 0.33$ ,  $0.48 \pm 0.07$  ng/ml for C, DHT and DHT+D<sub>3</sub>). DHT-treated animals had higher plasma insulin levels than the controls, and this difference was eliminated by the D<sub>3</sub> vitamin treatment ( $p < 0.001$ ). The fasting and 120 min postload insulin levels were significantly different only in the DHT group ( $p < 0.001$ ). Serum fructose amine was similar in all groups and within reference range indicating, that the animals did not develop diabetes or blood glucose elevation.

**Arteriole geometry.** The relaxed outer radii for the different groups were  $167 \pm 13$   $\mu\text{m}$  (C),  $193 \pm 6$   $\mu\text{m}$  (DHT) and  $166 \pm 10$   $\mu\text{m}$  (DHT + vitamin D<sub>3</sub>). The relaxed inner radii of the gracilis arterioles were  $96 \pm 3$   $\mu\text{m}$  (DHT),  $84 \pm 6$   $\mu\text{m}$  (C),  $83 \pm 5$   $\mu\text{m}$  (DHT+D<sub>3</sub>). DHT treatment increased both the outer and inner radii, which were diminished by a parallel vitamin D<sub>3</sub> treatment ( $p < 0.05$  for both comparisons). The wall thicknesses for the different groups in the Ca<sup>2+</sup>-free medium were as follows:  $20 \pm 2$ ,  $19 \pm 1$ , and  $20 \pm 1$   $\mu\text{m}$  in the control, DHT- and DHT + vitamin D<sub>3</sub>-treated groups, respectively. In a Ca<sup>2+</sup>-free solution, the cross section areas for the vessel walls were  $12080 \pm 1627$ ,  $12542 \pm 1090$ , and  $11348 \pm 660$   $\mu\text{m}^2$  for these groups, which were not significant.

**Arteriole elasticity.** The mechanical load on the vessel wall was measured, and the tangential wall stress was significantly higher throughout the entire pressure range for the DHT-treated group compared with the other two groups in both a Ca<sup>2+</sup>-free solution and during norepinephrine contraction ( $p < 0.05$ ). Vitamin D<sub>3</sub> treatment induced a partial reversal of wall stress in the NE-contracted segments and an overcompensation in the Ca<sup>2+</sup>-free solution (both for DHT vs. DHT + D<sub>3</sub> and the control vs. DHT + D<sub>3</sub>,  $p < 0.05$ ). Isobaric distensibility was significantly higher throughout the entire pressure range for the DHT group compared with the other two groups in both the Ca<sup>2+</sup>-free medium and during norepinephrine contraction ( $p < 0.05$ ). In response to a parallel treatment with vitamin D<sub>3</sub>, relaxed distensibility fully returned to the control in Ca<sup>2+</sup>-free solution. After NE contraction only a partial recovery was detected in DHT+D<sub>3</sub> group. Wall elastic moduli were not affected by DHT and vitamin D<sub>3</sub> treatment (in a Ca<sup>2+</sup>-free solution, the log elastic moduli were  $2.8 \pm 0.14$ ,  $2.7 \pm 0.16$  and  $2.4 \pm 0.11$  log(Pa) in the control, DHT- and DHT + vitamin D<sub>3</sub>-treated groups, respectively, ns.). There was no difference in the elastic moduli as a function of tangential wall stress.

**Arteriolar contractility** DHT treatment significantly reduced NE tone, which was almost fully restored by vitamin D<sub>3</sub> treatment ( $p < 0.01$  between the control and DHT,  $p < 0.05$  for the other comparisons). These segments do maintain substantial myogenic (spontaneous) tone. The tone was also lower following DHT treatment, and interestingly, co-administration of D<sub>3</sub> vitamin further decreased myogenic tone (the control and DHT-treated rats,  $p < 0.01$ , as well as the D<sub>3</sub> vitamin-treated and untreated DHT rats,  $p < 0.05$ , were significantly different). The sum of the two contractions, which represent the ability of the segments to fully contract (range of total pharmacological adaptation), was significantly lower after DHT treatment and was partially restored by vitamin D treatment (the control and DHT-treated rats,  $p < 0.01$ , as well as the D vitamin-treated and untreated DHT rats,  $p < 0.05$ , were significantly different).

**Endothelial dilation.** Segments contracted by NE were relaxed in response to 1  $\mu$ M acetylcholine, and this relaxation was significantly larger in the control compared with the DHT-treated group. ACh dilations were severely diminished in the testosterone-treated female animals. Vitamin D<sub>3</sub> treatment did not compensate this alteration but produced only a nonsignificant trend toward restoring relaxation. ( $p < 0.05$  for both treatments comparison with the controls). L-NAME induced substantial contraction in the control segments, which was missing in both DHT-treated groups.

**Insulin-induced vascular relaxation of gracilis arterioles** Insulin administered to NE-precontracted gracilis arteriole segments induced concentration-dependent relaxation in the control animals. This relaxing effect was much reduced in the DHT-treated group ( $p < 0.001$ ). The administration of vitamin D<sub>3</sub> partially restored the insulin-induced relaxation ( $p < 0.001$ ). Another significant effect of Vitamin D<sub>3</sub> treatment was revealed when we compared the NE-induced contractions of segments before and after in vitro treatment with elevating concentrations of insulin. Typically, NE-induced contractions were more profound after insulin treatment. This difference was reduced in vitamin D<sub>3</sub>-treated animals ( $p < 0.05$ ).

## DISCUSSION

As a model of human polycystic ovary syndrome, ten weeks of dihydrotestosterone treatment starting during adolescence in female rats induced significant metabolic and vascular alterations in our studies. Systemic IR without altered glucose metabolism was



developed after 70-day DHT treatment. The two hours insulin value of oral glucose tolerance test in the DHT-treated animals was nearly two times higher than in controls. Chronic DHT treatment of female rats, which is an animal model for human PCOS, induced significant alterations in the biomechanical and pharmacological properties of a skeletal muscle arteriole. We observed an increase in arteriolar diameter. The relaxed lumen was dilated but was not accompanied by an elevation in wall mass (eutrophic remodelling). This elevated wall stress was accompanied by an increase in isobaric distensibility. The elastic moduli did not change, which demonstrates that passive elastic element remodelling was limited. There was a substantial alteration in the contractility and spontaneous (myogenic) tone of the segments. The additional contraction generated by 1  $\mu$ M NE was reduced in the DHT-treated segments. Endothelial dilation was diminished in the DHT-treated groups. The present study demonstrated that insulin-induced vascular relaxation of arterioles was diminished. These loss of insulin dependent dilation of arterioles is the vascular form of IR. While increased IR in human PCOS disease and in DHT treated female animals has been described previously, the alteration of the well-known vasodilator activity of insulin was not studied.

Vitamin D<sub>3</sub> is an adjuvant therapy for PCOS, and the cardiovascular protective effect of low-dose vitamin D<sub>3</sub> therapy is widely accepted. In our study vitamin D<sub>3</sub> therapy was demonstrated to reverse systemic IR in our early PCOS model caused by DHT treatment. Vitamin D<sub>3</sub> supplementation entirely corrected IR. Chronic vitamin D<sub>3</sub> treatment restored vascular diameter. Our experiments demonstrate that vitamin D<sub>3</sub> can reverse morphological remodelling, elevation of wall stress and distensibility in a resistance vessel. It improved NE-induced contraction and myogenic tone was further reduced. However, loss of endothelial relaxation was unaffected. It was shown that changes after DHT treatment are partially counteracted by vitamin D<sub>3</sub>. Investigation of these basic mechanisms revealed that the first effect from a hyperandrogenic state on musculocutaneous gracilis arterioles is that NO-dependent relaxation is impaired. However, vitamin D<sub>3</sub> treatment has no effect on the NO-dependent abnormalities; other mechanisms are responsible for compensation. In animals treated with vitamin D<sub>3</sub>, the insulin-evoked relaxation returned to the control level in arterioles. In addition, the resistance artery effects of vitamin D<sub>3</sub> treatment, which improves both the alterations associated with PCOS and IR, had not been investigated yet.

Our results show basic changes in vascular biomechanics in response to DHT treatment; the arteriolar walls become more rigid with increased tangential wall stress compared with the controls. However, the tangential wall stress differences that were detected even in normotensive animals might be considered prehypertensive abnormalities. The enhanced mechanical load of the arteriolar wall is the local factor for the self-perpetuating, vicious cycle of emerging hypertension. For the decreased spontaneous tone observed in the vitamin D<sub>3</sub>-treated group, tangential wall stress is not elevated. This has a local effect against hypertension development. Thus, the mechanical load of the vessels increases. Under passive circumstances, vitamin D<sub>3</sub> treatment fully restores the biomechanical balance of the vessel wall. However, in response to norepinephrine, mechanical load of the vessel wall increases compared with the control group, although it does not reach the level of the DHT-treated group. As a result of the smooth muscle pharmacological reactivity changes, the total adaptation range for skeletal muscle perfusion significantly decreases in response to DHT treatment. Although vitamin D<sub>3</sub> treatment significantly improves this range, it does not reach the level of the control group. In studying the basis for this phenomenon, an obvious, significant decrease in NE response is noted after DHT treatment. This is almost restored by vitamin D<sub>3</sub> treatment. A decrease of relaxation is detected in response to DHT treatment. This decrease is even more significant when DHT is combined with vitamin D<sub>3</sub>. Another interpretation of these findings is that DHT treatment results in diminished adaptive reserve capacity in both directions. Vitamin D<sub>3</sub> applied with DHT significantly improves pharmacological reactivity but decreases the vessels' spontaneous tone even compared with the control.

In our experiments, vitamin D<sub>3</sub> treatment significantly decreased the vascular IR of arterioles caused by DHT. During NE-induced precontraction, DHT treatment decreased insulin-dependent relaxation, thus producing vascular IR. However, similar relaxation was observed in the control and vitamin D<sub>3</sub>-treated groups. In response to insulin, the NE sensitivity of the vitamin D<sub>3</sub>-treated group practically remained unchanged. In the control and DHT-treated groups, a 20% increase in contractility was observed after rinsing and during the second contraction compared with the first norepinephrine contraction. No differences were found between the first and second NE contractions in the vitamin D<sub>3</sub>-treated group, which suggests that vitamin D<sub>3</sub> treatment improves NE sensitivity. However, the most prominent insulin-dependent relaxation was detected in

the vitamin D<sub>3</sub>-treated group. Comparing the insulin-dependent relaxation to ACh relaxation, highly significant differences were noted. ACh caused stronger relaxation in the control group than in the two treated groups. Moreover, this difference remained the same or increased during L-NAME blockade. These results suggest that the relaxing effect of insulin is independent from NO in the gracilis arterioles and that vitamin D<sub>3</sub> restores normal vascular tone independently from the NO system.

## CONCLUSIONS

In this study we used an experimental animal model of PCOS to examine early metabolic and vascular lesions. Our results show that the hyperandrogenic state resulted in more rigid, less flexible artery walls. These arteries show reduced endothelium- and smooth muscle-dependent vasorelaxation and constriction as well as a complete loss of NO-dependent relaxation compared with the controls. In the relaxed vessels, concurrent vitamin D<sub>3</sub> treatment corrected wall mechanics to the control level. Herein, we provide this first data for the effect of vitamin D<sub>3</sub> on the mechanical properties of arteries in PCOS. This is the first study investigating the vascular adaptation and insulin sensitivity of gracilis arterioles in a PCOS model. This is the first study demonstrating vascular insulin resistance of the gracilis arterioles and the counteracting effect of vitamin D<sub>3</sub> in an experimental PCOS model. The observed alterations of resistance arteries form an important prehypertensive pathway of vascular damage in PCOS and might explain the observed clinical effectiveness of vitamin D treatment.

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## LIST OF PUBLICATIONS

**The thesis is written on the basis of the following publications:**

Sara, L., Antal, P., Masszi, G., Buday, A., Horvath, E.M., Hamar, P., Monos, E., Nadasy, G.L., Varbiro, S. (2012) Arteriolar insulin resistance in a rat model of polycystic ovary syndrome. *Fertility and Sterility*. 97(2): 462-468. IF 3,958 (2010)

Sara L., Nadasy Gy.L., Antal P., Monori – Kiss A., Szekeres M., Masszi G., Monos E., Varbiro Sz. (2012) Pharmacological reactivity of resistance vessels in a rat PCOS model – vascular effects of parallel vitamin D3 treatment. *Gynecological Endocrinology* DOI: 10.3109/09513590.2012.683079. Article in press. IF: 1,461 (2010)

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## **Other publications:**

Valent S, Oláh O, Sára L, Pajor A, Langmár Z. (2011) Ultrasonography in the diagnosis of ovarian and endometrial carcinoma [Az ultrahangvizsgálat szerepe a méhtest és a petefészek rosszindulatú daganatainak diagnosztikájában] *Orvosi Hetilap*. 152(47): 1887-1893.

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