Effect of hyperandrogenism and vitamin D deficiency on metabolic parameters and coronary artery function in female rats

PhD thesis

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Introduction

Polycystic ovary syndrome (PCOS) is reported to be the most common endocrine disorder that affects 8- 12% of the world's female population. Excess of androgen activity, oligo-anovulation and/or polycystic ovaries and exclusion of other disease or condition that would cause excess of androgen activity are necessary for the actual diagnosis of PCOS. Cardiometabolic disorders such as and insulin resistance (IR), diabetes, obesity, hypertension and higher prevalence of cardiovascular events are frequent complications. Approximately 75-80% of PCOS women suffer from IR, which is significantly worse than the age- and body mass index -matched female population

Vitamin D deficiency (VDD) affects approximately 30-50% of the world's population. Very low levels of vitD (<20 ng/ml) are reported to be present in 67-85% of PCOS women. Coexistence of IR, VDD and AE in PCOS women has been already described. Women with AE and PCOS have at least two times higher relative risk of coronary heart disease that healthy control. Recently, an inverse relationship was found between vitamin D (VD) serum levels and occurrence of coronary heart disease.

Rodent models are suitable to study PCOS and the parallel effects of VDD and AE on vascular reactivity. According to some previous data, aorta rings of hyperandrogen female PCOS rats showed increased norepinephrine induced contraction and reduced endothelial nitric oxide induced relaxation capacity. Moreover, calcitriol administration was able to improve vascular relaxation capacities of hyperandrogen animals. Nevertheless, in hyperandrogen PCOS more rigid, less adaptive vessels are detected, which is mostly interpreted as the first step towards hypertensive state and vascular remodeling. These negative effects could be diminished if VD supplementation was applied as eutrophic remodeling was less pronounced in rats with AE and PCOS.

Aims:

The aim of our study was to study the early biomechanical and metabolic alterations of coronary resistance arterioles in hyperandrogen PCOS and VDD with the help of a newly introduced chronic rat model.

1. Our aim was to create a new, easily reproducible hyperandrogen PCOS rat model with the use of transdermal testosterone (T) treatment. We wanted to reproduce all the well-known reproductive, metabolic and vascular alterations of PCOS with the help of this model.
2. We aimed to examine the separate and joint impact of VDD and AE on the severity of reproductive and metabolic complications and IR in PCOS.
3. We would like to investigate the typical changes of coronary biomechanics, which could possible put PCOS and VD deficient women at higher risk of CVD. We assumed that in both diseases, IR has a great importance regarding such functional disturbances as reduced dilatation capacity or coronary wall remodeling. It was also relevant for us to show whether there is any kind of interaction between VDD and hyperandrogen PCOS regarding coronary artery disease.

Materials and methods:

Chemicals: Transdermal T gel (Androgel 1% from Lab. Besins International S.A, Paris, France) was used to induce hyperandrogenic state. Cholecalciferol suspension (Vigantol oil 20.000 IU/ml from Merck/Merck Serono12, Mumbai, Maharashtra, India) was applied for the oral vitamin D supplementation. We used normal Krebs-Ringer solution for *in vitro* studies. To produce smooth muscle relaxation, calcium free Krebs solution was applied. Maximal contraction was achieved with U46619 (potent thromboxane A_2 receptor agonist from TOCRIS Bio-Techne, Bristol, UK). Adenosine (Adenocor, Sanofi-Aventis, Madrid, Spain) was used as potent vasorelaxant. Human-recombinant insulin (Actrapid pentafill 100 IU/ml from Novo Nordisk, Copenhagen, Denmark) was used for *in vitro* vascular tests.

For anesthesia, 45 mg/kg intraperitoneal Nembutal (Phylaxia-Sanofi, Budapest, Hungary) was used at the end of the study procedure.

Animals. 46 adolescent (21-28 day-old), female Wistar rats, weighing 90-110 grams were provided by the Animal Facility of Semmelweis University in agreement with Charles River. Animals were supplied ad libitum with tap water and with normal or VD deficient food (see below). No medical or toxic complication was observed during the 8-week-treatment period. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals*

published by the US National Institutes of Health (8th edition, 2011) and the EU conform Hungarian Law on Animal Care (XXVIII/1998). The institutional Animal Care Commission and Hungarian authorities accepted the protocol (PEI/001/820-2/2015).

Chronic treatment. Rats were randomly selected into 4 groups and treated for 8 weeks. Body weight was measured 5 times a week during the protocol. Body mass gain ratio (%) was calculated: final bodyweight/initial bodyweight *100%. Twenty-four animals received complete, normal diet (Ssniff Germany –SM Rat/mouse complete diet containing 1000 IU/kg vitamin D_3), from which 12 animals were treated with bodyweight adapted transdermal T 5 times a week. To ensure optimal VD serum levels (serum 25-hydroxicholecalciferol of 30ng/ml), bodyweight adapted oral VD supplementation was administered from the 2nd week. Oral cholecalciferol administration was as follows: at the beginning of the 2nd week, 500 IU Vigantol oil was used per os as a loading dose. From the 4th week, cholecalciferol was provided weekly up to 3000 NE/kg body weight to reach higher range normal vitD serum levels.

Twenty two animals were put on VD free diet (Ssniff Germany - EF Rat/mouse complete VD free diet: containing vitamin D_3 < 5 IU/kg). Additional VD intake was excluded. Half of VD deficient animals (n=11) were treated with transdermal T as well.

Testosterone gel was applied 5-times a week from the 2nd day of treatment. The dose was 0.033mg/g body weight, which ensured close to 10 times elevation in serum T levels in treated female animals (see Table 1). Back skin was regularly shaved before transdermal T treatment.

Oral glucose tolerance test (OGTT) and homeostatic assessment for insulin resistance (HOMA-IR). Oral glucose tolerance test was performed on the 6th week of treatment. After overnight fasting, a loading dose of per os 30% glucose solution (2g/ bodyweight kg glucose) was administered through a gauge. Blood sugar levels were measured at 0’ - 60’ - 120’, whilst insulin levels at 0’-120’ were detected by enzyme linked immunosorbent assay (Merck/Merck Millipore, Darmstadt, Germany/ Budapest, Hungary). HOMA-IR was calculated as fasting plasma insulin (in milliunits per liter) × fasting plasma glucose (in millimoles per liter)/22.5.

Sexual steroid, VD and leptin levels. Blood samples were taken on the 6th and 8th week of treatment from tail vein. Serum samples were obtained and 5-dihydrotestosterone, 5-

hydroxycholecalciferol, progesterone and T levels were analyzed with high performance liquid chromatography. Leptin levels were measured at 8th week of treatment with ELISA (Phoenix Pharmaceuticals/ Phoenix Europe GmbH, Karlsruhe, Germany).

Vaginal smear and ovarian morphology. Starting from the 6th week, daily vaginal smear examination was performed to assess the ovulatory cycle changes. Ovaries were collected at the end of the protocol for histological examination. After measuring their weight, they were fixed in formaldehyde solution and stained with hematoxylin –eosin. Representative samples of each group (n=6) was analyzed to detect polycystic ovarian morphology. The total number, the mean diameter and the total area of follicles and corpora lutea and the total area of ovarium were measured with AxioVision Panoramic Viewer software (3DHISTECH Ltd., Budapest, Hungary).

Transthoracic echocardiography and invasive arterial blood pressure measurement. Both examinations were performed at the 8th week of treatment under pentobarbital anesthesia. Long-axis B-mode ultrasound images of the left ventricle were obtained to calculate end-diastolic volume (EDV) and end-systolic volume (ESV) from left ventricular area (LVA) and left ventricular length (LVL) as $8(LVAd)^2/3\pi LVLd$ and $8(LVAs)^2/3\pi LVLs$, respectively (in the formulae d and s stand for diastole and systole, respectively). Arterial blood pressure was measured through the aseptic cannulation of an internal carotid artery.

Microangiometry. On the 8th week of treatment, animals were anaesthetized. The chest was opened in order to extract the heart and its weight was measured. It was perfused with non-heparinized normal Krebs-solution for 2 minutes, which was followed by micropreparation of a coronary arteriole segment from the intramural network of the left anterior descendent coronary artery with *in vivo* outer diameter of 100-150 micrometer. The coronary arteriole was microcannulated in normal Krebs solution in an organ chamber filled with saline and oxygenized at fixed 37 ˚C temperatures. The microcannulas were connected to the servo pumps (Living Systems, Burlington, VT, USA). Under no-flow conditions, the arteriole was intraluminal pressurized at 50 mmHg and extended to its normal, *in vivo* length. The setup was positioned in the light route of an inversed Leica microscope to allow the evaluation of inner and outer diameter changes of the arteriole. With the aid of a digital histologic Leica video camera (DFC 320) and Leica QWin software, magnified pictures of the

arteriole were obtained. Analysis of the vessel pictures was performed off-line with the help of Leica QWin image analyzing program.

The arteriole was allowed to equilibrate in oxygenized normal Krebs solution at fixed pressure (50mmHg) and temperature (37°C) for 30 minutes. Their steady-state diameter was measured in this state. Pressure-diameter profile curves were obtained by training (0-150-0-150 mmHg intraluminal pressure) and fraction elevation (10 MmHg) of the intraluminal pressure from 0 mmHg to 150 mmHg. In the end of 10 minutes incubation on 50 mmHg, the resting diameter intraluminal pressure was re-measured. As the next step, increasing dose of insulin (concentration of 30-100-300-600 mIU/ml; 1IU= 0.035mg insulin) was added to the bath and segments were incubated for 8 minutes, respectively. After registration each dose-response, the drug was washed out with slow continuous flow of oxygenized and heated normal Krebs solution. The vasoconstrictor properties of the vessel, was tested by a single high dose (10^{-6}M) of thromboxane A_2 receptor agonist (U46619). After 5 minutes of incubation, the intraluminal pressure was gradually increased with 10 mmHg from 0 to 150 mmHg and alteration in inner and outer diameter was registered. After resting 10 minutes on 50 mmHg, the inner and outer diameters were recorded again. Without washing out U46619, elevating dose of adenosine (10^{-9}M, 10^{-8}M, 10^{-7}M, 10^{-6}M), a potent coronary-relaxant agent was added to the organ bath. Each dose was equilibrated for 3 minute-period and changes in diameter registered. The maximal smooth muscle relaxant potential of the vessel was evaluated by changing the organ chamber's solution to calcium free Krebs solution, which was likewise heated and oxygenized. After 20 minutes of incubation, the passive biomechanical properties of the vessel were examined by the changes of inner and outer diameter to gradual intraluminal pressure elevation (0 MmHg to 150 MmHg). We used the Wild micrometer ethalon for calibration.

Biomechanical calculations. Full contraction of the segment (T_{Full}) is $T_{Full}= 100*(R_{cafree}-R_{U46619})/ R_{cafree}$ (%), where R_{cafree} is the radius measured in calcium free solution and R_{U46619} is the measured radius if TXA_2 agonist was added to the bath. Spontaneous (myogenic) tone was computed as follows: $T_{nKR}= 100*(R_{cafree}-R_{nKR})/ R_{cafree}$ (%), where R_{nKR} is the radius of the coronary segment in normal Krebs solution. Adenosine induced relaxation was calculated with the help of the radius parameter (R_{Ade}), measured after elevating concentration of adenosine was applied: $T_{Ade}= 100*(R_{Ade}-R_{u46619})/ R_{cafree}$ (%).Tangential stress was calculated according to the Laplace equation: $Tg_{stress}= P*R_{i\ nKR}/ h_{nKR}$, where Tg_{stress} is the tangential (circumferential) wall stress, P is the intraluminal pressure, Ri is the

inner radius and h_{nKR} is the wall thickness in normal Krebs solution Wall thickness was calculated as follows: $h= R_o-R_i$, where R_o is the outer radius. The circumferential incremental elastic modulus was computed with the following equation: $E_{inc}= (dP/dR_o)x\ 2(R_i\ x\ R_o^2)/\ (R_o^2-R_i^2)$ where E_{inc} is the incremental elastic modulus and dR_o is the change in outer radius in response to intraluminal pressure change of dP. Cross sectional area was calculated with the help of inner and outer radius in calcium free solution: $A_{cs}=(\ R_{o\ cafree}^2\ -\ R_{i\ cafree}^2)*\pi$. The remaining tone in insulin was expressed as percent of actual radius as percent of passive (fully relaxed) radius: $T_{Ins}= 100x\ (R_{cafree}-R_{Ins})/\ R_{cafree}$ (%), where R_{Ins} is the radius of the coronary arteriole, if insulin was added to the organ chamber.

Elastic fibre density of the vessel. Neighbouring segment of the examined coronary arteriole was taken for histological examination, fixed with formaldehyde and stained with haematoxylin-eosin and resorcin-fuchsine. Microscopic pictures and morphometric were taken and elastic fibre density measurements were made on scanned sections (Panoramic Viewer, 3DHISTECH Ltd., Budapest, Hungary) under identical conditions. As the magenta color of the RF stain suppresses the green color, RGB green intensities (0-255) were measured in the radial direction starting at the endothelial luminal surface.

Insulin and VD receptor (VDR) density of the vessel. Paraffin embedded tissue sections were stained against insulin receptor beta and VDR by BenchMark ULTRA Automated IHC/ISH slide staining system (Ventana Medical Systems, Inc., Tucson, AZ, USA) using monoclonal mouse anti insulin receptor beta (Santa Cruz Biotechnology, Dallas, TX, USA) and polyclonal rabbit anti VDR (Abcam, Cambridge, UK) antibodies. Visualization of specific labeling with diaminobenzidine (DAB) as colored substrate and hematoxylin counterstaining was achieved by UltraView Universal DAB Detection Kit (Ventana Medical Systems, Inc., Oro Valley, Arizona, USA). Microscopic images of the stained vessels were taken by Zeiss Axio Imager system (Zeiss, Oberkochen, Germany). Positively stained area as the percentage of total tissue area was measured in the intimal and medial layers of the vessel walls using the ImageJ software (NIH, Bethesda, Maryland, USA).

Statistical analysis. For statistical analysis GraphPad Prism 6.0 (GraphPad Software, Inc. San Diego, California, USA) was used. Two-way repeated-measures analysis of variance (ANOVA) was used for the statistical analysis of the curves (eg. cumulative concentration-diameter curve). Discrete parameters (eg. bodyweight) were compared with one-way ANOVA. Tukey test was used as a post hoc test and $p<0.05$ was uniformly accepted as the

threshold for statistical significance. Data are shown as mean ± SEM (standard error of the mean).

Results:

Phenotypical changes and serum hormone levels. At the 8th week of the protocol, bodyweight of transdermal testosterone treated animals (VD+/T+ 327.8 ±8.3 g; VD-/T+ 327.8 ±8.3 g) was significantly higher than of non-treated ones (VD+/T- 282.5 ±6.2 g and VD-/T- 296.9± 3.9 grams; $p<0.01$, respectively).

Vitamin D deficient diet produced about 5-times lower serum 25-hydroxicholecalciferol levels compared to the supplemented groups ($p<0.01$). Oral VD supplementation excluded VDD in the treated groups. Significantly elevated serum T (VD+/T- 0.31±0.16, VD-/T- 0.72±0.16, VD+/T+ 4.29±0.56 and VD-/T+ 5.50±0.56 ng/ml; $p<0.01$, respectively) and its active metabolite, 5-DHT (VD+/T- 0.1±0.01, VD-/T- 0.12±0.02, VD+/T+ 0.62±0.14 and VD-/T+ 0.60±0.16 ng/ml; $p<0.05$, respectively) were detected in transdermal T treated animals. Vitamin D supplemented, T treated animals had significantly elevated leptin levels, compared to the "double-control" ($p<0.05$).

PCOS morphology (high amount of small, primordial follicle, missing or low number of corpus luteum) and irregular ovulation pattern could be observed not only in T treated animals, but in VDD too. Dominant follicles and corpus luteum were detectable only in "double-control" rats. In non-T treated, VD supplemented females 3,64±0.15 intact estrus cycles were observed during the last 14 days of the treatment. In VDD, without T treatment significantly lower cycle number (2,00±0.23, $p<0.05$) was detected. Transdermal T treatment led to dramatic cycle reduction compared to the "double-control" (VD+/T- 0.90±0.21, VD-/T- 0.36±0.2; $p<0.01$ in both comparisons).

Transthoracic echocardiography and invasive arterial blood pressure. There was no significant difference in blood pressure values, left ventricle end diastolic volume, ejection fraction or other parameters sensitive to the left ventricular hypertrophic transformation (IVSD and EDV).

Geometry of the coronary arterioles and control of the smooth muscle tone. In VDD groups, significantly narrower passive lumina and lower inner radii were registered,

which was accompanied by higher wall thickness values ($p<0.01$ in all comparisons to VD+ groups, Fig. 1/A). The significant effect of VD availability on the morphological modeling of coronary arteriole is also shown by alterations in cross sectional area results (amount of wall material). While it was the highest in "double control" VD supplemented T -free rats, 20.1 ± 3.4 $10^3 \mu m^2$, lower values, 11.4 ± 1.05 and 10.9 ± 0.91 10^3 μm^2 were observed in the T -free and T treated VD deficient groups. Independently of VD supplementation, T treatment reduced cross sectional area results (to 14.3 ± 1.57 10^3 μm^2, $p<0.01$ in all these comparisons).

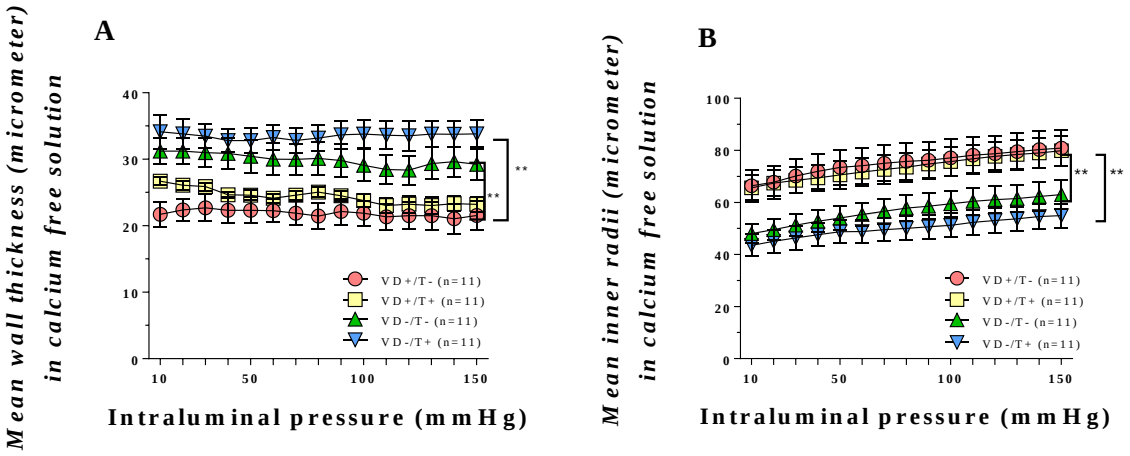

Figure 1. Geometrical properties of intramural coronary resistance arteries from VD supplemented and VD deficient, testosterone treated and untreated female rats. A. Wall thickness as a function of intraluminal pressure in calcium free solution (full relaxation). Wall thickness values of both VD- groups are significantly higher (** - $p<0.01$, in both comparison, respectively) than VD+ groups. **B**. Inner radius in the fully relaxed state as a function of intraluminal pressure. Here again, VDD caused significantly lower inner radii values in both groups, compared to VD+ groups **- $p<0.01$, in both comparison, respectively). These results suggest, that coronary arterioles had narrower lumina in VDD, regardless to T treatment.

Alone or in combination, VDD and T treatment resulted significant reduction of the myogenic tone compared to the "double-control" (Figure 2/A, $p< 0.01$, respectively). Additional substantial contraction was induced by the potent TXA_2 agonist, U46619 (1μmol/lit at 50 mmHg). In "double-control" (VD+/T-), 54.6±3.2% of inner diameter contraction was recorded and T treatment did not affect the substantial contraction capacity (VD+/T+: 54.1±4.1%). Developed tone was significantly reduced in VDD: 26.7± 4.7% in VD-/T- and 34.8 ± 1.9% in VD-/T+ groups, respectively ($p<0.01$ in comparisons of VD+ and VD- groups). Afterwards, adenosine was used to induce smooth muscle relaxation. Our "double control" segments produced significantly better relaxations than the "double noxa" segments. ($p<0.01$, Fig. 2/B)

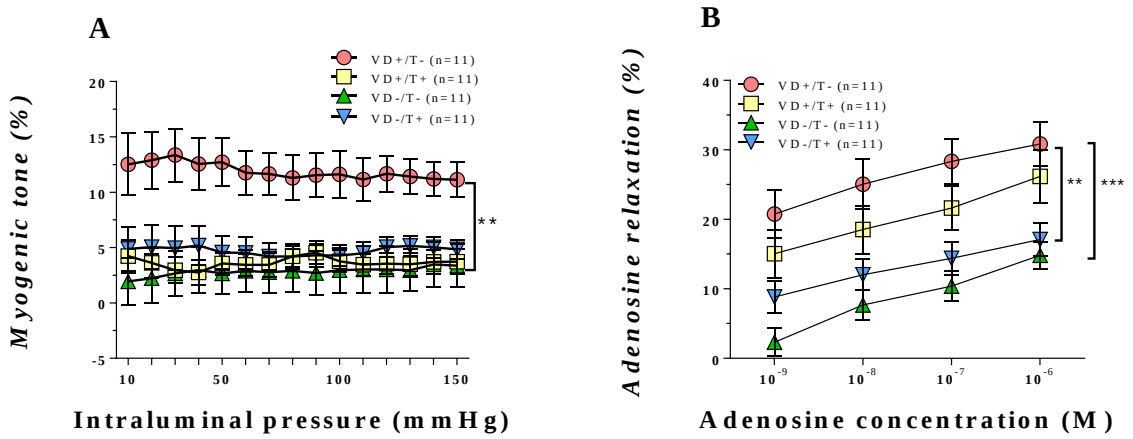

Figure 2. Contractile and relaxation properties of intramural coronary resistance arteries. A. Myogenic tone in nKR as a function of intraluminal pressure. In the presence of any of the applied noxa, significant myogenic tone reduction was calculated compared to the noxa free (VD+/T-) group (**- $p < 0.01$). **B.** Relaxation induced by increasing concentrations of adenosine at 50 mmHg pressure. Vitamin D deficiency alone (**- $p < 0.01$) or in combination with T treatment (*** - $p < 0.001$), resulted diminished adenosine induced relaxation of the arterioles, compared to the "double-control" group. Significances of ANOVA tests are shown. All values are expressed in mean ±SEM.

Elasticity of the coronary arteriole. Tangential wall stress was significantly diminished in both VD deficient groups compared to VD supplemented groups, regardless to T treatment ($p<0.001$ above 50mmHg intraluminal pressure, respectively). Tangential elastic modulus was plotted against tangential wall stress to judge the elasticity of wall material independently from geometrical properties. At 25 kPa (high stress), significantly reduced elastic modulus was found in VD-/T- and VD-/T+ groups compared to VD+/T- group $p<0.001$) and VD+/T+ ($p<0.01$ and $p<0.05$). Elastic modulus was plotted against intraluminal pressure too (Fig 3/C). On low pressure ranges, all three treated groups differed significantly from VD+/T- group ($p<0.05$ in all three comparisons). Increase in intraluminal pressure led to further significant value reduction in case of VD deficient, transdermal T treated group.

In the vessel wall of "double control" animals (VD+/T-) significantly less amount of elastic components was detected (compared to all other groups, $p< 0.05$).

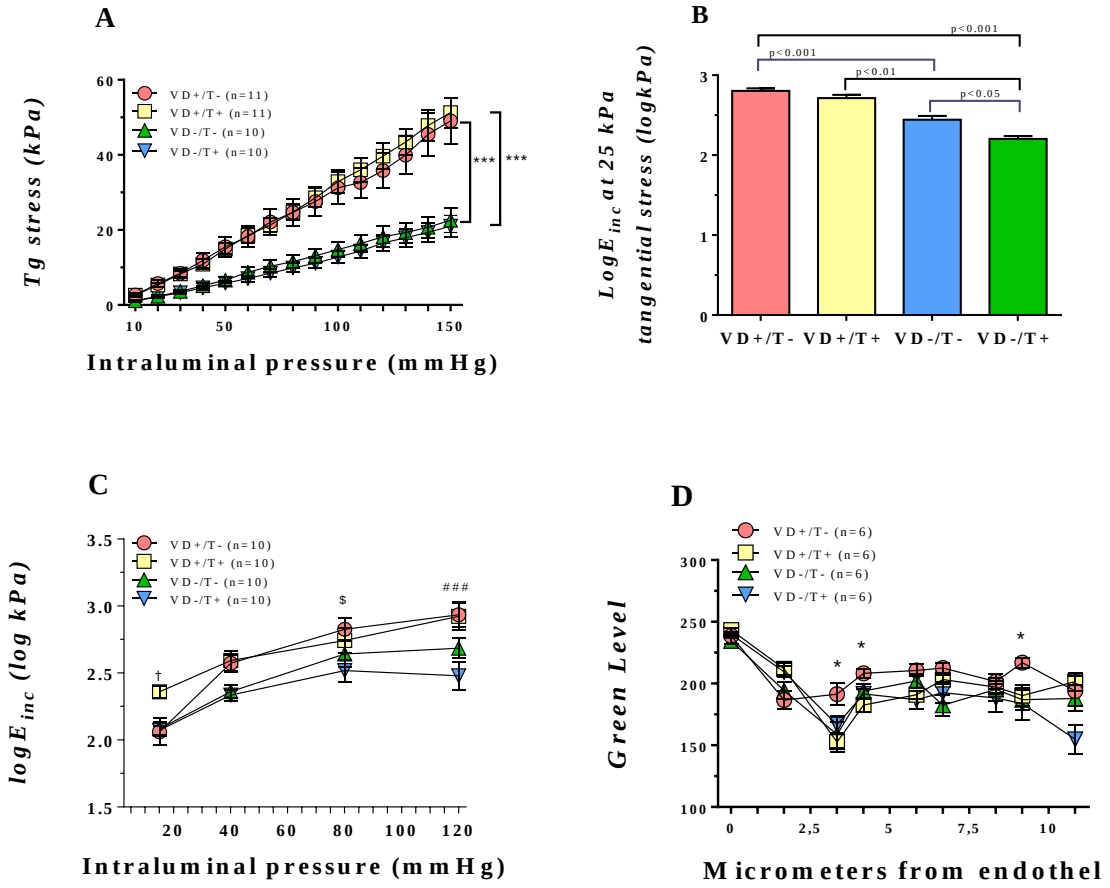

Figure 3. Tangential wall stress in normal Krebs solution and elastic properties of coronary arterioles A. Tangential wall stress in normal Krebs solution. Above 50mmHg intraluminar pressure, Both VD- groups differ significantly from both VD+ groups, respectively ($p<0.001$ in all comparison). **B.** Incremental elastic moduli ($logE_{inc}$) at 25 kPa tangential stress. Both VD- groups had reduced incremental elastic moduli values, compared to both VD+ groups. Significances of ANOVA tests are shown with numbers. **C.** Elastic modulus as a function of intraluminal pressure. On low pressure ranges, all three treated groups differed significantly from the "double control" ($p<0.05$ in all three comparisons). On higher intraluminal pressure ranges, VD-/T+ group showed further significant reduction compared to VD+ groups ($p<0.001$, respectively).

Evaluation of insulin resistance. Before OGTT, no significant difference could be detected in fasting glucose and insulin levels among the four groups. The 60-minute postload blood sugar levels of VD supplemented, T treated rats were significantly higher than of the "double control" ($p<0.05$, Fig. 4/A). This elevation was significant in the 120-minutes postload values too and was detectable in both T treated groups (Fig. 4/A, in comparison to both T –free groups, $p<0.05$ and $p<0.01$, respectively). The 120-minutes postload plasma insulin values of VD deficient, T-free group were significantly higher than the "double

control" counterparts (Fig. 4/B, $p<0.05$). Computed HOMA IR (insulin resistance) results of both VD deficient groups were significantly higher ($p<0.05$), compared to the "double control" (Fig. 4/C). In case of insulin induced relaxation of the coronary arterioles, one of the two noxa was enough to significantly reduce insulin induced relaxation ($p<0.01$ in all three groups, compared to "double control" animals, Fig. 4/D). Furthermore, on higher insulin concentration no relaxation could be observed at all.

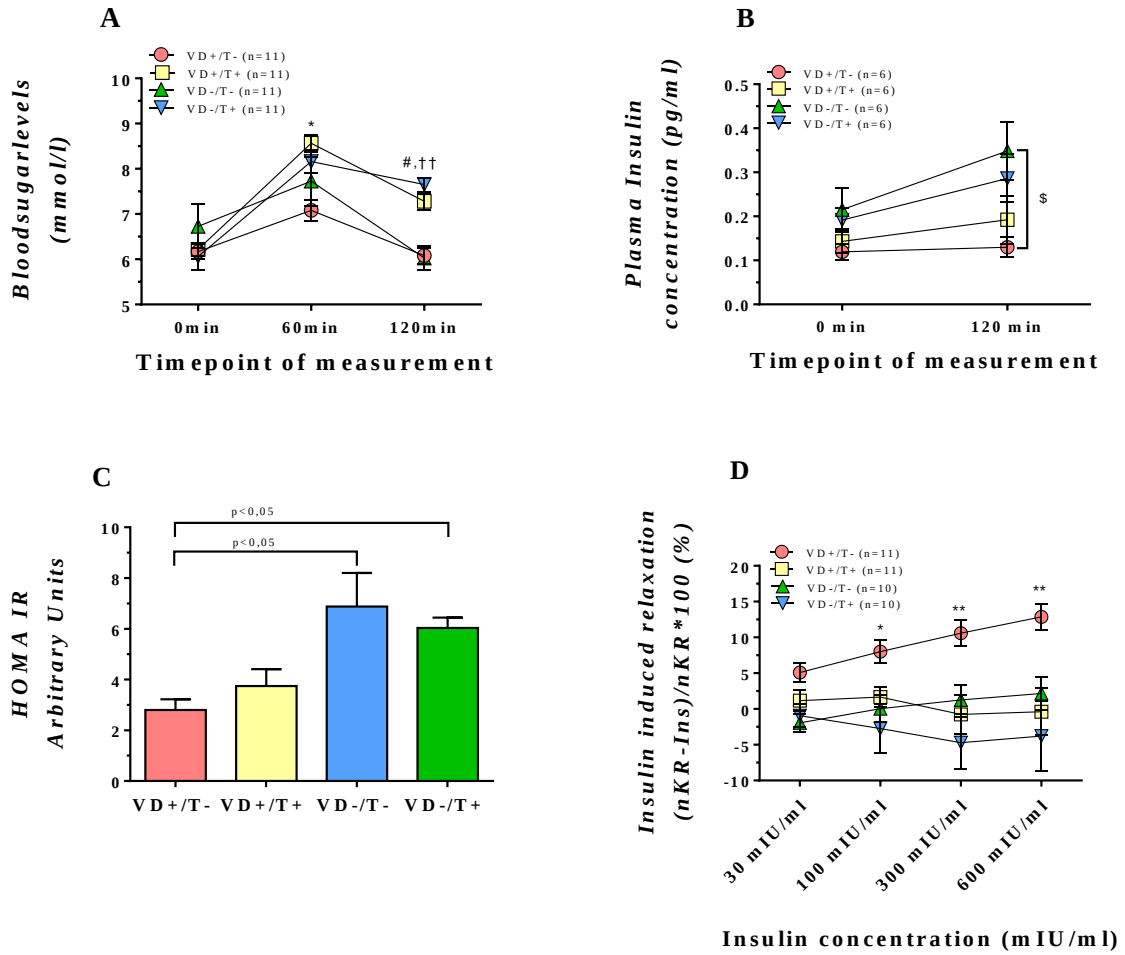

Figure 4. Results of OGTT and insulin induced relaxation of the coronary arterioles. A. Plasma glucose levels of the four groups following OGTT at the 6th week of treatment, n=11 for each group. **B.** Plasma insulin levels following OGTT, n=6. **C.** Calculated HOMA IR values, n=11 for each group. **D.** Insulin induced relaxation, n= 11. Both T+ groups had significantly elevated "120 minutes" blood sugar levels compared to T- groups ($p<0.05$), however these results were not accompanied by significant plasma insulin concentration changes. VD-/T- group had significantly higher "120 minutes plasma insulin concentration compared to VD+/T- group. HOMA IR results revealed that both VD- groups were insulin resistant.

Insulin and VD receptor (VDR) density of the coronary arterioles. Higher insulin receptor ($p<0.001$ in all VD supplemented comparisons) and VDR density was found in both VD deficient groups ($p<0.01$ and 0.001, in all VD supplemented comparisons).

Discussion:

Fenotypical changes. At the end of our study protocol, markedly elevated serum T, and its active metabolite, DHT levels were measured in treated animals. Untreated animals kept normal sex steroid levels, which also proved the efficiency of our transdermal testosterone regime. Hyperandrogen female rats developed phenotypic changes of PCOS as well, including higher bodyweight and body mass gain ratio, similar to the ones in human PCOS. Goal VD status was achieved in specific groups: oral VD supplementation ensured high normal levels meanwhile VD deficient nutrition resulted in VDD. According to various human studies in adult PCOS women, we concluded that our study succeeded to reproduce similar range of VD serum levels in rodents.

Transdermal T treated female rats have failed to develop regular estrus cycles, which entailed both low follicle rate and remarkably reduced number of corpus luteum. We detected, that VD deficient animals without hyperandrogenism had irregular estrus cycle patterns, elevated follicle and reduced number of corpus luteum. These findings could be explained by the multiple regulatory role of VD in ovarian follicular development and luteinisation. As VD affects signal transduction of Anti-Müllerian hormone, follicle stimulating hormone sensitivity and progesterone production of granulosa cells, PCOS-like ovarian morphology and ovulation disturbances are often observed in VDD.

Biomechanical properties of the arterioles. We observed, that not only AE (transdermal T treatment), but chronic VD withdrawal alone resulted fundamental changes in arteriolar geometry. Smaller inner radii and bigger wall thickness was observed in both VD deficient groups. In VDD, vascular remodeling is triggered by migration of leukocytes into the vessel wall, early vessel wall calcification and activation of endothelial cells and pericytes. The observed AE induced hypotropic remodeling might be based on the activation of different matrix metalloprotease subtypes and VSMC migration rate.

Thromboxane A2 agonist induced maximum contraction and adenosine induced vasodilation were also decreased in the presence of VDD (Fig. 2/B), which was partially

restored by VD supplementation in AE. VDD attenuated the adaption capacity of coronary arterioles; however T was responsible for mainly relaxation capacity changes. We conclude that both noxa deteriorate the vessel's capability to adjust local ventricular flow to higher metabolic demand. The long term consequences of such vascular tone disturbances are alteration in myocardium trophism and higher CV risk.

Elastic properties of the coronary arterioles are useful indicators to evaluate possible vascular remodeling. In both VD deficient groups elastic modulus parameters were reduced at the same tangential wall stress (on high wall shear stress value: 25kPa). Our elastica staining results correlated well with the changes in vessel biomechanics, while corresponding significant alterations were found under the above mentioned circumstances.

Different mechanism of insulin resistance: Although there was no difference between fasting glucose values in our study, transdermal T treatment led to elevated blood sugar levels (60min and 120min results), which was not followed by greater insulin secretion. In VDD, higher insulin output was recorded, however glucose levels remained unchanged. These observations suggest that two different ways of IR could be present at one time. In the case of T treatment serum insulin levels were unable to avoid developing hyperglycemia. This effect could be due to decreased beta-cell sensitivity and lead to worsening of hyperglycemia. If VDD was present, higher insulin levels could be able to normalize blood glucose levels. Both groups of VDD had high HOMA IR values If serum levels of VD were optimal, HOMA IR results of T treated animals were unaltered compared to transdermal T -free ones. In combination of the two noxa, marked elevation of plasma glucose levels were recorded, despite higher 120 min insulin levels. In "double noxa" group.

Only one of the two noxa was enough to induce dysfunctional of insulin induced relaxation. Only VD supplemented and T free animals showed normal relaxation profile. Normally, in the vasculature activation of insulin receptors could keep balance between phosphatidyl-inositol 3-kinase (PI3-K) and mitogen activated protein kinase (MAPK) dependent pathways. The PI3-K pathway regulates endothelial NO production and MAPK dependent one trigger the secretion of the vasoconstrictor ET-1. Possible inhibition of the PI3-K might lead to increased ET-1 production and vasoconstriction.

Conclusions:

We studied the metabolic and vascular effects of AE with and without VD deficiency in a rat model of PCOS. We also investigated the cardiometabolic effects of VD deficiency without additional AE.

- ***With 8-weeks of transdermal T treatment and VD –free diet, we were able to create a hyperandrogen and vitamin D deficient PCOS rat model:***

  The applied transdermal T treatment successfully produced the phenotypical changes, required for the diagnosis of PCOS. However VD supplementation in rats with AE, has partially restored ovulatory patterns, polycytic ovary morphology was still present. Although VDD alone did not affect the measured sexual steroid serum levels, typical changes of ovarian morphology were present, just like in the oligo-anovulatory, polycystic PCOS phenotype without AE. Moreover VDD in combination with AE produced the lowest estrus cycle rates, which also highlights the importance of VD supplementation in hyperandrogen PCOS women with history of conceiving problems.

- ***Major metabolic changes of VDD and AE could be observed at the same time:***

  Our study revealed that both factors (in combination or alone) were able to induce the major symptoms of metabolic syndrome as well as IR. We concluded that AE had much important role in body weight changes, which is supported by the fact that 75% obese PCOS patient suffer from AE too. Neither VD supplementation, nor VDD had any relevant impact on body weight gain ratio in our study. According to some findings, which showed that body mass index has an inverse correlation with circulating VD levels, it is tempting to speculate that VD insufficiency might be responsible for the development of dysfunctional adipose tissue and obesity.

- ***Vitamin D deficiency and T treatment induced a morphological and elastic remodeling of the wall:***

  Narrower passive inner lumina and higher wall thickness were detected in VD deficiency however the presence of T treatment even worsened wall properties. Although, we speculated earlier, that testosterone treatment would provoke wall hypertropism, our findings confirmed the opposite on coronary arterioles. The contractile-relaxation range of the arterioles was also reduced by VDD and AE. Damaged vascular adaptation range has negative impact on ventricular tissue blood flow if sudden adjustment is need to altered metabolic needs. Reduced spontaneous tone

and increased wall rigidity were both characteristics of VDD and T treatment. Regarding maximal relaxation capacity, the presence of both noxa led to the most disadvantageous changes. Alteration of the vessel's elastic components was observed in case of both VDD and AE, which also support the existence of early wall remodeling.

- ***Vitamin D deficiency and AE provoked IR with different mechanisms:***

  In case of T treatment and VD supplementation, AE elevated postprandial sugar, but generated neither hyperinsulinemia, nor HOMA IR elevation. Insulin induced relaxation of the coronary arterioles was also diminished despite unaltered insulin receptor expression. Nevertheless, VDD regardless to T treatment resulted higher postprandial insulin concentrations, elevated HOMA IR values and reduced insulin induced vascular response. Moreover, in case of VD elevated number of vascular insulin receptors was detected in both the endothelial and media layer of the vessel wall. This type of receptor number alteration was also measurable in VDR amount. Therefore we conclude that there might be two different ways for IR, which together significantly worsen the vascular response to insulin.

Our results confirmed the fact, that both AE and VD could provoke undesirable vascular and metabolic alterations on the coronary resistance arteries. We must also admit, that this study is aimed to demonstrate the early changes, hence possible long term interactions should be further considered. Our findings fit the previous reports, which suggested the importance of treating both AE and VDD in PCOS in order to minimize prevalence of metabolic and vascular complications.

List of Publication:

The thesis is based on the following publications:

1. **Hadjadj L**, Monori-Kiss A, Horváth EM, Heinzlmann A, Magyar A, Sziva RE, Miklós Z, Pál É, Gál J, Szabó I, Benyó Z, Nádasy GL, Várbíró S. (2019) Geometric, elastic and contractile-relaxation changes in coronary arterioles induced by Vitamin D deficiency in normal and hyperandrogenic female rats. Microvasc Res, 122: 78–84 IF: 2.604

2. **Hadjadj L,** Várbíró S, Horváth EM, Monori-Kiss A, Pál É, Karvaly GB, Heinzlmann A, Magyar A, Szabó I, Sziva RE, Benyó Z, Buday M, and Nádasy GL. (2018) Insulin resistance in an animal model of polycystic ovary disease is aggravated by vitamin D deficiency: Vascular consequences. Diab Vasc Dis Res, 15(4): 294-301 IF: 2.357

Other publications:

Hadjadj L, Pál É, Monori-Kiss A, Sziva RE, Korsós-Novák Á, Horváth EM, Benkő R, Magyar A, Magyar P, Benyó Z, Nádasy GL and Várbíró S. (2019) Vitamin D deficiency and androgen excess result eutrophic remodeling and reduced myogenic adaptation in small cerebral arterioles in female rats. Gynecol Endocrinol, 35(6): 529–534. IF: 1.406

Lajtai K, Nagy CT, Tarszabó R, Benkő R, **Hadjadj L**, Sziva RE, Gerszi D, Bányai B, Ferdinandy P, Nádasy GL, Giricz Z, Horváth EM, Várbíró S. (2019) Effects of vitamin D deficiency on proliferation and autophagy of ovarian and liver tissues in a rat model of polycystic ovary syndrome. Biomolecules, 9(9): 471-485. IF: 4.694

Pál É*, **Hadjadj L***, Fontányi Z, Monori-Kiss A, Lippai N, Horváth EM, Magyar A, Horváth E, Monos E, Nadasy GL, Benyó Z, Várbíró S. (2019) Gender, hyperandrogenism and vitamin D deficiency related functional and morphological alterations of rat cerebral arteries. PLoS One, 14(5): e0216951: 1-13. IF: 2.776

Pál É, **Hadjadj L**, Fontányi Z; Monori-Kiss A; Mezei Z; Lippai N; Magyar A; Heinzlmann A, Karvaly GB, Monos, E, Nádasy GL, Várbíró S. (2018) Vitamin D deficiency causes inward hypertrophic remodeling and alters vascular reactivity of rat cerebral arterioles. PLoS One, 13(2): e0192480, 1-16. IF: 2.776

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