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# **Influence of Vitamin D status on the vasoactive effects of aorta rings in a rat model of polycystic ovary syndrome**

**PhD thesis**

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## LIST OF ABBREVIATIONS

ABCA 1 - ATP-binding cassette transporter 1  
Ach – acetylcholine  
ACTH - adrenocorticotropic hormone  
AMH – anti – Müllerian hormone  
COX-2 - cyclooxygenase 2  
DHEA – dehydroepiandrosterone  
DHEA-S – dehydroepiandrosterone sulphate  
DHT – dihydrotestosterone  
eNOS – endothelial nitric oxide synthase  
ER $\alpha$  – estradiol receptor  $\alpha$   
EDHF - endothelium-derived hyperpolarizing factor  
FSH - follicle stimulating hormone  
GnRH - Gonadotropin-releasing hormone  
GPER - G Protein-coupled Estradiol Receptor  
HOMA -IR- Homeostasis Model Assessment - Insulin Resistance  
IR - insulin resistance  
MAPK - mitogen-activated protein kinase  
L-NAME - L-NG-nitro-arginine methyl ester  
LH – luteinizing hormone  
NE – norepinephrine  
NIH - National Institute of Health  
PCOS – polycystic ovary syndrome  
PLA2 - phospholipase A2  
PM - pressure myography  
VDD – vitamin D deficient  
VDRE - Vitamin D response element  
VSM - vascular smooth muscle

# 1. INTRODUCTION

## 1.1. Polycystic ovary syndrome (PCOS)

### 1.1.1. Definition and diagnostic criterions

The first definition for PCOS was given by the NIH (National Institute of Health) in 1990. According to this definition two symptoms are needed for the diagnosis: anovulation and clinical or biochemical signs of hyperandrogenism. This definition did not take ovarium morphology into consideration. To complete this definition another consensus was accepted in Rotterdam, in 2003, which contained three criteria where two of them need to be fulfilled: oligo – anovulation, the clinical or biochemical signs of hyperandrogenism, or polycystic ovarian morphology (1). Naturally, other diseases which are associated with hyperandrogenism, must be excluded. Most studies apply the Rotterdam criteria.

### 1.1.2. Epidemiology

The prevalence of PCOS is 8 – 13% in women of reproductive age, and 6% in adolescent girls (2). Although the number of the cases depend on race, ethnicity and diagnostic criteria. A study made in India reported about a prevalence of 3.7 – 22.5 % depending on the diagnostic criteria used (3). In another example three studies from different countries were compared: the Mexican – American project showed a 13% prevalence, an Australian study showed an even higher number (15,3%), while in China a lower (2.2%) prevalence was reported (4). In Hungary about half a million women may be affected with PCOS (5).

### 1.1.3. The pathomechanism of PCOS

#### *Changes concerning menarche*

The maturation of the adrenal cortex (adrenarche) starts at girls at the age of 6 – 8, and it is marked by rise in the levels of DHEA (dehydroepiandrosterone), DHEA-S (dehydroepiandrosterone sulphate) and androstendion. It seems, that this is a key moment before the gonadarche. Due to several common properties, puberty seems to be a PCOS

– like status: adolescent girls have hyperinsulinemia, hyperandrogenism and frequently anovulatory cycles. The connection between gonadarche and the androgen level of the adrenal cortex shows that menarche starts later in girls with Addison ‘s– disease (5).

### *Hyperandrogenism*

Hyperandrogenism is a major contributor to polycystic ovary syndrome in the majority of instances. But the underlying reason for the hyperandrogenism may be different. One theory involves increased cortisol degradation by the  $5\alpha$  – reductase enzyme or decreased  $11\beta$  – hydroxysteroid dehydrogenase activation. Both of them lead to lower cortisol serum levels, and a consecutive increase in the production of ACTH (adrenocorticotrophic hormone) (6, 7).

It is well known, that the appropriate rhythm of the excretion of GnRH (Gonadotropin-releasing hormone) is necessary for the physiological function of the hypophysis (8). The increased GnRH pulsatility promotes the excretion of LH (luteinizing hormone) and inhibits FSH (follicle stimulating hormone) secretion. The increased LH/FSH ratio inhibits the genesis of the follicles, elevates excreted androgen levels in the ovarium, and even leads to typical lab test anomalies of PCOS. LH enhances both oestrogens and androgens, but testosterone inhibits the sensitivity of the hypophysis regarding negative feedback. Lower estradiol levels inhibit LH and FSH secretion (the FSH more specifically) (9), while elevated oestradiol levels lead to elevated LH excretion and effect an LH – surge in the middle of the oestrus circle.

On the other hand, hyperandrogenism may be caused by a genetic anomaly. In the theca cells of ovaries carrying the PCOS the gene CYP11A and CYP17A were more heavily expressed. Due to the enhanced function of the CYP11A gene, precursors of sexual steroid hormones are accumulated. The CYP17A gene is responsible for the enzyme (P450c17 $\alpha$ ), which controls the velocity of androgen hormone synthesis (10). The heightened activity of the CYP17A enhances the synthesis of androgen hormones.

In a quarter of cases the hyperandrogenaemia is of adrenal origin, with the underlying reason being elevated cortisol levels. The androstendion – which originates in the ovarium and in the adrenal gland – is transformed into testosterone and dihydrotestosterone by the  $5\alpha$  – reductase enzyme. A heightened activity of the skin  $5\alpha$  – reductase enzyme has been detected in women with PCOS who have hirsutism (5, 11).

#### 1.1.4. Animal models of PCOS

The etiology of PCOS is investigated almost sixty years but the exact pathomechanism is not fully revealed. Furthermore, the phenotypes are really heterogenic. To understand the mechanism which causes the PCOS or PCOS-like anomalies, many PCOS animal models have been developed.

In this topic the most laboratory animals are mainly rodents, but other mammals were also investigated: e.g., sheep's or rhesus monkeys.

The other question is: how the PCOS-like change could be reached? One of the methods induced PCOS by indirect hormonal interventions.

Desire for completeness there are some methods below (12):

- genetic animal models (13) (Zucker fa/fa/ rat, or New Zealand obese mice)
- ER $\alpha$  KO mice (14)
- aromatase KO mice(15)
- diet – mediated PCOS – like phenotypes(16)
- environmental changes induce PCOS – like phenotypes(17)
- chemically induced PCOS – like phenotypes (18)

Direct hormonal interventions could be applied in many different ways (19). One point of view is the date: prenatal, neonatal, peripubertal and adult models. Most often used treatments are testosterone, DHT, letrozole and DHEA. Neonatal testosterone and DHT treatment were unable to trigger the appropriate PCOS - like model.

Caldwell et al. studied 4 mice groups: prenatal DHT treatment, long - term postnatal DHT treatment (90 days from 21 day of age), postnatal DHEA and letrozole treatment. Because of long – term DHT treatment induced unhealthy antral follicles, also induced adipocyte hypertrophy, hypercholesterinaemia and increased body and fat pad weights. Based on these criteria they find this for an ideal model, to investigate PCOS pathogenesis (20).

On day 1 or 5 postnatal DHT treatment had no effect on ovarian follicles by histological examination and on cyclicity (21). However, - as Manneras et al. described – although the ovary weight was reduced, it showed absolutely the typical PCOS appearance after the continuous – 90 day long – DHT treatment of 21 – day – old rats



(22). Beyond the lesion of ovaries elevated body fat, abdominal fat, elevated cholesterol levels and insulin resistance have been detected too (23, 24).

Sára et al. employed 70 day – long DHT treatment, by Masszi et al. the DHT pellet was 90 day long in subcutis, but both described the characteristic metabolic disorders even with insulin resistance and without hypertension (25, 26).

Layer of theca cells of DEHEA induced PCOS -like ovaries are thin, LH levels are not so coherent and even the presence of associated metabolic disorder (24, 27).

## 1.2. Vitamin D

Vitamin D is the general name for fat-soluble steroids. Ergocalciferol (vitamin D<sub>2</sub>) and cholecalciferol (vitamin D<sub>3</sub>) are its two primary forms, whose main effect is to increase the absorption of calcium in the intestine.

Vitamin D is one of the most ancient hormones, which phytoplankton, plants and animals living 750 million years ago were able to produce when they were exposed to sunlight. Its synthesis in humans is also dependent on sunlight exposure. As a result of UVB (ultraviolet B) radiation, previtamin D<sub>3</sub> (cholecalciferol) is produced from 7-dehydrocholesterol in the skin.

A part of this precursor is then transformed into an active vitamin by the heat of the skin, while another part is further transformed into an inactive isomer. This is also why hypervitaminosis D<sub>3</sub> cannot develop due to excessive sun exposure.

Depending on the angle of incidence of the sun's rays, it is absorbed in the ozone layer around the Earth. The rate of absorption is highest in the early morning and in the afternoon and evening hours, so we are exposed to reduced exposure at these times regardless of which part of the planet we are in.

Due to the shape of the Earth above the 35th degree of latitude (also in our country), the angle of incidence of the sun's rays is much smaller from October to March, which is why cholecalciferol production is also minimal. Additional sources include foods that contain a lot of vitamin D (e.g. cholecalciferol (D<sub>3</sub>): fish oil, salmon, herring; and ergocalciferol (D<sub>2</sub>): some types of mushrooms (28).

Cholecalciferol ingested with food or synthesized in the skin is in the liver, it is further transformed into 25-hydroxycholecalciferol (calcidiol - prohormone). From there, it is

bound to alpha-globulins and transported to the kidneys, where it becomes the already active 1,25-dihydroxyvitamin D<sub>3</sub> (calcitriol, hereinafter vitamin D).

Today it has already been recognized that this process does not only take place in the kidney, but to a lesser extent the colon, brain, prostate, pancreatic  $\beta$ -, vascular smooth muscle cells and macrophages are also capable of synthesizing vitamin D (28-30).. It exerts its effect through the active vitamin D receptor (VDR). By forming a heterodimer with the retinoid X receptor (RXR), VDR mediates the transcription of many genes. These genes influence processes such as cell division or regulation of the immune system.

Renal vitamin D synthesis is determined by serum parathyroid hormone (PTH), calcium, and phosphate levels. The main task of vitamin D is to regulate the body's calcium (Ca) and phosphate (P) metabolism, which is primarily influenced by absorption in the intestine (31). Vitamin D is necessary for the absorption of around 60% of the calcium in the diet. When vitamin D was administered, these values increased to 30-40% for calcium and 80% for phosphates (32, 33).

At a conference held in Warsaw in 2012, a guideline for the treatment and prevention of vitamin D deficiency was adopted for Central European countries, in which the following recommendations were formulated: the optimal serum vitamin D concentration is 30-60 ng/ml. In addition, maintenance vitamin D treatment is also necessary (especially in the period from October to March). Between 20-30 ng/ml is suboptimal, while below 20 ng/ml we are talking about absolute vitamin D deficiency. In the latter case, a therapeutic amount of vitamin D should be administered (34).

A serum concentration above 100 ng/ml is already considered toxic. In this case, it is recommended to suspend vitamin D supplementation until the serum concentration returns to the target range. The upper limit of safe daily vitamin D supplementation is 1000 IU for newborns, 2000 IU for children under ten years of age, and 4000 IU for children over 10 years of age, adults and pregnant women (34).

Since it is a fat-soluble vitamin, the daily requirement of obese adults can be up to 10,000 IU. In case of vitamin D deficiency, the daily therapeutic dose is 1000 IU for newborns, 1000-3000 IU up to one year of age, 3000-5000 IU under the age of 18, while for adults 7000-10000 IU/day (depending on body weight), or 50000/week. In case of deficiency, it is necessary to check the amount of serum vitamin D, alkaline phosphatase,

phosphorus, calcium and the amount of calcium excreted in the urine every one to three months (34).

Vitamin D deficiency can be considered a global problem, especially in developed industrialized countries, where the number of hours spent in the sun has significantly decreased due to lifestyle.

This phenomenon is further aggravated by the damage to the ozone layer and the use of high-factor sunscreens. Europeans - where the intake of vitamin D from food is very low - are particularly at risk. In India, Turkey, Lebanon and Australia, 30–50% of the population suffers from vitamin D deficiency (35-37). The characteristic symptom of vitamin D deficiency was felt by many children living in Great Britain during the industrial revolution, and the name (English disease, rickets) also comes from this. Its counterpart in adults is osteoporosis.

Due to the low vitamin D level, the serum calcium level decreases. The body replaces this from the bones under the control of PTH, thereby causing a breakdown of the bone's inorganic stock (osteomalacia). In addition to the weakening of the bone structure, pathological fractures may also become more frequent. In the absence of vitamin D, the incidence of muscle weakness and certain myopathies also increases, which can lead to bone fractures due to falls (38, 39). Vitamin D receptors (VDRs) have been detected in many cells, through which vitamin D is directly or indirectly involved in the regulation of about 200 genes, thus influencing cell proliferation, differentiation, apoptosis and angiogenesis.

It reduces the division of both healthy and cancerous cells, and its active metabolite is successfully used to treat psoriasis. The role of long-term vitamin D deficiency has been demonstrated in many chronic diseases. Colon, pancreatic, ovarian, prostate, breast cancer and Hodgkin's lymphoma also occurred more frequently in patients with reduced serum vitamin D levels. The risk of autoimmune diseases, diabetes and even schizophrenia and depression are increased with vitamin D deficiency. Three times weekly UV-B treatment for three months increased vitamin D levels in hypertensive patients by 180%, and vitamin D values measured after therapy returned to the normal range (28, 40).

According to a survey, 67-85% of PCOS women had serum vitamin D concentrations below 20 ng/ml. Due to its effect on the immune system, vitamin D improves the chances

of pregnancy and implantation. Through its receptor (VDR, vitamin-D receptor), it affects the blood levels of LH, SHBG, testosterone and insulin (41). In animal experiments, the regression of PCOS morphology was demonstrated in response to vitamin D administration (42). Based on these, it can be concluded that vitamin D plays a fundamental role in the development of pregnancy through the regulation of carbohydrate and calcium metabolism and follicular maturation.

### 1.3. Vitamin D and PCOS

There is mounting evidence that vitamin D has a role in the pathophysiology of polycystic ovary syndrome (PCOS) by influencing a number of its metabolic and reproductive characteristics.

In humans, VDR is expressed in the ovaries and the placenta. In terms of ovarian steroidogenesis, the presence of calcitriol enhanced the generation of progesterone, estradiol, and estrone by human ovarian cells. Vitamin D stimulated an increase in 3 $\beta$ -HSD messenger (mRNA) levels and progesterone secretion in cultured human granulosa cells. Vitamin D increases dehydroepiandrosterone sulfotransferase (SULT2A1), as was previously observed in animal models and was confirmed by in vitro research in human models. Ovarian reserve indicators, especially AMH, are correlated with vitamin D levels. An interesting follow-up investigation showed that a functioning Vitamin D response element (VDRE) is present in the human AMH promoter (43).

It's worth noting that hyperinsulinemia and insulin resistance (IR) play a major part in the etiology of polycystic ovary syndrome (PCOS), influencing the intensity of clinical characteristics regardless of weight. Several hypotheses have been offered to explain the connection between vitamin D and IR: (i) via increasing insulin receptor expression and boosting insulin responsiveness for glucose transport, vitamin D increases insulin action; (ii) critical for insulin-mediated effects in insulin-responsive tissues is calcium, which is regulated by vitamin D both inside and outside of cells (44); (iii) the anti-inflammatory effects of vitamin D; and (iv) the human insulin gene contains the vitamin D response element (VDRE) in its promoter, and vitamin D stimulates transcription of this element. However, the vast majority of PCOS sufferers are overweight. Due to reduced sun exposure and the lipophilic vitamin's sequestration in adipose tissue, those who are overweight tend to have lower levels of 25(OH) vitamin D (43).

Using a DHT-induced PCOS rat model, researchers have shown the precise mechanism by which insulin-dependent vasorelaxation of aortic rings deteriorates. DHT therapy decreased insulin-dependent aortic ring relaxation in rats, as shown by Massi et al (25). Vascular insulin resistance is characterized by this impaired ability to dilate in response to insulin. In the aortic rings, vitamin D treatment had no effect on the vascular insulin resistance generated by a hyperandrogenic condition, in contrast to the systemic insulin resistance and the local insulin-dependent relaxation in small arteries, i.e., arterioles. It has been shown that aging also causes a separation of metabolic and vascular insulin resistance, therefore this phenomenon is not unprecedented. Large vessel wall reactivity may have been decreased as a residual direct impact of chronic DHT therapy due to the differences in vitamin D's effects on micro- and macro-vessels. But vitamin D protected against insulin-induced arteriole relaxation, which may have been linked to the resolution of systemic insulin resistance in DHT-treated rats (26). Vitamin D had no effect on insulin-induced vasorelaxation in the aorta of DHT-treated rats.

Our team have previously reported that pre-contracted aortic rings from hyperandrogenic rats have a higher contractility to norepinephrine (NE) and a lower instantaneous relaxation to estradiol therapy. Vitamin D injection alongside DHT decreased NE reactivity but did not re-establish relaxation in response to estradiol. Ovarian and blood leukocyte Poly-(ADP-ribose) (PAR) staining in DHT rats exhibited increased DNA damage, which was mitigated by concurrent vitamin D treatment. The endothelium and vascular smooth muscle cells of the aortic rings of hyperandrogenic rats surprisingly showed less PAR staining. Thus, PCOS is associated with a shift in vascular tone toward vasoconstriction and a reduction in vasorelaxation, as well as vascular dysfunction and altered PARP activity (45).

Vitamin D has been linked, either directly or indirectly, to improved reproductive health in women, according to data from animal research. Overall fertility in female rats depleted of vitamin D through diet was reduced by 75% compared to fertile rats with adequate vitamin D levels. The offspring of vitamin D-deficient rats were born prematurely and showed stunted growth. Fewer conceptions and more pregnancy difficulties were blamed for the decline in fertility (46). The VDR mutant animals showed remarkable uterine weight increases in response to estrogen treatment, suggesting a potential role for VDR in estrogen signaling (47).

Women with PCOS who are infertile and have 25(OH) vitamin D levels of 30 ng/ml or more are more likely to ovulate than those whose levels are lower than 20 ng/ml, according to a new retrospective cohort research. Additionally, women who gave birth successfully had higher levels of 25(OH) vitamin D than those who did not. Therefore, a woman's ability to ovulate and become pregnant while suffering from PCOS may hinge on her 25(OH) vitamin D status (48).

#### 1.4. Theoretical background of studying vascular function

Ex-vivo vascular function testing has long been a favourite method of researchers in mapping the mechanism of vascular function affecting circulation. The blood vessel section removed from the experimental animal retains its viability for a long time in a solution with a suitable composition, while certain blood vessel functions (e.g. constriction, relaxation) can be excellently studied. In order to clarify the various pharmacological effects and signal transmission mechanisms, processes that are often difficult to separate from each other can be observed separately with appropriate stimulating and inhibitory substances.

The initial vasoconstriction is often created with a solution containing potassium. During this time, we can get an idea of how damaged the given blood vessel section was during the organ removal. The extent of this vasoconstriction can be compared to that of other constrictors (e.g. norepinephrine), and since in the case of potassium non-receptor-dependent smooth muscle contraction occurs, the smooth muscle function of the vessel wall can be examined separately, independently of the endothelium.

After the pre-contraction, the vascular function can be tested with various vasoactive drugs with known effects. Since adrenaline also plays a central physiological role in regulating blood pressure, it can also be used ex-vivo to induce vasoconstriction. The  $\alpha_1$ -receptors of adrenaline bind to the Gq protein, which, through the activation of phospholipase C (PLC), raises the free calcium level within the cell, which causes smooth muscle contraction. Other vasoconstrictors include serotonin, endothelin-1, thromboxane A<sub>2</sub> (TXA<sub>2</sub>), and prostaglandins G<sub>2</sub> and H<sub>2</sub> (PGG<sub>2</sub> and PGH<sub>2</sub>) (49).

It was an important discovery that most vasodilators exert their effect only through the endothelium - indirectly. The recognition is linked to the detection of the endothelium-derived relaxation factor (EDRF), which, according to our current knowledge, is nitric

oxide (NO) (Louis Ignarro: 1998, Nobel Prize) (50, 51). Ligands bound to the appropriate receptor of the endothelium trigger the release of NO through the activation of the endothelium-derived nitric oxide synthase eNOS, which results in an increase in cyclic guanosine monophosphate (cGMP) levels in the smooth muscle and consequent smooth muscle relaxation (52). The increased shear force acting on the endothelium, with the activation of surface calcium channels, also triggers the phosphorylation of eNOS and the release of NO (Figure 8) (53). Endothelial dysfunction can essentially be caused by reduced production or increased use of NO.

The former can be caused by various endothelial damages, the latter by increased superoxide formation and oxidative stress (e.g. in atherosclerosis, hypercholesterolemia). Thus, during measurements with substances that trigger NO-dependent relaxation - acetylcholine, histamine, bradykinin, vasopressin, estrogen, insulin, oxytocin (54) - we can get an accurate picture of the condition and function of the endothelium.

By using certain inhibitors, an even more detailed study of endothelial dysfunction can be carried out. The NO pathway can be blocked with L-NG-nitroarginine methyl ester (L-NAME) through the inhibition of NOS, so the effect of other vasodilator substances can also be measured, and elements with previously suppressed constrictor effects can also become effective. Indomethacin, which inhibits the cyclooxygenase (COX) enzyme, can be used to investigate prostanoid dysfunction.

The disadvantage is that it blocks both the synthesis of prostaglandins, which usually act as vasodilators, and thromboxane, which has a significant vasoconstrictor effect, so a more selective antagonist is needed for their more detailed examination.

To studying the above-mentioned vascular function, there are two established methods: the wire and the pressure myograph systems.

Vascular reactivity and functional responses in arteries with internal diameters from 600  $\mu\text{m}$  to 10 mm can be measured with an *ex vivo* approach called wire myography. In 1976, Mulvany and Halpern conducted studies to explore the contractile responses of isolated tiny resistance arteries, which led to the development of the myography technique (55). Both pharmacology and vascular physiology benefit from this flexible method. Small and big arteries, veins, and lymph vessels from a wide range of species and transgenic models can be used, which is a major benefit of this method. Hypertension, atherosclerosis, diabetes, senescence, ischaemic heart disease, and heart failure are only

few of the pathophysiological disease states that can be studied (56). Isometric procedures involve dissecting and cleaning the vessel before mounting it on a four-channel myograph. Then, the maximal active tension development in each vessel is normalized. This allows for the starting experimental conditions to be standardized, which is crucial for comparing vessels pharmacologically (57). The isometric contractility of vasculature in rodents can be studied in response to stimuli such as vasoconstrictors, inhibitors, and medicines using the myograph technique. We can learn more about the mechanics of blood pressure maintenance and the pathophysiology of disorders related to vascular smooth muscle if we can successfully monitor contraction (58).

When used in conjunction with pharmacological characterization, the pressure myograph provides a novel approach to characterizing endothelial function by revealing the endothelium's autoregulatory properties in response to changes in blood flow (59), this method might be used in the vessel range of 30-800 micrometers. The impact of transmural pressure on vascular diameter was initially described by Bayliss in 1902 (60). Pressure changes induced vasodilation in small resistance arteries taken from rabbits, cats, and dogs, while pressure changes induced vasoconstriction. Myogenic reaction is the term for this occurrence. In isobaric settings, Bayliss and other researchers found that small resistance arteries produce a prolonged constriction known as myogenic tone. Pressure myography (PM) is a technique that measures both myogenic reaction and myogenic tone. Vasoactivity of smaller arteries, veins, and other vessels can be measured using PM. PM, as the name suggests, is used to evaluate both the influence of vasoactive substances on vascular diameter and the effect of intravascular pressure on vascular diameter. Software advancements in the previous few decades have facilitated video microscopy and glass pipette pulling, lowering the barrier to entry for PM. The ability to examine microvascular function in near-physiological settings is greatly aided by the use of ex vivo pressured preparations of tiny resistance arteries. This is accomplished by applying pressure to a pair of small glass cannulas through which a recently isolated, undamaged section of a small resistance artery (diameter 260  $\mu\text{m}$ ) has been mounted. These artery preparations allow for real-time vascular tone evaluation and maintain several features seen in vivo (61).

Pressure and wire myographies allow us to examine different levels of vascular tree and help us distinguishing local vascular properties and systemic regulator effects. That



was the reason why we used these methods for describing different level vascular effects of vitamin D deficiency in a PCOS model.

## 2. OBJECTIVES

The primary goal of our research was to investigate the function of the aortic vessel in an early PCOS rat model. This early PCOS was created with a relatively short (8 week) course of transdermal testosterone, which does not result in hypertension like the longer models.

In PCOS, vitamin D deficiency is a frequent comorbidity. In consequence we aimed to study in parallel, the potential effects of vitamin D deficiency and supplementation on vessel function.

This model aims to examine the earliest alterations of vessel function in order to understand the initial steps of vascular damage – further down this may lead to the establishment of preventative measures in the medical practice. In humans there is no way to study these alterations in an ethical manner, therefore this animal model was created. However, we aim to use this knowledge in human daily practice, as a future perspective.

Our aim is to examine how aorta vessel function changes in these different groups:

1. Could we detect the hypothetical damage of the relative contractile ability of the aorta in female rats which is tested with norepinephrine?
2. Are there any changes in vessel relaxation, while using acetylcholine and estradiol, both in cumulative concentrations?
3. What kind of different effects of NO and prostanoids are on vasorelaxation, while L – NAME, and COX-2 inhibitor were used?
4. How alter the ER $\alpha$  (estradiol receptor  $\alpha$ ), eNOS, and COX-2 enzymes amount in the aorta of female rats, which could be the reason of the changed vascular function?

### 3. METHODS

#### 3.1. Chemicals

Ex vivo evaluation of aortic function was performed in Krebs-Ringer (KR) solution (in mmol/l): NaCl 119, KCl 4.7, NaH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 1.17, NaHCO<sub>3</sub> 24, CaCl<sub>2</sub> 2.5, glucose 5.5, and EDTA 0.034. The solution was prepared daily; the temperature was maintained at 37°C. By bubbling in a combination of gases, the pH was maintained (containing O<sub>2</sub> 20%, CO<sub>2</sub> 5%, and N<sub>2</sub> 75%). Norepinephrine (NE) and acetylcholine (Ach) were obtained from Sigma-Aldrich (St Louis, MO, USA). 17-β -estradiol was bought from TOCRIS Bio-Techne (Bristol, UK), while testosterone, eNOS inhibitor (L-NAME, 10<sup>-4</sup>) and COX-2 inhibitor (NS398, 10<sup>-5</sup>) was purchased from Sigma-Aldrich (St Louis, MO, USA).

#### 3.2. Animals

The research followed the Protocol for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (8th edition, 2011) and the EU conform Hungarian Law on Animal Care (XXVIII/1998). Hungarian authorities and the institution's Animal Care Commission both approved the study's procedures (IRB: 8/2014, PEI/001/1548-3/2014).

From Charles River, we obtained 48 female Wistar rats ranging in weight from 100 to 140 grams and in age from 21 to 28 days (Charles River Ltd., AnimaLab, Vác, Hungary) and kept at the Animal Facility of Semmelweis University. The following are the four experimental groups that were formed through random assignment: hyperandrogenic vitamin D deficient group (T + D<sup>-</sup>, N = 12), hyperandrogenic, vitamin D supplemented group (T + D<sup>+</sup>, N = 12), vitamin D deficient group (T-D<sup>-</sup>, N = 12), and vitamin D supplemented group (T-D<sup>+</sup>, N=12).

#### 3.3. Chronic Treatment of the Rats

The hyperandrogenic state was induced by a transdermal testosterone treatment lasting eight weeks. 0,0333 mg/g of Androgel (50 mg/5 ml gel by Lab. Besins International S.A.)

was applied five times weekly to a 3 cm by 3 cm region of the animals' backs that had been shaved.

The vitamin D shortage in the animals was induced by feeding them Vitamin D Free Lab Rat/Mouse Chow (ssniff Spezialdiäten GmbH, Soest, Germany), which contains less than 5 IU/kg vitamin D<sub>3</sub>. Vitamin D-supplemented rats were given a regular diet with 1,000 international units per kilogram of vitamin D. Additionally, the following oral vitamin D supplements were given: On week two, 500 IU of cholecalciferol (Vigantol (cholecalciferol) 20000 IU/ml, Merck/Merck Serono, Darmstadt, Germany) was given via gavage cannula, and on weeks five through seven, a weekly dose of 140 IU/100 g was given. Human vitamin D supplementation guidelines recommend a serum 25-OH-cholecalciferol level between 25 and 50 ng/ml as the optimal range [8, 9]. The rats were furnished with the proper quantity of rat food and tap water. Rats were confined at room temperature at all times (22° C ± 1° C) in 12 h/12 h light-dark cycle. There were two rodents in each cage.

After eight weeks of treatment, intraperitoneal Nembutal anesthesia (45 mg/kg) was administered. Before isolating the thoracic aorta, the cardiovascular system was infused for two minutes with heparinized KR. The aorta segment in the chest was removed and cut into nine pieces, each measuring around 3 millimeters in length. A typical wire myograph setup (610-M MultiMyograph System; Danish Myo Technology A/S, Hinnerner, Denmark) was used to perform functional tests on these 8. Formaldehyde and paraffin were used to preserve the surviving aortic annulus.

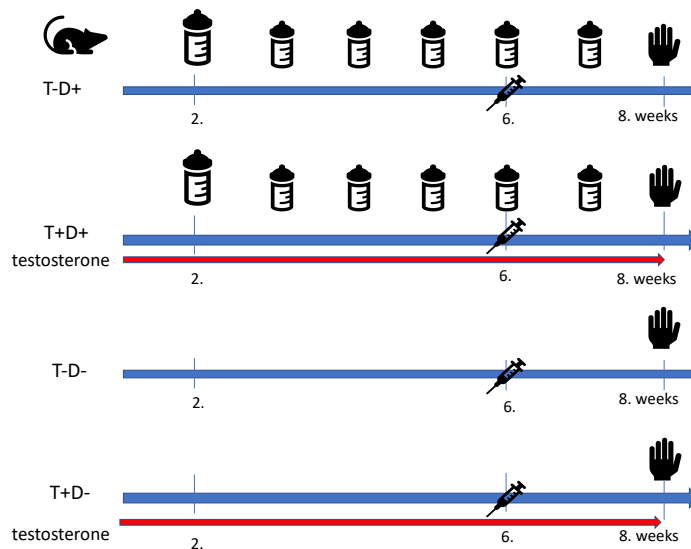


Figure 1.

**Timeline of chronic treatment.** Wistar adolescent rats were divided into two groups: those given transdermal testosterone (0.0333 mg/body weight grams 5 times weekly) for 8 weeks (T+D+, T+D-). Animals were divided into two groups: those that got testosterone treatment and those that did not (T-D-, T+D-), and those that received appropriate vitamin D supplementation (monthly 1,4 NE/body weight grams per os on the third, fourth, fifth, sixth, and seventh week following a 500NE saturation on the second week) (T-D+, T+D+). After 8 weeks of treatment, the animals were euthanized and ex vivo studies were conducted (62).

### 3.4. Wire myograph protocol

Using wire myography, the isometric tension of isolated thoracic aortic rings was measured. The organ chambers were flooded with eight milliliters of KR solution. The temperature was held at 37°C. 15 mN pretension was reached in a progressive fashion. After achieving stable pretension, 124 mmol/L K<sup>+</sup> was administered for 3 minutes to confirm the vessels' contractile ability and establish a maximal value for contraction force. Then, K<sup>+</sup> was washed out with KR solution, and the contraction force after cumulative concentrations of NE (10<sup>-9</sup>, 10<sup>-8</sup>, 10<sup>-7</sup>, and 10<sup>-6</sup> mol/L) was measured. After maximal contraction, we investigated the vasorelaxant potential of raising concentrations of Ach (10<sup>-8</sup>, 10<sup>-7</sup>, 10<sup>-6</sup>, and 10<sup>-5</sup> mol/L). After another equilibration with KR, norepinephrine newly generated precontraction (5 × 10<sup>-8</sup> mol/L). The relaxation of estradiol was evaluated at three distinct doses (10<sup>-7</sup> – 10<sup>-5</sup> mol/L) after 5 × 10<sup>-8</sup> norepinephrine precontraction.

Before the second and third precontraction, the following inhibitors were administered to identify putative endothelium-dependent relaxing pathways (L-NAME  $10^{-4}$  M and NS398,  $10^{-5}$  M).

### 3.5. Immunohistochemistry

Antibodies against estrogen receptor alpha (ER), endothelial nitric oxide synthase (eNOS), and cyclooxygenase 2 (COX-2) were used to stain paraffin-embedded tissue slices. Antigen retrieval was performed by heating the slides in citrate buffer (pH=6) after deparaffinization. The 10%  $H_2O_2$  in  $dH_2O$  solution inhibited endogenous peroxidase activity. The usage of 2.5% normal horse serum (Vector Biolabs, Burlingame, CA, U.S.A.) was implemented to forestall the occurrence of aspecific labeling. Overnight at  $4^{\circ}C$ , the primary antibodies were applied (COX-2: 1:500; eNOS: 1:50; Abcam, Cambridge, UK). Secondary labeling was performed utilizing an HRP-linked anti-mouse or anti-rabbit polyclonal horse antibody (Vector Biolabs, Burlingame, CA, U.S.A.). To see the specific labeling, we utilized the brown dye diamino-benzidine (DAB; Vector Biolabs; Burlingame, California, United States). Hematoxylin, a blue dye made by Vector Biolabs in Birmingham, California, was used to counterstain the slides. The BenchMark ULTRA Automated IHC/ISH slide staining system (primary antibody 1:100) (Ventana Medical Systems, Inc., Tucson, AZ, U.S.A.) was used to conduct the ER immunohistochemistry. DAB as the colored substrate and hematoxylin counterstaining with the UltraView Universal DAB Detection Kit (Ventana Medical Systems, Inc., Oro valley, AZ, U.S.A.) allowed for the visibility of particular labeling. To capture the images, we used a Zeiss Axio Imager system (Zeiss, Oberkochen, Germany) for light microscopy. Using ImageJ (NIH, Bethesda, MA, U.S.), we quantified the positively stained area in the intimal and medial layers of the vessel walls as a percentage of total tissue area or as the uncalibrated optical density of particular staining.

### 3.6. Statistics

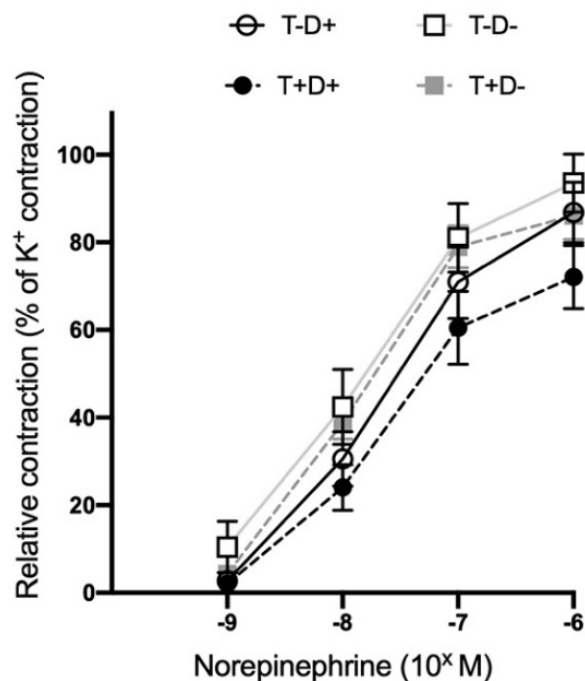
Two-way ANOVA with Tukey's post-hoc test in Prism 8 (GraphPad Software, USA) was used to assess the impact of testosterone therapy and vitamin D status. Using repeated-measures two-way ANOVA with Bonferroni's post hoc test, we studied vascular function curves. The critical value of  $p < 0.05$  was agreed upon by all researchers.

## 4. RESULTS

### 4.1. Vascular function

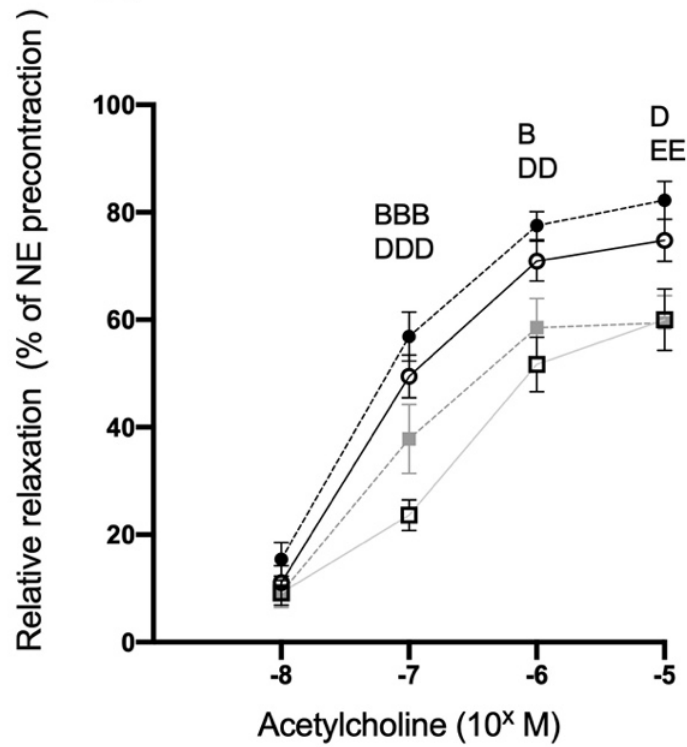
#### 4.1.1. Contraction and Ach mediated vasorelaxation of isolated thoracic aorta segments

Contractile capacity of the aorta was maintained in all experimental groups, this was demonstrated via intact NE-induced vasoconstriction of the isolated vascular segments (**Figure 2**). This indicates that the aortic wall's smooth muscle cell function is well preserved. In vitamin D-deficient groups, however, impaired endothelial function could be detected as early as the measurement of attenuated Ach-mediated vasorelaxation. The T-D- group demonstrated substantially less dilatation than the D+ groups. At concentrations of  $10^{-7}$  M Ach, the difference reached statistical significance, while it remained constant at higher concentrations. The T+D- group was substantially less relaxed than the T+D+ group at  $10^{-5}$  M **Figure 3**.



**Figure 2. Norepinephrine-induced vasoconstriction**

Relative contractile ability of the aorta in female rats following induction by cumulative concentrations of NE. After being exposed to 124 mmol/L of  $K^+$  for 3 minutes, the percentage of maximum contraction was determined. Norepinephrine concentration had no effect on the degree of vasoconstriction that was induced in any of the treatment groups.  $N = 10 - 11$  in each group. Two-way ANOVA, Tukey's post hoc test. Data are presented as mean  $\pm$  SEM (63).



**Figure 3. Acetylcholine-mediated vasorelaxation.**

Dilation was compared to pre-contraction induced by  $5 \times 10^{-8}$  M norepinephrine. In VDD groups, relaxation was significantly reduced beginning at a concentration of  $10^7$  Ach and staying significantly reduced at higher doses.  $N = 9 - 11$  in each group. Two-way ANOVA, Tukey's post hoc test. BBB: T-D- vs. T-D+  $p < 0.001$ , B: T-D- vs. T-D+  $p < 0.05$ , DDD: T-D- vs. T+D+  $p < 0.001$ , DD: T-D- vs. T+D+  $p < 0.01$ , D: T-D- vs. T+D+  $p < 0.05$ , EE: T+D- vs. T + D+  $p < 0.01$ . Data are presented as mean  $\pm$  SEM (63).

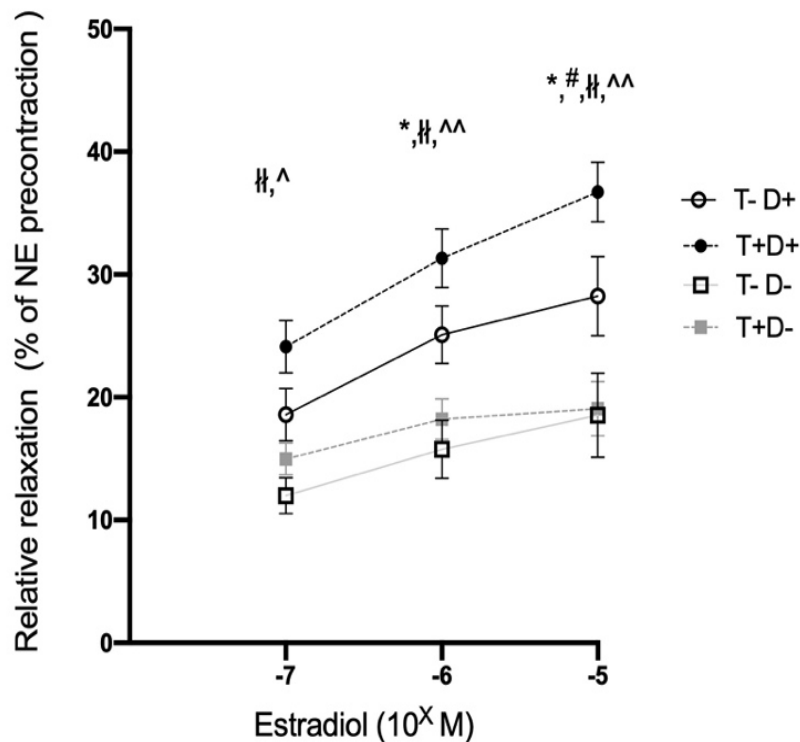


## 4.1.2. Estradiol induced relaxation of isolated thoracic aorta segments

The increasing concentration of estradiol ( $10^{-7}$  –  $10^{-5}$  M) lead to the relaxation of the pre-contracted ( $5 \times 10^{-8}$  norepinephrine) thoracic aorta segments in all experimental groups. Regardless of their androgenic status, the aortic segments of VDD rats revealed considerably reduced estradiol-induced relaxation (**Figure 4**).

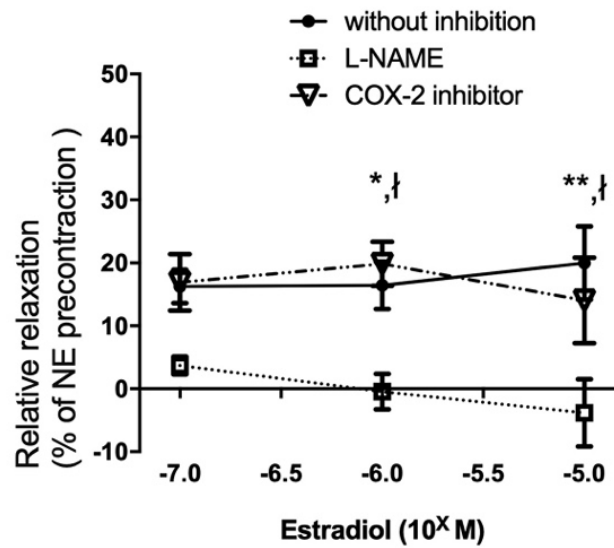
The inhibition of cyclooxygenase 2 (COX-2) further decreased the reduced estradiol-dependent relaxation of vitamin D-deficient aortas, whereas COX-2 inhibition had no effect on the vessels of vitamin D-supplemented rats (**Figure 5 and 6**).

The estradiol-induced relaxation was eliminated across all groups when eNOS was inhibited by pretreatment with L-NG-Nitro arginine methyl ester (L-NAME) (**Figure 5-8**).



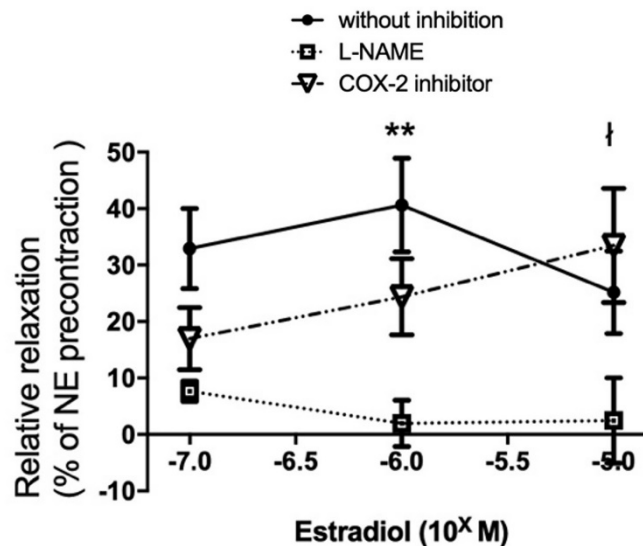
**Figure 4. Estradiol-dependent vasorelaxation**

In response to rising estradiol levels, all previously constricted sections of the thoracic aorta widened. Significantly lower levels of estradiol-induced vasorelaxation were recorded in vitamin D deficient groups, and this effect appeared to be androgen-independent. Repeated measures two-way ANOVA using Bonferroni's post hoc test: Data are presented as mean  $\pm$  SEM. \*:  $p < 0.05$  T-D+ vs. T-D-, #:  $p < 0.05$  T-D+ vs. T+D-, H:  $p < 0.01$  T+D+ vs. T-D-, ^:  $p < 0.05$  T+D+ vs. T+D-, ^^:  $p < 0.01$  T+D+ vs. T+D- (62).



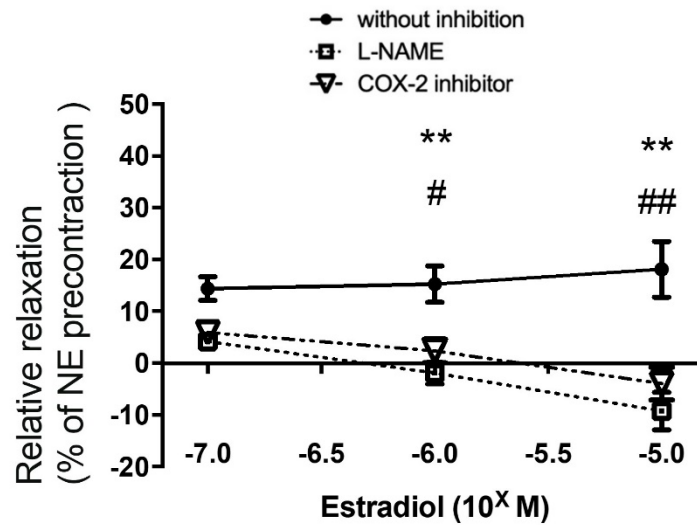
**Figure 5. Estradiol-induced vasorelaxation in the presence of eNOS (L-NAME) or COX-2 (NS398) inhibitors in T-D+ group**

Estradiol-dependent vasorelaxation decreased significantly after L-NAME incubation. Data are presented as mean  $\pm$  SEM. Repeated measures two-way ANOVA using Bonferroni's post hoc test: \*:  $p < 0.05$  L-NAME vs. without inhibition, \*\*:  $p < 0.01$  L-NAME vs. without inhibition, †:  $p < 0.05$  L-NAME vs. COX-2 inhibitor (62).



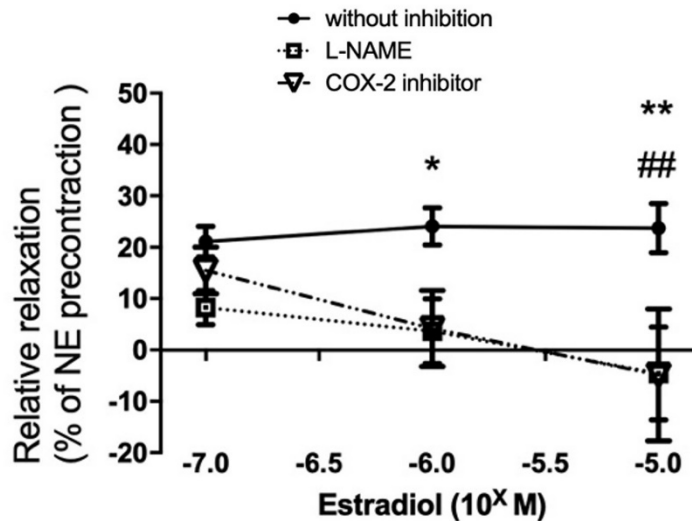
**Figure 6. Estradiol-induced vasorelaxation in the presence of eNOS (L-NAME) or COX-2 (NS398) inhibitors in T+D+ group**

Following L-NAME incubation, estradiol-dependent vasorelaxation significantly decreased. Data are presented as mean  $\pm$  SEM. Repeated measures two-way ANOVA using Bonferroni's post hoc test: \*\*:  $p < 0.01$  L-NAME vs. without inhibition, †:  $p < 0.05$  L-NAME vs. COX-2 inhibitor (62).



**Figure 7. Estradiol-induced vasorelaxation in the presence of eNOS (L-NAME) or COX-2 (NS398) inhibitors in T-D- group**

Following L-NAME incubation, estradiol-dependent vasorelaxation significantly decreased. Following incubation with COX-2 inhibitor, vasorelaxation decreased only in the vitamin D deficient groups. Data are presented as mean  $\pm$  SEM. Repeated measures two-way ANOVA using Bonferroni's post hoc test: \*\*:  $p < 0.01$  L-NAME vs. without inhibition, #:  $p < 0.05$  COX-2 inhibitor vs. without inhibition, ##:  $p < 0.01$  COX-2 inhibitor vs. without inhibition (62).

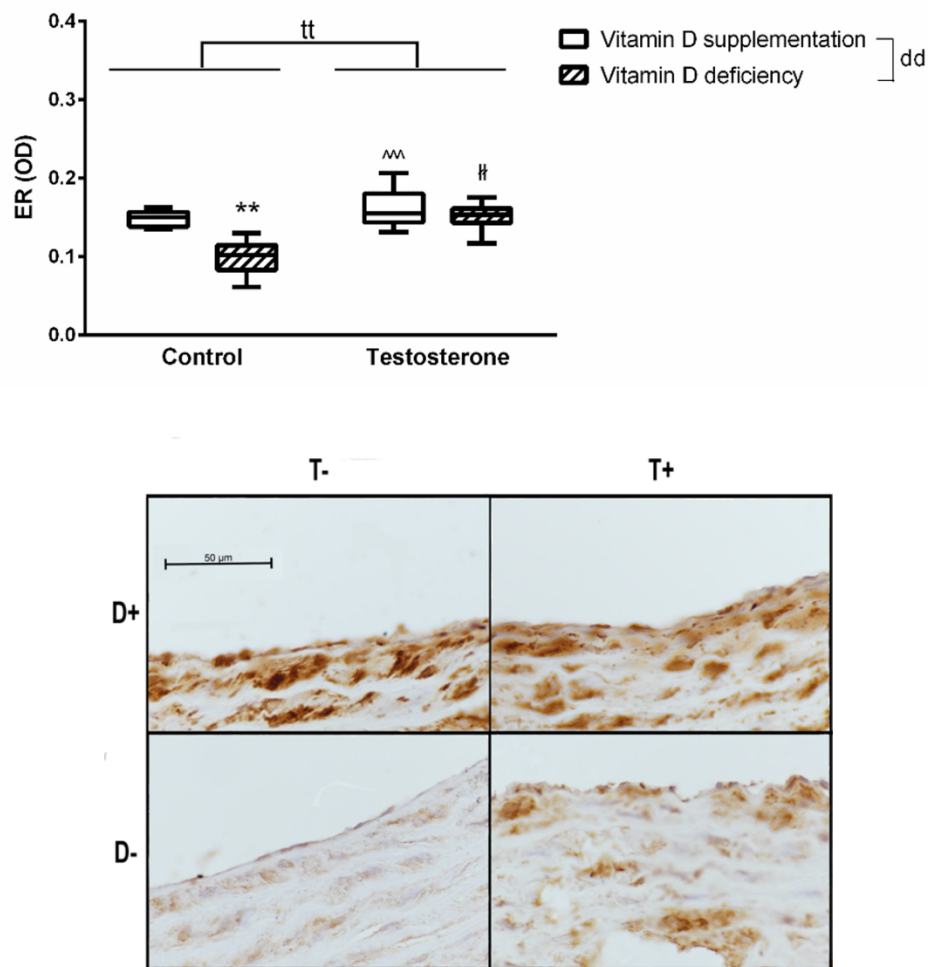


**Figure 8. Estradiol-induced vasorelaxation in the presence of eNOS (L-NAME) or COX-2 (NS398) inhibitors in T+D- group**

Following L-NAME incubation, estradiol-dependent vasorelaxation significantly decreased. Following incubation with COX-2 inhibitor, vasorelaxation decreased only in the vitamin D deficient groups. Data are presented as mean  $\pm$  SEM. Repeated measures two-way ANOVA using Bonferroni's post hoc test: \*:  $p < 0.05$  L-NAME vs. without inhibition, \*\*:  $p < 0.01$  L-NAME vs. without inhibition, ##:  $p < 0.01$  COX-2 inhibitor vs. without inhibition (62).

## 4.2. Immunohistochemistry

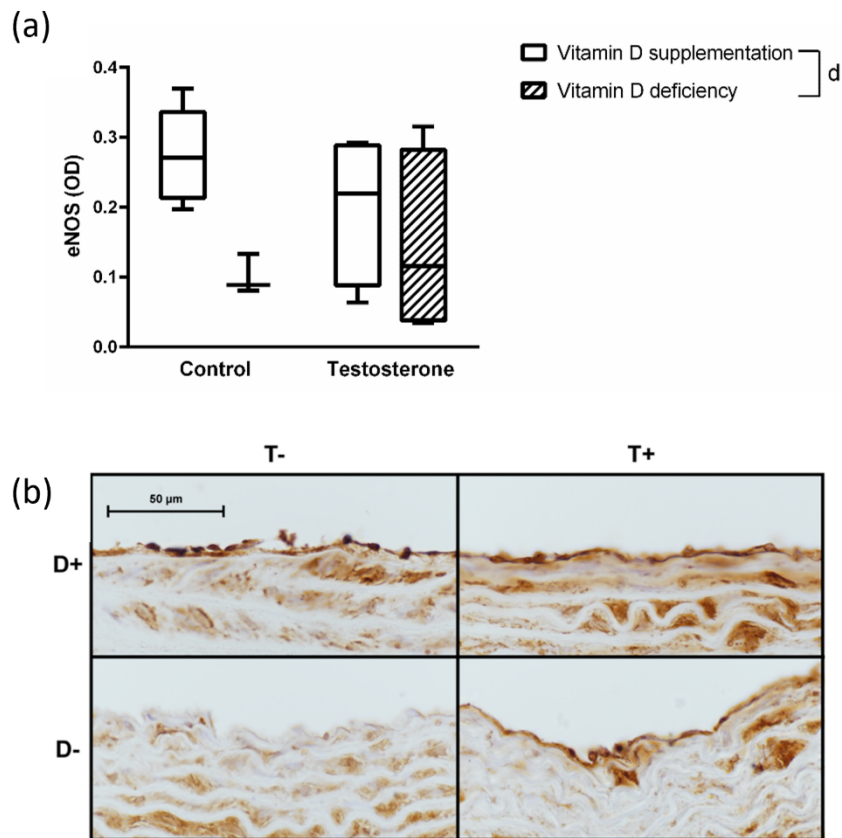
In the aortic intima, ER specific labeling was found to be higher after testosterone treatment and lower after VDD. The T-D- group had the lowest ER staining when the post hoc test was performed compared to the other groups (**Figure 9**). Two-way ANOVA results showed that VDD considerably decreased eNOS specific staining intensity in the analyzed vessels. However, therapy with testosterone had little effect. There were no obvious differences between the groups, according to a post hoc analysis (**Figure 10**). COX-2 specific labelling showed significant increase following testosterone treatment (**Figure 11**). Post hoc testing showed a statistically significant increase in the T+D+ group compared to the control animals.



**Figure 9. Immunohistochemical staining of the aortic wall with ER $\alpha$  antibody**

Panel (a). Non-calibrated optical density was measured. ER specific staining was dramatically reduced in vitamin D deficiency while greatly elevated in testosterone treatment. The T-D- group's staining was the least intense of any of the experimental groups. Data are presented as mean  $\pm$  SEM. Two way (testosterone treatment and vitamin D status) ANOVA; Tukey's post hoc test, \*\*:  $p < 0.01$  T-D+ vs. T-D- group, ^^:  $p < 0.005$  T-D+ vs. T+D+ group, #:  $p < 0.01$  T-D- vs T+D- group; tt:  $p < 0,01$  T-vs T+, dd:  $p < 0,01$  D+ vs D-.  $N=5-6$  in each group.

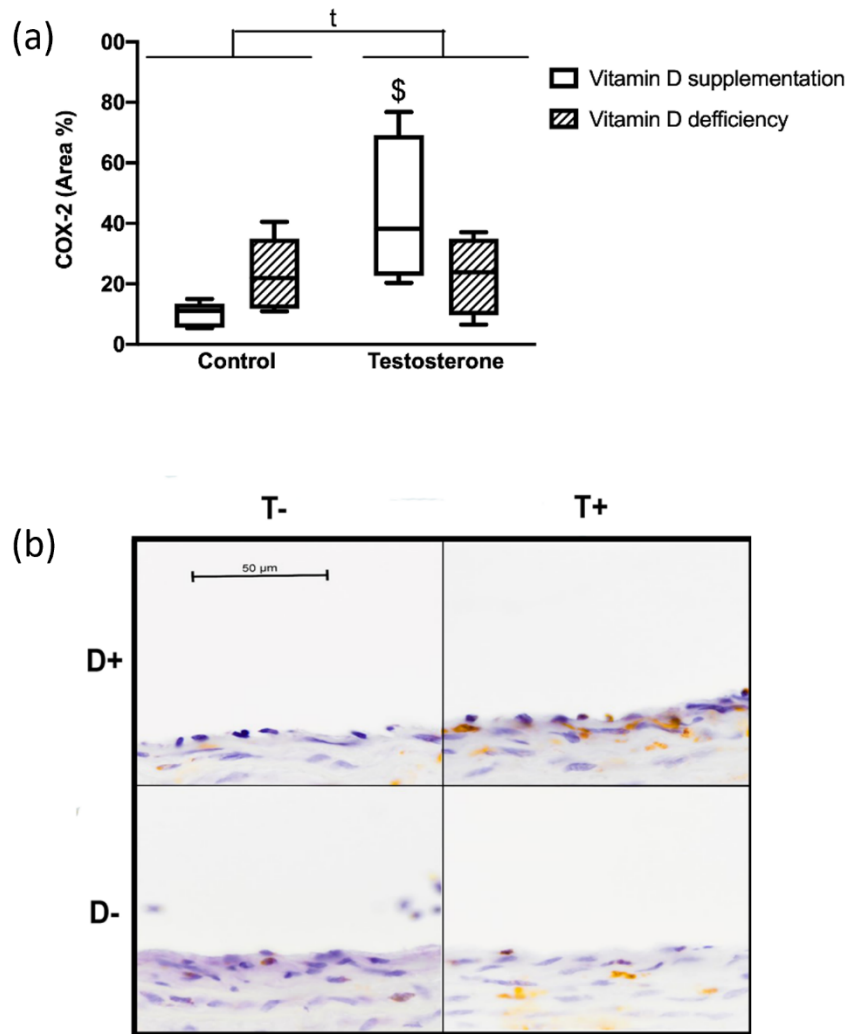
Panel (b) shows representative images of ER $\alpha$  staining- specific staining is visualized by brown colored DAB, while blue colored hematoxylin serves as counterstaining (62).



**Figure 10. Immunohistochemical staining of the aortic wall using eNOS antibody**

Panel (a). Immunostaining for eNOS was drastically reduced in those with vitamin D deficiency, although testosterone therapy had little effect. Data are presented as mean  $\pm$  SEM. Two-way (testosterone treatment and vitamin D status) ANOVA; d:  $p < 0,05$  D+ vs D-. N=4-6 in each group.

Panel (b) are representative images of eNOS staining (62).



**Figure 11. Immunohistochemical staining of the aortic wall using COX-2 antibody**

Panel (a). Positively stained areas were compared to total tissue areas to determine the percentage. Testosterone treatment significantly increased COX-2 staining in our experiment. Greater amounts of staining were seen in the T+D+ group than in the control group. Data are presented as mean  $\pm$  SEM. Two-way (testosterone treatment and vitamin D status) ANOVA; Tukey's post hoc test, \$:  $p < 0.05$  T-D+ vs. T+D+ group, t:  $p < 0.05$  D+ vs D-. N=4-5 in each group.

Panel (b) shows COX-2 staining in the different groups (62).

## 5. DISCUSSION

### 5.1. The hyperandrogenic model

There are many rat models to be found in the literature, wherein the effects of the hyperandrogenic milieu have been researched following various forms of chronic treatment (10, 64-71). In contrast to the latest models, we used a relatively short, 8 week - long testosterone treatment, to observe the parallel effects of VDD, in the early phase of possible vessel function damage causes by higher androgen levels. Establishing vitamin D deficiency in a hyperandrogenic state (group T+D-) was appropriate to examine a PCOS-like conditions (22, 72). Patients with PCOS are at greater cardiovascular risk, which is caused by damage of the endothel function (28-30).

In the 90 - day model used by Yanes et al. several metabolic disorders were described such as hypertension, significantly increased serum fasting insulin, and glucose levels accompanied by increased body weight (10). Our prior early PCOS model – 70-day treatment with a subcutaneous DHT pellet – showed insulin resistance without hypertension. It would appear that the observed anomalies in the hyperandrogenic group (heightened NE induced contraction, decreased Ach mediated relaxation) may be interpreted as pre-hypertensive disorders. D vitamin treatment restored the enhanced reactivity to NE but had no significant effect on Ach mediated vasorelaxation, which could be the reason for increased constrictor prostanoid levels (67).

In our latest 8 week - long PCOS rat model the transdermal testosterone treatment led to significantly elevated bodyweight and there was no difference regarding blood pressure values. The hyperandrogenic status was established (significantly increased testosterone and DHT levels) and furthermore in this group the missing luteal phase of the ovarian cycle led to decreased progesterone levels.

The characteristic PCOS phenotype may be established in our testosterone-treated rat model (hyperandrogenism, ovulatory dysfunction and PCO morphology of the ovaries) (63).

The hyperandrogenism caused by this testosterone treatment has been certified by Hadjadj et al, a piece: T-D+:  $0,311\pm 0,16$ , T+D+:  $4,292\pm 0,56$ , T-D-:  $0,720\pm 0,16$ , T+D-:  $5,495\pm 0,56$  (ng/ml, mean $\pm$ SEM) (73). In the hyperandrogenic groups the typical PCO morphology has involved published by Pal et al (74). A vitamin D-restricted diet led to



vitamin D deficiency (serum levels below 20 ng/ml), while vitamin D supplementation brought about the desired range of 30-50 ng/ml [(75),(76)]. In these ovaries no estrus cycles were detected. The other analysed effect, VDD has a more complex influence on the metabolism: increased insulin levels, and HOMA-IR, slightly elevated testosterone level and ovarium dysfunction (73).

## 5.2. Estradiol induced vasorelaxation and vitamin D deficiency

In this study, we analyzed the effects of estradiol on vasorelaxation in the thoracic aorta of female rats. Mechanisms by which estrogen causes the endothelium to relax vascular smooth muscle (VSM): activation of mitogen-activated protein kinase (MAPK), enhanced gene transcription, endothelial cell proliferation, and increased synthesis of endothelial nitric oxide synthase (eNOS) all result from estrogen binding to endothelial cytosolic/nuclear estrogen receptors (ER) via genomic pathway occurs. Beyond the nuclear ER receptors (ER $\alpha$  and ER $\beta$ ), estradiol can act through a non-genomic pathway, via G Protein-coupled Estradiol Receptor (GPER) as a result of vasorelaxation (77). Both COX-1 and COX-2 enzymes attend PGI<sub>2</sub> synthesis, but COX-2 directly regulated by estrogen. As Bucci et al described, both higher PGI<sub>2</sub> levels and eNOS phosphorylation through Akt/Pkb linked to estradiol - induced vasorelaxation (78). Endothelium-derived hyperpolarizing factor (EDHF) is produced in response to elevated ER, and its activation of K<sup>+</sup> channels leads to hyperpolarization and blockage of Ca<sup>2+</sup> influx via Ca<sup>2+</sup> channels, ultimately resulting in relaxation of the vascular smooth muscle (79).

It has also been found that endothelial cells and VSM express testosterone or androgen receptors. It indicates that the gender and gonad status influence the expression of androgen receptors in VSM. Female cells have lower concentrations of the androgen receptor protein than male cells do (80). While treatment with estradiol alone had little to no effect on androgen receptor expression in primate smooth muscle, treatment with estradiol in combination with testosterone significantly increased androgen receptor mRNA levels (79, 81).

Our results showed that the VDD groups had significantly reduced estradiol-dependent vasorelaxation (T-D-, T+D-). This level of the hyperandrogenic state, - which was detected in our 8 week – long testosterone treated groups, - had no significant effect on the estradiol dependent vasorelaxation. In contrast to this, in the studies conducted by

Masszi et al., there was already a significant decrease in relaxation due to estradiol as a result of 10 weeks of treatment (45). In addition to the duration of the treatment, another difference was that the hyperandrogenic state was achieved by subcutaneous implanted DHT pellets, the active metabolite of testosterone. This effect was independent from the conversion rate of testosterone. T-direct antioxidant, PARP blocker/ DHT – increases oxidative stress/ induce PARP activity (82-84).

Estrogen – induced vasodilation is gender related and vascular endothel plays an important role to mediate it. Additionally, female spontaneously hypertensive rats exhibit enhanced endothelium-dependent relaxation of isolated aorta compared to their male counterparts (85, 86). We recently found that vascular contraction is not different between castrated and intact male rats, but is significantly enhanced in ovariectomized females compared with intact females, suggesting that estrogens, rather than androgens, are responsible for the gender differences in vascular tone (87, 88). Data also points to interactions between different hormone receptors in the vasculature and sex hormones as a possible cause of the sex-based changes in vascular tone observed between the sexes.

In our study, VDD caused endothelial cells to express less ER compared to T-D+ rats, and this difference was statistically significant. The vitamin D receptor (VDR) mediates vitamin D's biological functions and is found in many different tissues, including the skeleton, parathyroid glands, and reproductive organs. The vitamin D receptor in the nucleus forms a heterodimer with the retinoid X receptor after being activated by vitamin D. Target gene promoter regions contain a vitamin D sensitive element, which is bound by this. Calcium-binding proteins and coactivator proteins are two examples of transcription integrators that interact with the vitamin D receptor. It takes hours to days for this genomic route to result in altered gene transcription. However, by interacting with a cell surface receptor and second messengers, a response time of seconds to minutes can be attained (28, 89, 90).

Some of vitamin D's physiological effects include promoting follicular maturation and selection and increasing synthesis of ovarian steroid hormones such progesterone, estradiol, and estrone. Calcitriol regulates HOXA10 expression in human endometrial stroma cells, which is critical for endometrial development to allow the uterus to be receptive to implantation (91).

Certain genes involved in testicular development in mice have been demonstrated to be upregulated by vitamin D therapy. Among these genes was ATP-binding cassette transporter 1 (ABCA1), - a potential male fertility regulator - because of its expression mostly in Sertoli cells. Mice lacking the gene ABCA1 produce much less testosterone and fewer sperm than their wild-type counterparts (92, 93). On the other hand, testosterone downregulates the VDR in testis cells (94).

Vitamin D insufficiency has been demonstrated to decrease mating success and fertility in female rats. Female rats on a vitamin D-deficient diet can still reproduce, but their chances of becoming pregnant and having a healthy offspring are significantly lower. Restoring normal calcium levels in vitamin D-deficient female rats has no significant effect on this condition. Incomplete spermatogenesis and degenerative changes were detected in the testis of VDD male rats, but – contrary to females – replacement of calcium was enough restoring fertility (95, 96). Kinght et al. described an inverse association between 25(OH) vitamin D levels and estradiol as well as progesterone (97). This could be the reason, why having a high 25(OH) vitamin D level is correlated with a lower chance of developing breast cancer.

Amenorrhea is caused by polycystic ovary syndrome (PCOS) in 90% of cases (98). Vitamin D levels link with ovulation likelihood in polycystic ovary syndrome (68% chance below 20 ng/mL) (48). Both granulosa cell VDR expression and follicular fluid vitamin D levels are reduced in PCOS, suggesting that vitamin D supplementation may have a benefit in the treatment of infertility in PCOS (99, 100).

L-NAME was utilized to inhibit eNOS and investigate the primary mechanism of estradiol-dependent vasorelaxation. All groups showed reduced vasorelaxation after L-NAME incubation, demonstrating the critical role of NO generation in this process. According to our findings, VDD dramatically decreased eNOS staining.

The vasorelaxation caused by acetylcholine was enhanced by a COX-2 inhibitor in female rats. In addition, PCOS rats had elevated COX-2 levels in their ovaries (101). There are variances in the extent to which selective and nonselective COX inhibitors relax blood vessels depending on the subject's gender. Female rats respond significantly to the COX-2 inhibitor, but male rats respond similarly to indomethacin (which serves as a broad inhibitor of COX isoforms). Previous research indicates that estrogen mediates the upregulation of COX-2 expression, which could explain this occurrence (102-104).

Although our investigation failed to find an increase in COX-2 staining in VDD due to the clear parallel effect of hyperandrogenic condition, the decreased estrogen-induced vasodilation during COX-2 inhibition in the VDD groups revealed the enhanced relevance of the COX-2 pathway. Vitamin D regulates the production and activity of phospholipase A2 (PLA<sub>2</sub>), which in turn affects the concentration of COX-2 products (105). The expression of COX-2 in reproductive organs was shown to be elevated in female rodents with hyperandrogenism (106, 107). In our research, we observed that testosterone therapy had a similar effect on aortic endothelial cells. However, inhibiting COX-2 had no negative effects on the vasodilation caused by estrogen. TXA<sub>2</sub> is just one of many vasoconstrictors that originate from the COX-2 pathway. Chronic testosterone therapy increases TXA<sub>2</sub> synthase expression in the cerebral arteries of female rats (108). Our findings may be explained by a shift in the balance between vasoconstrictor and vasodilator eicosanoids.

Although the applied testosterone treatment achieved the criteria of PCOS, in case of vitamin D supplementation the earlier described vascular dysfunction could not be demonstrated in great vessels. In case of physiological vitamin D level, the contraction for NE treatment neither the Ach, nor the insulin mediated vascular relaxation did not change significantly. Despite in VDD groups – even in normo – or hyperandrogenic state – endothel dysfunction could be detectable. The previous context was investigated in a VDR knockout mouse model (109).

Whereas vitamin D plays an important role to control the antioxidant processes, VDD causes higher nitrative and oxidative stress (110). The phosphorylation of eNOS is mediated through the PI3K/Akt. On the other hand, AC/PKA pathway, additionally the PIP<sub>2</sub>/IP<sub>3</sub> and PIP<sub>2</sub>/DAG pathways are activated by VDR which lead to an increased intracellular Ca<sup>2+</sup> levels. This is even a key contributor for eNOS activation. The above described context result is therefore that VDD is responsible for elevated nitrative and oxidative stress (111).

Our experiment strengthens these: significantly decreased eNOS expression was detected in the aorta rings of the VDD groups. Maintaining an adequate vitamin D serum level is critical for proper vascular function. It's common knowledge that VDD raises the odds of developing hypertension and cardiovascular disease (112).

At the currently applied testosterone treatment and vitamin D supplementation did not emerge the vascular function anomaly and nitrative stress in T+D+ group. Testosterone treatment beside VDD (T+D-) did not caused worse endothel dysfunction, as we supposed based on our latest studies, but the negative effect maintained and prevented nitrative stress. As Khalil at al described: vitamin D may increase aromatase activity in several cell line, thereby reduce the androgen production. The measured moderately elevated serum testosterone level in our VDD group correspond this theory, which could be a reaction to the damages give rise by the VDD. Estradiol has vasodilatory effect on female aorta, and that could be also converted from testosterone by aromatase (113). Not only the antioxidant effect of vitamin D but testosterone has been described in male too (114).

Beside the positive effects of testosterone, the negative consequences should be taken into consideration especially in case of a long term - treated model (73). 10-week-long DHT treatment obviously ruined the Ach mediated vasorelaxation in female rat aorta (115). The type of the androgen used for the treatment could be another reason for the detected differences between the above mentioned two models. However, we have to mention that in our model, testosterone related decrease in vasorelaxation was observed on resistance vessels – on coronary arterioles impaired insulin relaxation was found (73). So we can conclude that testosterone dependent vascular damage starts from arterioles and resistance arteries to the aorta which will be the last vessel affected by hyperandrogenic damage.

## 6. CONCLUSIONS

In our rodent model of PCOS we examined the possible early vascular changes of the disease, and the possible interplay of hyperandrogenism and vitamin D deficiency.

1. There was no difference in the developed – norepinephrine induced-vasoconstriction amongst the treatment groups.
2. As the effect of VDD (in both VDD groups), significantly reduced relaxation was measured while using acetylcholine and estradiol.
3. Following L-NAME incubation, estradiol-dependent vasorelaxation significantly decreased in each group. Following incubation with COX-2 inhibitor, vasorelaxation decreased only in the vitamin D deficient groups which was not modified by testosterone treatment.
4. Vitamin D deficiency significantly decreased, while testosterone treatment increased ER $\alpha$  expression by immunohistochemistry. T-D- group had significantly lighter staining compared to all other experimental groups. Vitamin D deficiency significantly decreased eNOS specific staining while testosterone treatment did not alter this result. Testosterone treatment significantly increased COX-2 staining in our experiment. T+D+ group demonstrated significantly larger stained areas compared to control.

Vascular dysfunction was significantly exacerbated by VDD, a typical co-morbidity of PCOS. Reduced ER and eNOS immunostaining paralleled with endothelial dysfunction.

Beside short-term testosterone treatment, endothel dysfunction did not evolved, however, other initial alterations were observed causing endothelial dysfunction and play key roles in target organ damages observed in PCOS, eg. increased COX activation. Testosterone increased estradiol receptor expression, via this mechanism reached similar level of aortic estradiol relaxation comparing to testosterone free animals.

Further investigation needed to determine the positive and the less favorable effect of testosterone to vascular function and to determine the ideal interventions involved.

The development of endothelial dysfunction and the subsequent rise in cardiovascular risk may be postponed with treatment of VDD. Our findings suggest that, in addition to regulating the menstrual cycle, sufficient vitamin D supplementation may be fundamental in relieving the abnormal vascular adaptation in early PCOS.

## 7. SUMMARY

Increased cardiovascular risk has been linked to polycystic ovarian syndrome (PCOS). In women with polycystic ovary syndrome (PCOS), cardiovascular disease may occur early on due to vascular dysfunction. The etiology of polycystic ovary syndrome (PCOS) is complicated by vitamin D insufficiency (VDD), a common comorbidity. Vascular dysfunction may be a result of the elevated oxidative stress seen in both PCOS and VDD.

Our objective was to determine the effect of vitamin D status on aortic function. Female rats were administered transdermal testosterone for eight weeks to induce polycystic ovary syndrome, and adequate vitamin D status was attained through diet. Using a wire myograph apparatus, contraction and estrogen-induced vasorelaxation of segments of the thoracic aorta were measured with or without inhibition of endothelial nitric oxide synthase (eNOS) or cyclooxygenase-2 (COX-2). Using immunohistochemistry, the distribution of estrogen receptor (ER), eNOS, and COX-2 in the aortic wall was determined.

The norepinephrine-induced contraction of the aortas of distinct groups did not differ, whereas the VDD groups exhibited reduced acetylcholine relaxation.

VDD aortas exhibited substantially diminished estradiol-induced relaxation regardless of androgenic status, which was further diminished by COX-2 inhibition. Inhibition of COX-2 had no effect on vessel function in D+ rats. Inhibiting eNOS eliminated the relaxation induced by estradiol in all subjects. Significantly diminished ER and eNOS staining accompanied alterations in vascular function in VDD. VDD induced vascular dysfunction, compromised estrogen-dependent vasodilation, and alterations in ER and eNOS immunostaining, whereas short-term chronic hyperandrogenism did not.



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## 9. BIBLIOGRAPHY OF PUBLICATIONS

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**Tarszabó R**, Bányai B, Ruisanchez É, Péterffy B, Korsós-Novák Á, Lajtai K, Sziva RE, Gerszi D, Hosszú Á, Benkő R, Benyó Z, Horváth EM, Masszi G, Várbiro S.

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