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ALTERATIONS OF ARTERIOLAR REACTIVITY IN A RAT POLYCYSTIC OVARY SYNDROME MODEL – EFFECTS OF PARALLEL VITAMIN D_3 ADMINISTRATION

PhD thesis

Levente Sára MD

Basic Medicine Doctoral School
Semmelweis University Budapest

Supervisor: Szabolcs Várbíró MD, PhD

Official reviewers:
Attila Szabó MD, PhD, Dsc
Sándor Alföldi MD, PhD

Head of the Final Examination Committee:
János Rigó Jr. MD, PhD, Dsc

Members of the Final Examination Committee:
Ádám László MD, PhD
Péter Studinger MD, PhD

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Abbreviations

ACh	Acetylcholine
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of Variance
Apo	Apolipoprotein
BMI	Body Mass Index
C	Control
CAH	Congenital Adrenal Hyperplasia
cAMP	Cyclic Adenosine Monophosphate
COX	Cyclooxigenase
CRP, hs-CRP	C-reactive protein, high sensitive C-reactive protein
CVD	Cardiovascular disease
DHEA, DHEAS	Dehydroepiandrosterone, Dehydroepiandrosterone Sulfate
DHT	Dihydrotestosterone
DHT+D3	Dihydrotestosterone + Vitamin D3
E1, E2	Oestrone, Oestradiol
FSH	Follicle Stimulating Hormone
GLUT 4	Glucosetransporter Type 4
GnRH	Gonadotropic Releasing Hormone
Grb	Growth factor receptor - bound receptor
GSK3	Glycogen synthase kinase 3
HA	Hyperandrogenemia
HAF rat	Hyperandrogenic female rat
HAIRAN-syndrome	Hyperandrogenism, insulin resistance and Acanthosis nigricans syndrome
HCG	Human Choriogonadotropin
HDL, HDL-C	High Density Lipoprotein / -Cholesterol

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HOMA-IR	Homeostasis Model Assesment of Insuline Resistance
IGF	Insulin-like Growth Factor
IGT	Impaired Glucose Tolerance
IL-6	Interleukin 6
INR, INSR	Insulin Receptor, Insulin Receptor Subunit
IR	Insulin Resistance
IRS	Insulin Receptor Substrate
L-NAME	Nitro-L-Arginine Methyl Esther
LDL	Low Density Lipoprotein
LH	Luteotropic Hormone
LOD	Laparoscopic Ovarian Diathermy
LPL	Lipoprotein lipase
MAPK	Mitogen-activated protein kinase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NE	Norepinephrine
NIH	National Institute of Health
nKR	normal Krebs-Ringer
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
OC	Oral Contraceptive
OGTT	Oral Glucose Tolerance Test
PC	Membran glycoprotein
PCOS, PCO	Polycystic Ovary Syndrome
PGE	Prostaglandin E
PI3K	Phosphatidylinositol 3-kinase
PKA, PKC	Protein kinase A, C
PPAR	Proliferator-Activator Receptor
SHBG	Sex Hormone Binding Globulin

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Shc	Homology domain transforming protein
SHHF	Spontaneously Hypertensive, Heart Failure-Prone rat
SHR rat	Spontaneously Hypertensive rat
T1DM, T2DM	Type 2 Diabetes Mellitus
TA	Thoracic Aorta
TC	Total cholesterol
TG	Triglyceride
TGF	Transforming Growth Factor
TNF, TNFα	Tumour Necrosis Factor α
TZDs	Thiazolidinediones
VDR	Vitamin D Receptor
VLDL	Very-Low Density Lipoprotein
WKY rats	Wistar-Kyoto rats

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1. Background

Polycystic ovary syndrome (PCOS) is one of the most common endocrine diseases and the most frequent disorder in women of reproductive age. Nowadays, PCOS is in the focus of research because of its increasing prevalence. The aetiology of this complex and heterogenous disorder is still uncertain. Environmental factors such as physical inactivity, malnutrition, obesity and insulin resistance (IR) have crucial role in development of the disorder [*Baranova et al. 2011*] PCOS might be a multifactorial and polygenic disorder, but gene variants of the CYP11A, the insulin gene, and the follistatin gene are suspected to contribute to PCOS. There is no gene identified as a substantial cause of PCOS. [*Legro et al. 2002, Diamanti-Kandarakis et al. 2005*]. The inheritance of PCOS is suggested to be autosomal dominant, which is transmitted to male and female offsprings , but the phenotype occurs only in women [*Lunde et al. 1989*]. The most common features of PCOS, called the "Rotterdam criteria", are menstrual disorders (amenorrhoea) such as oligo- or anovulatory menstrual cycles; polycystic, large ovaries as detected by ultrasound; and clinical or laboratory signs of excess androgen. It often associates with obesity, acne, hirsutism, cardiovascular disorders (CVD) and obstructive sleep apnea [*Nitsche et al. 2010*]. There is a wide variability in phenotypes; symptoms and severity vary among affected women. PCOS may present at one end of the spectrum with a single diagnosis of polycystic ovarian morphology and at the other end with obesity, menstrual disorders, hyperandrogenism and hypertension. PCOS is the leading cause of female infertility [*Boomsma et al. 2008*]. A reduced oocyte quality and response after stimulation was found in patients with PCOS; additionally, there is an increase in the failure rate of implantation after in vitro fertilisation [*Dumesic et al. 2005, Homburg et al. 1988*]. Women with PCOS have a higher prevalence of early-onset atherosclerosis, metabolic syndrome, IR and type 2 diabetes

mellitus (T2DM), and they may develop hypertension during their reproductive years. PCOS also alters cardiovascular function through various mechanisms [*Dokras A. 2008*]. The mechanisms underlying this increased risk and its possible therapeutic approaches are still under investigation. PCOS has been reported to affect behaviour and social activity, resulting in masculinisation and defeminised behaviour [*Abbott et al. 2008*].

1.1. PCOS epidemiology

PCOS affects approximately 4-10% of fertile women around the world [*Hart et al. 2011, Knochenhauer et al. 1998*], but some researchers estimate the overall rate to be between 4-25% [*Homberg R. 2002*]. The difference in the above mentioned rates can be attributed to the different criteria used for the diagnosis of the syndrome and the different prevalence rates among races and ethnicities. The incidence of PCOS is significantly greater among South-Asian and Mexican women [*Rodin et al. 1998*] than Caucasian women. The highest rate was found in South Asian immigrants in Great Britain. The PCOS prevalence was 52%, and approximately 49% of those women had menstrual irregularity [*Rodin et al. 1998*]. Balen A. et al. reported that patients of South Asian origin tend to present with more severe symptoms at an earlier age with higher incidence of IR than their Caucasian British counterparts [*Balen A. et al. 2004*]. The severity and features of PCOS largely differ among different populations. Kauffman et al. reported that Mexican American women suffering from PCOS have a higher prevalence of IR than white American women [*Kauffman et al. 2002*]. Asian populations have a higher risk for PCOS and IR, however the metabolic syndrome is less frequent among Chinese patients compared to patients of other ethnicities [*Ni et al. 2009*].

Environmental factors such as sufficient or insufficient nutrition and lifestyle management such as physical exercise or domesticity might influence the expression of PCOS. Individuals who have tendency to be obese can preserve their ability to be fertile

by maintaining a threshold weight; however they have a greater survival potential during starvation. A better nutrition and more comfortable environment and lifestyle can contribute to the development of PCOS [*Balen et al. 2002*]. The prevalence of PCOS in Hungary is approximately 5-7% among fertile women (up to half million women might be affected); however, cohort studies investigating the associated symptoms and risks of complications, especially cardiovascular problems, are still lacking [*Speer G. 2009*] .

1.2. Definition of PCOS

The polycystic ovarian morphology was described by Chereau and Rokitanszky in 1844. PCOS was first described in 1935 by Irving Stein and Michael Leventhal [*Stein IF., Leventhal ML. 1935*]. However, the specific phenotype of the disorder was known since the XVIII century, when Stein and Leventhal first discovered the relationship between ovarian morphology and amenorrhoea [*Speer G. 2009*].

The first international consensus on the definition of PCOS was established by the National Institute of Health (NIH) in 1990. At the conference held in the United States, three important criteria for PCOS were nominated and accepted: 1. Oligoovulation 2. Hyperandrogenism (excess androgen activity - clinical or biochemical alterations) and 3. Exclusion of related disorders (i.e., hypothalamic amenorrhoea, hyperprolactinaemia, hyperandrogenism-insulin resistance-acanthosis nigricans (HAIRAN)-syndrome, primary ovarian failure, congenital adrenal hyperplasia (CAH), cushing syndrome, androgen-secreting tumours, exogenous androgen overdose, hypo/hyperthyreosis). The diagnosis of PCOS should be made when patients meet all three criteria. [*Azziz et al. 2006*].

In 2003, the Rotterdam consensus introduced a new concept for the definition of PCOS. Polycystic ovarian morphology detected by ultrasound was accepted by experts as a new criterion. [*Rotterdam ESHRE/ASRM Sponsored PCOS CWG. 2004.*, *Merino et al. 2011*].

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The consensus established the four different phenotypes of the disorder (Table 1.). This consensus was organised by the European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM). Based on the consensus, PCOS should be defined by the presence of two from the previous three criteria: 1. Oligo- and/or anovulation 2. Excess androgen activity and 3. Polycystic ovarian morphology (by ultrasound). The Rotterdam definition was much border than that of the NIH Consensus, which included patients without androgen excess. The diagnosis of polycystic ovaries using an ultrasound remains controversial [*Porter MB. 2008*].

Table 1.: **The different phenotypes of PCOS in terms of the Rotterdam criteria.** AO: anovulation. HA: clinical and/or biochemical hyperandrogenism. IR: insulin resistance. PCO: polycystic ovaries. PCOS: polycystic ovary syndrome. The phenotypes defined by NIH are highlighted in black, and the ones determined later by the Rotterdam Consensus are highlighted in blue. Based on *Lakatos et al. In: Lakatos P, Speer G. Polycystic Ovary Syndrome. Budapest. Semmelweis Kiadó, 2009. p.56.*

The phenotypes of polycystic ovary syndrome

Phenotypical groups	Classic PCOS	4th type PCOS	non AO PCOS	non HA PCOS
Menstrual disorders (oligo- / anovulation)	✔	✔		✔
Clinical and/or biochemical hyperandrogenism (HA)	✔	✔	✔	
Polycystic ovaries (PCO)	✔		✔	✔
Hyperinsulinaemia / IR	✔	✔	✔	
Prevalence in PCOS	61%	7%	16%	16%

The two consensuses mentioned above are commonly used in clinical practice [*Azziz et al. 2006*]. In 2006, the Androgen Excess Society suggested an unofficial modification to the diagnosis. Based on these recommendations, the following three criteria are required

for the diagnosis of PCOS: 1. Hyperandrogenism (clinical e.g. hirsutism and/or hyperandrogenaemia), 2. Ovarian dysfunction (oligo- and/or anovulation and/or polycystic ovarian morphology) and 3. Exclusion of other entities that would cause excess androgen activity [*Lakatos et al. 2009*]. The Androgen Excess and PCOS Society published the latest guideline for PCOS definition in 2009 as follows: 1. Hyperandrogenism (clinical and/or biochemical), 2. Ovarian dysfunction (oligo - anovulation and/or polycystic ovaries), and 3. Exclusion of related disorders [*Azziz et al. 2009, Wild et al. 2010*].

1.3. Diagnosis of PCOS

A history of menstrual disorder, hirsutism, obesity, and acne are strong predictors for PCOS [*Pedersen et al. 2007*]. These four criteria can be used to diagnose PCOS with a sensitivity of almost 80% and specificity of 93%. The anamnestic data and observation of clinical signs are the primary indicators for identifying the disorder, while an ultrasound and laboratory tests should be used to confirm the exact diagnosis. The role of ultrasound as a sole diagnostic method is controversial [*Porter MB. 2008*] because of the high rate of false positives. The latest guideline recommends stringent criteria, such as the presence of numerous small follicles (11-19 or more) and a large ovary with increased stroma (>10 cm^3). The follicles are oriented at the periphery of the ovary, and their diameter is not greater than 9 mm (2-9 mm). [*Balen AH. et al. 2003, Dewailly et al. 2011*]. In the future, a three-dimensional ultrasound might confer a technological advantage, thus providing better chances for a correct diagnosis [*Porter MB. 2008*]. More than 80% of PCOS women have androgen excess during or before adrenarche. Another 20-30% of patients present with adrenal androgen excess, which manifests as elevated dehydroepiandrosterone (DHEA), dehydroepiandrosterone-sulfate (DHEAS), androstenedione levels and specific hyperandrogenic responses to adrenocorticotropic hormone (ACTH) [*Abbott et al. 2008*]. An elevated androgen hormone level, especially that of free testosterone, is a strong predictor for PCOS [*Huang et al. 2010*]. Additionally,

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luteotropic hormone (LH) hypersecretion has a low prognostic prevalence for PCOS, while serum anti-Mullerian factor (>5 ng/ml) has a higher prognostic prevalence for the syndrome [*Dewailly et al. 2011*]. Although a laparoscopy procedure is not routinely used for PCOS diagnosis, it may be used for providing incidental findings of PCOS during an operation.

It is necessary to exclude the related diagnoses of hypophysis disorders, adrenocortical diseases, ovarian and thyroid problems. An irregular menstruation cycle can be caused by hypogonadotropic hypogonadism, cushing syndrome, hyperprolactinaemia and hypothyreosis. A hyperandrogenemia (HA) can be caused by CAH, cushing syndrome, androgen-secreting adrenocortical, ovarian tumours, persisting follicle, and anti-epileptic drugs (i.e., Valproat) [*Lakatos et al. 2009*].

1.4. Aetiology of PCOS

The aetiology of PCOS appears to be both polygenic and multifactorial, thus explaining the multiple phenotypes, which seem to vary from patient to patient. The exact mechanism of PCOS is still debated. Genetic studies have identified a relationship between insulin resistance and PCOS. It suggests that PCOS might be a phenotypic expression of a complex genetic trait disorder [*Franks et al. 2001*]; however, it is challenging to determine the genotype due to its multiple variations. This is because different symptoms of PCOS may link to different genetic variants [*Balen A. 2004*].

Others have hypothesised that androgen excess during the intrauterine life or during early adolescence is a predisposing factor for developing PCOS [*Abbott et al. 2008, Motta AB. 2010*]. Although external or environmental effects are possibly factors, prior androgen excess seems to be essential. Prepubertal HA was found to increase the number of T lymphocyte cells that can infiltrate ovarian tissue, retroperitoneal and

axillar lymph nodes [*Luchetti et al. 2004*]. Additionally, the ratio of CD8+ T lymphocytes to CD4+ T cells increases locally in ovaries in hyperandrogenism. The elevated production of cytotoxic T lymphocytes in HA might contribute to oxidative stress in the ovarian tissue [*Luchetti et al. 2004*]. Prepubertal hyperandrogenism is also reported to increase serum tumour necrosis factor alpha (TNFα) levels; additionally, a mutation of the TNF receptor in association with hyperandrogenism has been demonstrated. [*Luchetti et al. 2004., Peral et al. 2002*].

Luchetti et al. reported increased ovarian lipid peroxidation, decreased glutathione content and catalase activity in hyperandrogenism. This means that there is a higher risk for the accumulation of reactive oxygen species and nitrogen-derived products, thus leading to impaired ovarian function [*Luchetti et al. 2004*]. This vicious cycle might be caused by androgen excess rather than hyperinsulinaemia. Corbould et al. did not find any difference *in vitro* in basal glycogen synthesis or glucose transport between PCOS and controls in a preadipocyte culture, therefore demonstrating that other factors are involved and not just the intrinsic defects in the insulin sensitivity of the adipocytes. *In vivo,* they proposed that external factors, such as high circulating androgen levels, are a linkage of the association between hypertrophic adipocytes and insulin resistance [*Corbould et al. 2007*⁎]. Corbould also showed *in vitro* that androgens can induce insulin resistance in subcutaneous adipose cells [*Corbould A. 2007*⁎⁎] In humans as well as in primates, it is suggested that a prenatal androgen excess might contribute to PCOS programming in the foetus [*Abbott et al. 2008*]. The different timing of this androgen excess during pregnancy may cause different phenotypes and outcomes: androgen exposure during implantation resulted in embryo resorption [*Sander et al. 2005*]. The various phenotypes of PCOS were determined after prenatal androgen exposure in rhesus monkeys, according to their gestational age. Late-treated (second half of the pregnancy) prenatally androgenised Rhesus females presented with anovulatory and behavioural traits resembling those of human PCOS, while early-treated animals exhibited not only PCOS traits but virilised genitalia [*Eisner et al. 2000, Dumesic et al. 2002*].

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There are several mechanism that may lead to hyperandrogenism during pregnancy, such as: placental aromatase deficiency [*Simpson et al. 1997*]; elevated free testosterone levels due to maternal PCOS [*Sir Petermann et al. 2002*]; adrenal 21-hydroxylase deficiency [*Barnes et al. 1994*] or 17,20 lyase excess [*Azziz et al. 1998*]; maternal IR [*Sir Petermann et al. 2002*]; foetal hyperandrogenism due to foetal ovarian hyperplasia; or genetic factors. Barbieri et al. reported that maternal hyperinsulinemia induces excessive placental human choriogonadotropin (HCG) secretion, which contributes to foetal ovarian hyperplasia and hyperandrogenism. [*Barbieri et al. 1986*] In conclusion, the pathophysiological processes of PCOS seem to start prenatally or in adolescence due to androgen excess; however, concomitant HA is not required for the development of PCOS.

The most important feature of PCOS is menstrual disturbance amongst all the other symptoms such as HA, oligo-amenorrhoea and obesity/metabolic syndrome. A hyperandrogenic state (clinical and/or biochemical) and menstrual disturbance are essential to the diagnosis of PCOS. However, elevated levels of serum androgen were only detected in a third to a half of the women with PCOS. [*Legro et al. 1998, Balen A. 2004*]. The IR is associated with the syndrome in 20-40% of patients [*Speer G. 2009*]. An elevated serum LH concentration can be detected in 40-60% of women with PCOS [*Balen AH.et al. 1995*]. An increased LH concentration is found to be associated with a high risk of miscarriage and infertility [*Balen AH. et al. 1993*], which is independent of HA or IR. The roles of the hypothalamic-pituitary-ovarian axis and increased LH secretion are still unclear. A correlation between the decrease of circulating LH concentration after laparoscopic bilateral ovarian diathermy or drilling (LOD) and the ovarian response by ovulation was reported (*Balen A. 2004*]. Although the mechanism of ovulation induction by LOD is unclear, it suggests that injury to the ovaries contributes to the induction of a local cascade of factors. A LOD procedure is commonly used as therapy in Hungary after unsuccessful attempts to stimulate the ovaries hormonally.

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1.5. Pathophysiology of PCOS

1.5.1. Hypothalamic-pituitary-ovarian axis

LH excess has been considered to play an essential role in development of ovarian HA and PCOS. LH hyper-secretion independently or along with insulin was demonstrated to contribute to ovarian hyperthecosis and elevated androgen levels [*Dumesic et al 2007, Lima et al. 2006*]. Elevated LH pulse amplitude and increased LH pulse frequency can be observed in two third of PCOS patients due to altered GnRh pulsatility, which causes a three-fold elevation in circulating LH versus FSH levels [*Dumesic et al. 2007*]. It was demonstrated in the 1990s that the ovaries played a primary role in the development of HA, instead of hypothalamic-pituitary system, in PCOS [*Balen A. 2004*]. These findings contradict the initial role of LH in the production of excess androgen in PCOS. LH secretion of PCOS patients shows a reduced hypothalamic sensitivity to progesterone negative feedback [*Marshall et al. 1999*], which can be restored by flutamide (androgen receptor blocker) [*Eagleson et al.2000*]. Exogenous dopamine has been reported to influence increases in LH levels through GnRh pulsatility, while naloxone, β-endorphin, and metoclopramide did not alter the circulating levels of LH. This suggests a deficiency in endogenous dopaminergic inhibition and an underlying hypothalamic defect in opioid control [*Cumming et al. 1984*]. The plasma inhibin and androstenedione concentrations are shown to be correlated, and women with PCOS have elevated levels of serum inhibin-B. Inhibin stimulates androgen production, and in response, androgen stimulates inhibin secretion. This explains the low concentration of follicle-stimulating hormone (FSH) compared to that of LH in anovulatory PCOS women [*Anderson et al. 1998*]. However, central hypothalamic-pituitary disturbances were determined to be secondary to peripheral ovarian factors [*Balen A. 2004*] and prepubertal hyperandrogenism seems to have initial role in reduced hypothalamic negative feedback with rapid GnRh pulsatility [*Chhabra et al. 2005*].

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1.5.2. Hyperandrogenemia

HA is one of the main features of PCOS, and the classical phenotype involves an increase in androgen production by ovarian cells. The definition of PCOS involves clinical and/or biochemical signs of HA. The primary clinical sign is hirsutism; however, the exact diagnosis is highly dependent on a screening method [*Tehrani et al. 2011*] because the assessment is often subjective and standardised scores (for different races) are rarely used [*Balen A. 2004*]. It is controversial to use the presence of acne and androgenic alopecia as indicators of HA [*Balen A. 2004*]. HA can be a biochemical marker for determining PCOS. Balen A. et al. reported that a third of patients with PCOS had elevated levels of serum testosterone in a study with over 1700 women [*Balen A. 2004*]. The measurements of androstenedione, DHEA, DHEA-S, sex hormone-binding globulin (SHBG), free testosterone or free androgen index (FAI) are also used.

The theca interna cells of the ovaries and zona fasciculata of the adrenal cortex synthesise androgens from cholesterol under the control of LH in the ovaries and ACTH in the adrenal cortex (*Fig. 1.*). Both glands secrete androstenedione in significantly greater quantities than testosterone. The rate-limiting factor in androstenedione production is the gene expression of P450c17, which is dependent on trophic hormones. Normally, elevation in the level of LH causes a down-regulation of the LH receptors; reduces cholesterol side-chain cleavage activity and 17,20 lyase activity; reduces the activity of 17-hydroxylase; and decreases androgen levels. The effects of trophic hormones can be modified by small peptides such as insulin and insulin-like growth factors (IGF) [*White DW. et al. 1995*]. In theca cells, the elevated expression of the LH receptor, insulin receptor (INR), lipoprotein receptor (HDL and LDL), steroidogenic acute regulatory protein (StAR), P450 side-chain cleavage (P450scc), 3β.hydroxysteroid dehydrogenase (3β-HSD), and cytochrome P450c17 leads to excess production of progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone [*Diamanti-Kandarakis et al. 2006⁂,2008*].

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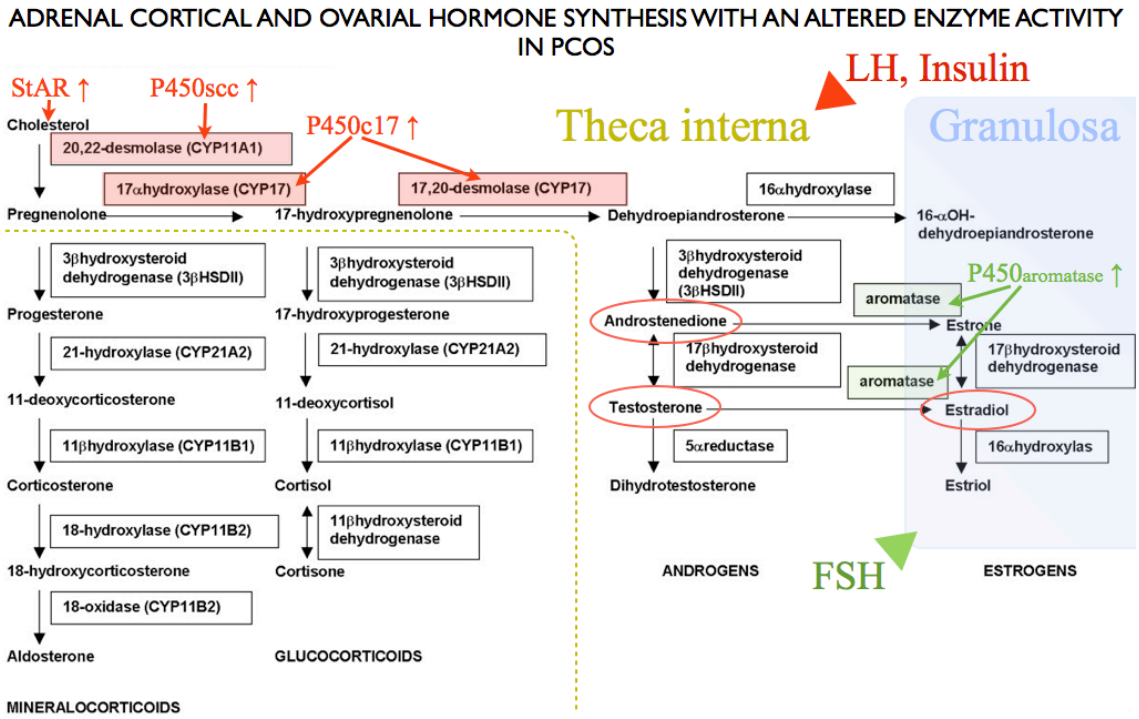

Figure 1.: **Steroid biosynthesis and altered enzyme activity of the adrenal cortex and the ovary in PCOS**. Cholesterol enters the mitochondria with the assistance of steroidogenic acute regulatory protein (StAR). In the mitochondria cholesterol is converted to pregnenolone by the cholesterol side chain cleavage enzyme (P450scc). Pregnenolone and Hydroxypregnenolone are converted to DHEA by microsomal P450c17. The increased expressions of these enzymes lead to higher levels of androstenedion and testosterone in hyperandrogenic PCOS patients. Oestradiol (Estradiol) level is also elevated due to increased expression of P450 aromatase enzyme. Over-expressions of enzymes highlighted in red contribute to androgen excess. Increased activity of aromatase enzymes highlighted in green tries to compensate hyperandrogenism. Applied and modified from: *MRCOG Facts. http://img.medscape.com/pi/emed/ckb/pediatrics_surgery/933425-940347-2132.jpg*

The level of oestradiol (E_2) increases in PCOS due to the availability of androgens for aromatase enzyme and hyper-reaction to FSH. The granulosa cells have been reported to lose FSH responsiveness and produce low quantities of progesterone [*Mason et al. 1994*]. Insulin acts as a co-gonadotropin and enhances the effects of testosterone by decreasing serum SHBG concentrations. The suppression of insulin secretion by a somatostatin analogue was demonstrated to lower both serum LH and androgen levels in women with PCOS [*Prelevic et al. 1990*].

Hyperandrogenemia has multiple effects on different tissues. HA leads to local pro-inflammatory stages in the ovaries and the endometrium. In contrast with the increase of the CD8+/CD4+ T lymphocyte cell ratio in the ovaries, an elevation in the number of CD4+ T lymphocytes and a parallel increase of tissue apoptosis was described in the endometrium [*Motta AB. 2010*]. In the ovarian pro-inflammatory stage, increased PGF2α levels, enhanced expression of cyclooxygenase-2 (COX2), nitric oxide synthase (NOS), and decreased levels of PGE, catalase and superoxide dismutase were described [*Elia et al. 2009*]. HA was found to induce morphological changes similar to precancerous endometrial structures [*Motta AB. 2010*]. These alterations of endometrial tissue are likely linked with infertility and recurrent miscarriage in PCOS.

Adipocyte cells pretreated with testosterone showed significantly impaired glucose uptake in response to insulin. This decrease was associated with a decreased insulin-stimulated phosphorylation of protein kinase C (PKC), which was reversible with anti-androgens such as cyproterone and partly reversible with flutamide [*Corbould A. 2007*⁂].

An impairment of the muscle glycogen synthase activity and minor changes in the muscle fibre composition were described in skeletal muscle cells after chronic testosterone treatment in rat model [*Corbould et al. 2008*]. Manneras et al. developed an animal model for human PCOS by administrating DHT to adolescent female rats. After ninety days, this treatment altered ovarian morphology (*Fig. 2.*) and vital parameters; early impairment of glucose metabolism was also found. They have suggested that the DHT model is suitable for studies of metabolic and ovarian features of the syndrome [*Manneras et al. 2007, Yanes et al. 2011*].

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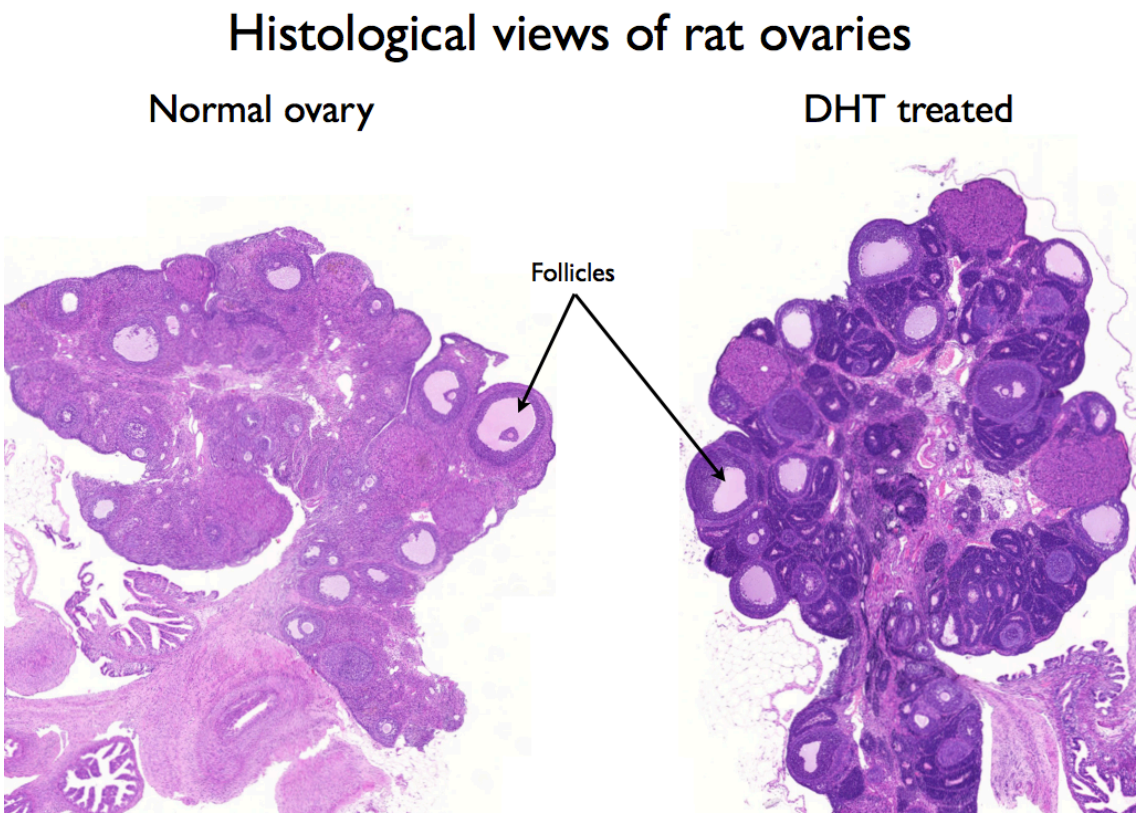

Figure 2.: **Histological views of whole rat ovaries.** A normal ovary is shown on the left side, and a DHT treated for ten weeks with PCO morphology (DHT 6. animal) is shown on the right side. Numerous premature follicles can be seen side by side peripherally in the ovary of the DHT-treated animal. From the experimental histological sections of the study.

1.5.3. Metabolic syndrome

Metabolic syndrome affects approximately 6% of women in the 20-29 year age group and 15% of 30-39 year age group. Among these women the prevalence of PCOS is twice to four times that of the normal population [*Moran et al. 2010*]. PCOS is often accompanied by metabolic syndrome, and the possibility of developing metabolic syndrome increases with age. The prevalence of metabolic syndrome was reported in 33-46% of women with PCOS [*Ehrmann et al. 2006*] The World Health Organization (WHO) criteria for metabolic syndrome were presented in 1999. The definition of metabolic syndrome requires the presence of one of the following: diabetes mellitus,

impaired glucose tolerance, impaired fasting glucose or IR, and two of the following: blood pressure ≥ 140/90 mmHg; dyslipidaemia (triglycerides ≥ 1.695 mmol/L and high-density lipoprotein cholesterol ≤ 1.0 mmol/L); either central obesity (waist-hip ratio > 0.85, or body mass index > 30 kg/m2); and either microalbuminuria (urinary albumin excretion ratio ≥ 20 μg/min or albumin: creatinine ratio ≥ 30 mg/g) [*Meigs JB. 2002*].

A prenatal exposure to androgen was reported to produce metabolic syndrome in adult female rats, which is similar to the PCOS model described earlier [*Demissie et al. 2008*]. Mehrabian et al. presented results on studies of Iranian women with PCOS and demonstrated a strong relationship between hyperandrogenism, IR and metabolic syndrome [*Mehrabian et al. 2011*]. The presence of central obesity in PCOS seems to be one of the most important independent factors leading to an approximately 14-fold higher risk for metabolic syndrome [*Ehrmann et al. 2006*]. Obesity associated with PCOS can be characterised by an increase in the size of fat cells (hypertrophic obesity) rather than an increase in the number of fat cells (hyperplastic obesity) [*Villa et al. 2011*]. In PCOS, adipocyte enlargement is strongly associated with IR [*Manneras et al. 2011*]. The lipolytic and storage capacity of the adipocyte cells is altered due to cellular hypertrophy. An increase in lipolysis and expression of the transmembrane proteins of fatty acid transport were detected [*Villa et al. 2011, Seow et al. 2009, Ek et al. 2002*] A significant decrease in lipoprotein lipase activity was demonstrated in subcutaneous adipocyte cells [*Manneras et al. 2011*].

1.5.3.1. Insulin resistance

Approximately 20-40% of women with PCOS, including obese and non-obese patients, have impaired glucose metabolism [*Speer G. 2009*]. IR also occurs in metabolic syndrome, T2DM, and PCOS. There are two types of IR syndrome that may develop independently in females. The type A is caused by an insulin signal defect; it affects

young women and is characterised by hyperinsulinemia, hyperandrogenism and obesity. Type B affects middle-aged women; it is characterised by signs of hyperandrogenism, autoimmune disorder and hyperinsulinemia. In Type B, the autoimmune antibodies block insulin binding or stimulate insulin receptors, leading to intermittent hypoglycaemia [*Powers AC. 2008*]. IR observed in PCOS is defined as a distinct entity of ovarian dysfunction but is similar to the type A IR syndrome. Although obesity is a common feature associated with IR, in PCOS, decreased insulin sensitivity is an independent component of body weight. The IR in PCOS is present at an incidence 50-70%, independent of obesity [*Mukherjee et al. 2010*]. The obese individuals with PCOS are more insulin resistant than their BMI counterparts without PCOS.

Insulin has a wide range of pleiotropic actions on the target tissue via different signalling pathways. Insulin affects the cellular metabolism, differentiation and growth of the target cells. After the binding of insulin to the α subunit of insulin receptor (INSR), the intrinsic tyrosine kinase is activated and its β subunits are auto-phosphorylated. The activated INR phosphorylates a number of substrates such as the insulin receptor substrate family (IRS1-4), Gab-1, Cb1, APS, homology domain transforming protein (Shc) isoforms and signal regulatory protein (SIRP) family members, which can bind to INSR [*Pessin et al. 2000*]. The phosphorylated IRS protein acts through several docking proteins such as the growth factor receptor-bound receptor 2 (Grb2), NcK and p85 subunit of phosphatidylinositol 3-kinase (PI3K). PI3K mediates metabolic actions such as glucose transport, glycogen synthesis, protein synthesis, and GLUT 4 translocation (helps in the rapid entry of glucose into cells); additionally, it activates Akt (AS160 substrate involved in GLUT4 translocation) and the atypical protein kinase C (PKC) isoforms [*Pessin et al. 2000, Hojlund et al. 2008*]. Insulin also has mitogenic actions mediated through the binding of phosphorylated IRS1/2 or Shc with the Grb-2/SOS complex, leading to p21Ras and Raf-1 activation of the mitogen-activated protein kinase pathway (MAPK). PI3K also facilitates the mitogenic response (*Fig. 3*.) [*Venkatesan et al. 2001*].

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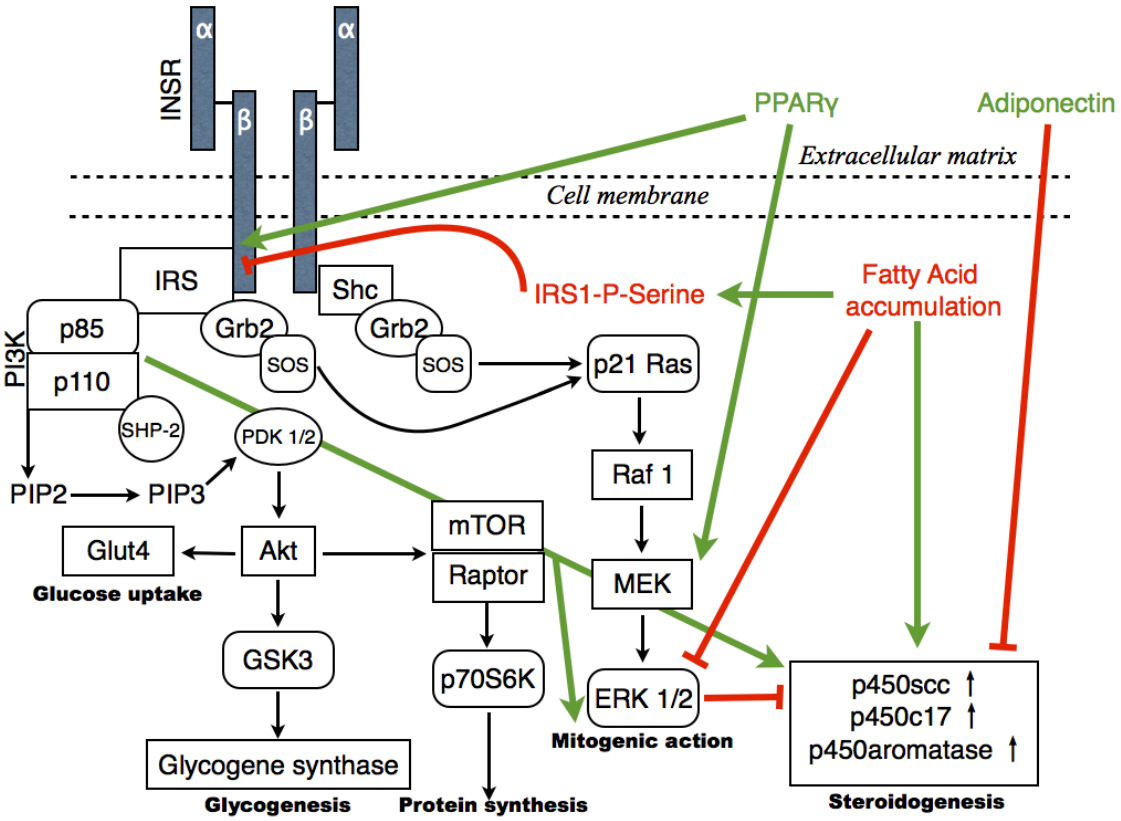

Figure 3.: **Illustration of major signalling pathways of insulin action and its modifying factors in PCOS**: Binding of insulin to its receptor (INSR) results in autophosphorylation and tyrosine kinase activation of the receptor. It phosphorylates other downstream mediators [insulin receptor substrate (IRS) and Src homology domain-containing transforming protein 2 (Shc)]. Various downstream signalling proteins are activated by these mediators. Phosphatidylinositol 3-Kinase (PI3K) regulates glucose transport, glycogenesis and protein synthesis. The Grb2/SOS (growth factor receptor-bound receptor 2/ Son of sevenless) complex activates the mitogen-activated protein kinase pathway (MAPK), playing a crucial role in cell proliferation. Another pathway that is not described yet may regulate steroidogenesis. Factors in red and green alter these pathways in PCOS. Red arrows show the inhibiting effects and green arrows the activation of cascades. Abbreviations: Akt-serine/threonine-specific protein kinase, PIP2-phosphatidylinositol 4,5-bisphosphate, PIP3- phosphatidylinositol 3,4,5-triphosphate, mTOR-mammalian target of rapamycin, Raptor-regulatory associated protein of mTOR, Glut4- glucose transporter type 4, GSK3-glycogen synthase kinase 3, PDK1/2-phosphoinositide dependent kinase1/2, p70S6K- ribosomal protein S6 kinase, MEK- mitogen-activated protein kinase, ERK1/2- extracellular signal regulated kinase, SHP-2- SH2 domain containing protein tyrosine phosphatase. On the basis of *Mukherjee et al. Indian J Med Res. 2010;131:743-60.*

A post-receptor binding defect in the insulin signalling pathway appears to play the primary role in the aetiology of selective IR. There is evidence that the tyrosine kinase auto-phosphorylation of the insulin receptor β subunit decreases and the serine-phosphorylation increases in PCOS. The β subunit can phosphorylate IRS-1 protein, which can activate PI3K and GLUT4 [*Pessin et al. 2000, Mukherjee et al. 2010, Speer G. 2009*]. Decreased auto-phosphorylation, tyrosine activation and the parallel increase in

serine-phosphorylation influence the intracellular signal transduction of insulin and diminish glucose uptake. The serine-phosphorylation of the β subunit can stimulate the lyase activity of the citochrom-P450c17α enzyme [*Bremer et al. 2008*](*Fig. 3.*).

The reduced insulin-receptor level and the increased levels of biologic markers (e.g., free fatty acid, leptin, TNFα, retinol-binding protein) secreted by adipocytes may play a secondary role in development of IR in PCOS, as well as impaired fatty acid oxidation, which generates reactive oxygen species such as lipid peroxides. The elevated levels of free fatty acids and reactive oxygen species have a lipotoxic effect on beta cells in the pancreas [*Powers AC. 2008*]. Both TNFα and PC1 membrane glycoprotein were reported to decrease tyrosine kinase activity [*Spaczynski et al. 1999*, *Speer G. 2009*]. The accumulation of lipids in myocytes impairs mitochondrial oxidative phosphorylation and reduces the insulin-stimulated production of mitochondrial ATP [*Powers AC. 2008*]. Other researchers have found a correlation between lipin 1β protein and IR [*Mlinar et al. 2008*]. Lipin 1 plays a key role in triglyceride (TG) and phospholipid synthesis and acts as a transcription co-activator in fatty acid oxidation. Lipin 1 was shown to have an insulin sensitising effect on tissues [*Peterfy et al. 2001*]. Lipin 1β is the main secreted isoform of mature adipocytes and increases the expression of genes involved in TG and fatty acid synthesis [*Peterfy et al. 2005*]. The lipin 1 concentration in the adipose tissue and the liver is inversely related to obesity and IR [*Croce et al. 2007*]. Mlinar et al. demonstrated that lipin 1β level are inversely correlated with BMI, waist circumference and HOMA-IR [*Mlinar et al. 2008*] in visceral adipose tissue. A positive correlation was demonstrated between lipin 1β level and HDL-cholesterol.

A low level of adiponectin was detected in patients with PCOS (*Fig. 5.*). This decrease was independent of BMI, but showed a strong correlation with IR [*Toulis et al. 2009*].The messenger molecule (adipokine) has a direct insulin-sensitising effect, and lower levels of adiponectin can contribute to IR. These pathways can elucidate the increased severity of IR in obesity (*Figs. 4. and 5.*). Adipocyte production and decreased levels of adipokines contribute to an inflammatory state, which explains the elevated levels of

hsCRP and IL-6 often seen with IR and T2DM [*Powers AC. 2008*]. Antilla et al. studied obese and non-obese women with PCOS and found that the non-obese women had elevated serum concentrations of bioactive LH related to hyperinsulinaemia. These changes were independent of androgen concentrations and ovarian androgen production. Antilla proposed that the degree of hyperinsulinemia and the severity of IR directly affected the glycosylation of LH (modifying the bioactivity of LH isoforms) [*Antilla et al. 1991*].

The altered response of insulin to different tissue cells was investigated in PCOS. In adipocytes, basal autophosphorylation of the IR β-subunit was normal and insulin-dependent autophosphorylation was significantly diminished, but receptor-kinase activity was normal [*Futterweit W. 1999*]. Decreases in levels of IRS-1 and IRS-2 tyrosine phosphorylation as well as in the expression of GLUT4 were also demonstrated [*Diamanti-Kandarakis et al. 2006*⁎⁎]. These changes show that there is no defect in the binding of insulin molecules to INR. The diminished insulin sensitivity in PCOS cannot be explained by defective binding at the receptor level. Thus, a step downstream of receptor binding is supposed to act in the insulin signal-transduction pathway, that is defective in IR [*Diamanti-Kandarakis et al. 2006*⁎⁎]. In fibroblast cells, basal autophosphorylation was increased in approximately 50% of the cultures obtained from PCOS patients [*Dunaif et al. 1995*]. A serine kinase associated with a PKA-regulated pathway is related to IR in cultured fibroblasts of PCOS women [*Diamanti-Kandarakis et al. 2006*⁎⁎]. In these cultured cells, IRS-1 tyrosine phosphorylation and insulin-dependent glycogen synthesis were decreased [*Diamanti-Kandarakis et al. 2006*⁎⁎], while IRS-1 expression and PI3K levels were normal. The decrease in the glycogen synthesis might have been caused by the reduced GSK-3 phosphorylation [*Venkatesan et al. 2001*]. In adipocytes, similar pathways have been observed. In skeletal muscle cells, a decreased IRS-1-associated PI3K activity was demonstrated *in vivo,* which was not completely compensated by elevated IRS-2 expression; therefore, the insulin-mediated glucose uptake remained decreased [*Dunaif et al. 2001*]. In contrast, a higher expression of IRS-1 and GLUT1 and reduced activity of the IRS-1-PI3K and IRS-2-PI3K complex

were detected *in vitro* [*Corbould et al. 2005*].

An increase in the prevalence of PCOS and its phenotypic features has been demonstrated in twin and family studies [*Diamanti-Kandarakis et al. 2005*]. The mode of inheritance is still not clear, but several gene variants are proposed to interact with each other and the environment in the manifestation of the syndrome. A strong linkage has been observed between the susceptibility to the disease and a dinucleotide marker on chromosome 19p13 [*Urbanek et al. 2005*]. The possible gene variants related to PCOS are depicted in *Table 2*.

IR increases the risk for developing glucose intolerance, T2DM, hypertension, dyslipidaemia and cardiovascular abnormalities in PCOS patients [*Lambrinoudaki I. 2011, Agarwal et al. 2010, Nakka et al. 2011*]. The severity of obstructive sleep disorders, which is often observed in PCOS, presents a strong correlation with IR than with the BMI [*Nitsche et al. 2010*]. IR can be reversed by lifestyle modifications as a first line of therapy [*Karakas et al. 2010*]. Other agents such as insulin sensitisers are recommended when cardiovascular risk factors persist in spite of lifestyle modification [*Katsiki et al. 2010*]. The drug metformin is a biguanide (N,N-dimethyl-biguanide) and can improve insulin sensitivity by increasing glucose uptake in peripheral tissues, stimulating glycolysis, decreasing liver glucose production and inhibiting intestinal glucose absorption [*Katsiki et al. 2009*]. Its effects on lipid metabolism, atherosclerosis and inflammatory markers of metformin are controversial [*Katsiki et al. 2010*]; however, it is reported to increase adiponectin level [*Agarwal et al. 2010*]. The use of metformin treatment alone or in combination with clomiphen-citrate improves menstrual irregularities and infertility. The positive effect of metformin on the cardiovascular system of patients with PCOS was also reported [*Agarwal et al. 2010, Palomba et al. 2010, Nakka et al. 2011*]. The drug significantly decreases the level of total testosterone, free testosterone and androstendione, while it increases the level of SHBG without affecting the levels of FSH, LH or DHEAS.

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Table 2.: **Gene variants related to insulin resistance in PCOS.** INR: insulin receptor, IRS1/2: insulin receptor substrate 1-2, ENPP1: Ectoenzyme nucleotide pyrophosphate phosphodiesterase(PC-1) gene, PPARγ: Peroxisome proliferator activated receptor gamma gene, CAPN10: Caplain-10(cystein protease) gene, PON1: Paraoxonase-1 gene, ADIPOQ: adiponectin gene, RETN: resistin gene, LEP and LEPR: leptin and leptin receptor genes, VNTR: variable number of tandem repeats. From: Mukherjee S. Molecular & genetic factors contributing to insulin resistance in polycystic ovary syndrome. *Applied and modified from: Mukherjee et al. Indian J Med Res. 2010;131:743-60.*

Gene	Variant/Locus	Phenotypic traits
Insulin	VNTR	PCOS, Hyperandrogenemia
INR	D19S884, His1058,+176447	PCOS
	Cys1008	PCOS, Insulin sensitivity
IRS1/2	Gly972Arg, IRS-1	PCOS,↑Fasting glucose, obesity, IR
	Gly1057Asp, IRS-2	PCOS, ↓↑2h- glucose,
	Ala513Pro, IRS-1, Gly1057Asp, IRS-2	PCOS
ENPP1	K121Q	PCOS
PPARγ	Pro12Ala	PCOS,↑Obesity,↑ Fasting insulin,↓IR
	1431 C/T	PCOS, ↑ BMI, ↑serum leptin
	His447His in exon 6	↓ Testosterone,↓ Insulin and IR
CAPN 10	Genotype 112/121	PCOS, ↑ fasting insulin
	Haplotype 111, diplotype 111/121 and 111/111	PCOS
	Haplotype 112, diplotype 112/121	↓PCOS risk
	UC SNP-44 Haplotype 1121	PCOS
	UC SNP-45, Haplotype 2111 and 1221	PCOS
PON1	-108C/T, L55M, Q192R	PCOS
ADIPOQ	T45G, G276T	PCOS,↑Serum adiponectin, ↓Insulin
	-11377	PCOS
RETN	-420C/G, -179C/G	PCOS
LEP	Coding region	PCOS
LEPR	K109R, Q223R, K656N, 3′ UTR	PCOS, low insulin
	Q223R	PCOS

Metformin treatment in combination with oral contraceptives (OCs), pioglitazone and flutamide has a stronger modulatory effect on hormone levels [*Katsiki et al. 2010*]. However, metformin is used worldwide without a full understanding of the mechanism involved. The thiazolidinediones (TZDs) are ligands of the peroxisome proliferator-activator receptor-gamma (PPARγ), a nuclear transcription factor, which is mainly expressed in adipose tissue, intestinal, pancreatic beta cells, macrophages, vascular endothelium and skeletal muscle [*Perry et al. 2002*] The two best-known agents in this group, rosiglitasone and pioglitasone, were reported to improve IR (HOMA-IR and insulin levels); restore menstrual cycles; induce ovulation; increase adiponectine, HDL-C and SHBG levels; decrease androgen, TC, TG, LDL and hs-CRP levels; and improve

hirsutism [*Ortega-Gonzalez et al. 2005, Glintborg et al. 2005*]. TZDs compared with OCs have superior effects in improving IR but inferior effects in reducing hyperandrogenism [*Katsiki et al. 2010*]. The role of TZDs, as a regulatory mechanism of ovarian function, indicates a relationship between insulin signalling, steroidogenic productions and PPARγ [*Mukherjee et al. 2010*]. The possible action of PPARγ on insulin signalling and the mitogenic cascade is depicted in *Fig. 5*. A new insulin sensitiser, BGP-15 (O -[3-piperidino-2-hydroxy-1-propyl]nicotinic-amidoxime-dihydrochloride), has been reported to have a non-PPAR agonist mechanism of action[*Literati-Nagy et al.2009*]. This new molecule, a co-inducer of heat shock proteins, might be a better agent for PCOS therapy. It has been suggested that vitamin D therapy also has positive effects on carbohydrate metabolism [*Yiu et al. 2011, Kotsa et al. 2009, Pittas et al. 2006, Hypponen et al. 2001*] and can prevent cardiovascular complications [*Wong et al. 2010, Przybylsky et al. 2010*].

1.5.3.2. Dyslipidemia

Based on ethnicity, genetic factors and lifestyle, 30 to 70% of PCOS patients present with abnormal levels of serum lipids [*NCEP 2002*]. PCOS patients with IR show a higher prevalence of dyslipidemia than their counterparts without IR (81 to 65%, respectively). The possible pathways of altered adipose tissue function due to IR were mentioned above. IR contributed to elevated triglyceride (TG), increased low-density lipoprotein-C (LDL-C) and very low-density lipoprotein (VLDL); decreased high-density lipoprotein-C (HDL-C) levels due to the impaired functions of the adipose tissue [*Brunzell et al. 2003, Wild et al. 1985*]. The changes in lipoprotein levels are strongly accompanied with an altered predominance of LDL-III and LDL-IV (small and very-small subspecies of LDL) and decreased ratios of the anti-atherogenic HDL_2 subtype in PCOS. Androgens also affect lipoprotein metabolism. Testosterone lowers HDL levels by increasing the expression of the genes involved in HDL catabolism; scavenger receptor B1 (SR-B1) mediates the selective uptake of HDL to hepatocytes,

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and hepatic lipase increases the clearance of HDL by catalysing HDL_2 conversion to HDL_3 [*Diamanti-Kandarakis et al. 2007*](*Fig. 4.*).

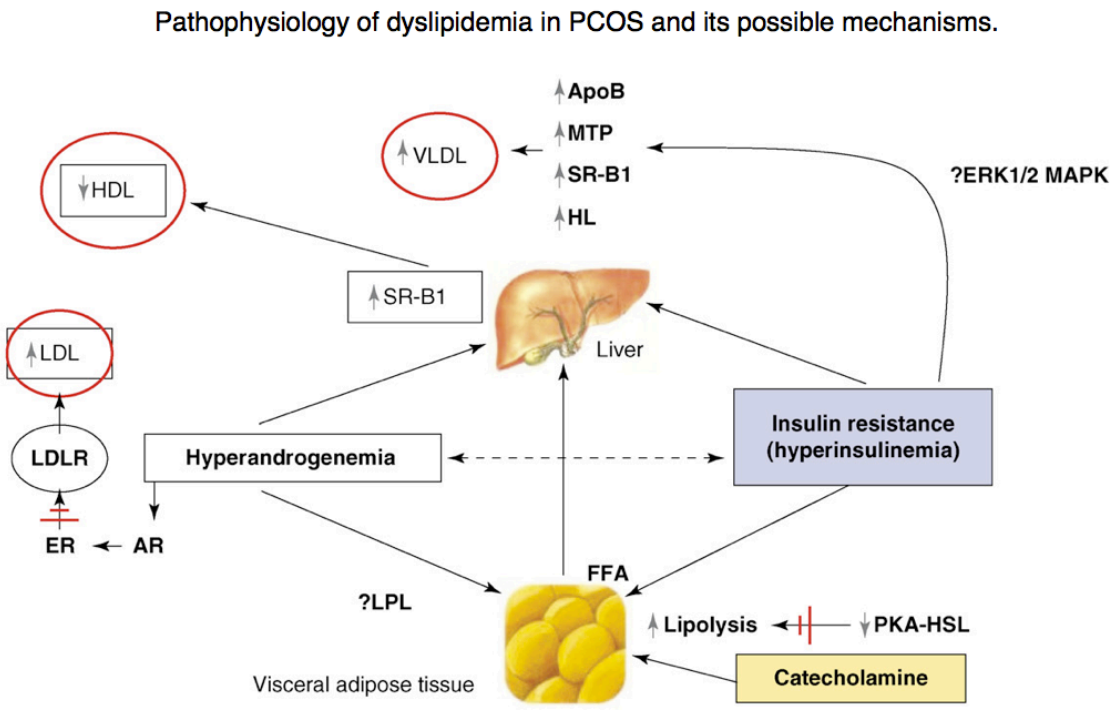

Figure 4.: **Pathophysiology of dyslipidaemia in PCOS and its possible mechanisms.** The main serum lipid abnormalities in PCOS are indicated in red circles. Broken arrows represent potential interaction. ↑ activation; ↓ deactivation; ≠ inhibition. Abbreviations: ApoB, apolipoprotein b; AR, androgen receptor; ER, estrogen receptor; ERK1/2 MAPK, extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase; FFA, free fatty acids; HL, hepatic lipase; LDLR, low density lipoprotein receptor; LPL, lipoprotein lipase; MTP, microsomal triglyceride protein; PKA-HSL, protein kinase a-hormone sensitive lipase complex; SR-B1, scavenger receptor-b1; VLDL, very low density lipoprotein. Adopted *from Diamanti-Kandarakis E. et al. Endocrinol Metab. 2007;18:280-5.*

IR increases the expression of microsomal triglyceride protein (MTP), which in combination with excess TG contributes to the overproduction of apolipoprotein B (ApoB) and a subsequent increase in VLDL production [*Diamanti-Kandarakis et al. 2007, Taghibiglou et al. 2000*](*Fig. 5.*). Apolipoproteins act on the surface of the circulating particles and helps direct these particles in metabolic and clearing patterns. An elevated ApoCIII/ApoCII ratio was described in PCOS [*Wild et al 2012*]. ApoCIII/CII regulates

lipoprotein lipase activity and responsible for VLDL metabolism. Additionally, VLDL has a primary role in TG metabolism. ApoCIII can block CII, ApoB and E (delay VLDL lipolysis), which results in greater amounts of circulating LDL in the plasma [*Wild et al. 2012*]. Serum levels of ApoB might be a better atherogenic predictor than non-HDL cholesterol levels [*Wild et al. 2012*].

A decrease in lipolysis and a reduction in chatecholamine-stimulated PKA/hormone-sensitive lipase complex activation were observed in subcutaneous fat tissue but not in visceral fat adipocytes after chronic testosterone treatment [*Dicker et al. 2004, Villa et al. 2011*]. These adipocyte lipolytic function impairments can be attributed as a secondary cause of hyperandrogenism [*Villa et al. 2011*]. Androgens regulate lipoprotein lipase activity. which was detected to be positively correlated with plasma free testosterone levels [*Iverius et al. 1988*]. Dihydrotestosterone was demonstrated to increase Lipoprotein lipase (LPL) levels [*Anderson et al. 2002*]. These patterns of dyslipidemia and IR might play a primary role in atherosclerosis in PCOS.

The adipose tissue secretes different types of signalling molecules. In PCOS, the production of leptin is controversial. The overexpression of this messenger protein can induce atherosclerosis and endothelial dysfunction [*Reilly et al. 2004, Knudson et al. 2005*] and influence hypothalamic GnRH secretion [*Walters et al. 2012*]. Elevated levels of plasma leptin were reported to positively correlate with Retinol binding protein 4 (RBP4) and asymmetric dimethylarginine in young women with PCOS, however these factors have a poor predictive value in PCOS. [*Yildizhan et al. 2011*]. The association between elevated levels of leptin and IR or obesity is still debated. Adiponectin was demonstrated to be an insulin sensitising and anti-inflammatory messenger molecule [*Weyer et al. 2001*]. Adiponectin levels are known to be decreased in obesity. Adiponectin produced by adipocytes is considered a useable marker for metabolic syndrome in PCOS because its level inversely related to adipocyte mass [*Groth SW. 2010*]. Low adiponectin levels measured in the morning and evening are usually associated with obstructive sleep apnea [*Nitsche et al. 2010*] in PCOS. Lipin 1 is a nuclear protein that is

essential for adipocyte metabolism. Mlinar et al. studied lipin 1β levels in subcutaneous and visceral fat adipocytes from patients with PCOS and controls patients and found that lipin 1β was negatively correlated with BMI, waist circumference and IR [*Mlinar et al. 2008*]. The level of lipin 1 was significantly lower in visceral adipose tissue than in subcutaneous adipose tissue after adjusting for BMI. Lipin 1 polymorphisms might also determine the development of PCOS. Lipin 1 gene variations were shown as possible factors in cardiometabolic complications of PCOS [*Mlinar et al. 2011*].

1.5.3.3. Hypertension

Hypertension is not sine qua non but is a very common symptom in PCOS, often due to developing metabolic syndrome, which starts mainly in the fourth - fifth decade of life. Increased arterial stiffness in PCOS accompanying hemodynamic changes with ageing contributes to high systolic blood pressure and pulse pressure and an increased ventricular load. The relationship between IR/hyperinsulinemia, hyperandrogensim and hypertonia is still under research. Some reports explained that hypertension seen in PCOS is a consequence of obesity and increased sympathetic activation [*Luque-Ramirez et al. 2007*⁎]. Hypertension was reported to develop in 2-years-old female sheep, which had been prenatally treated with Testosterone-propionate for 60 days [*King et al. 2007*]. Manneras and Yanes have presented a rat PCOS model in which hypertonia also evolved after chronic dihydrotestosterone treatment (12 weeks) [*Manneras et al. 2007, Yanes et al. 2011*]. These animal models can prove that prenatally or postnatally induced PCOS correlates with hypertension, however human cohort studies are controversial.

1.5.4. Connection between Hyperandrogenism and Insulin Resistance

Glucose metabolism is not altered in insulin-independent tissues or insulin-dependent

pathways, which might sometimes impair mitogenic-activated protein kinase signals that control cell growth and differentiation. The acceleration of these hyperinsulinaemia pathways may cause hyperandrogenism and atherosclerosis [*Powers AC. 2008*]. In recent studies, both hyperandrogenemia and hyperinsulinemia were established as the principal factors of PCOS. The cause and effect relationship between these features is still debated [*Dunaif A. 1997, Bremer et al. 2008*]. There is evidence that insulin can augment steroidogenesis in the ovaries and may reduce the liver production of sex hormone-binding globulin (SHBG) directly or through elevated levels of LH [*Poretsky et al. 1999, Bremer et al. 2008*]. There are two hypothesis regarding androgen synthesis in ovaries by insulin. It was demonstrated that insulin stimulates testosterone biosynthesis and acts directly via its receptor at physiological concentrations in cultured polycystic ovary theca [*Nestler et al. 1998*] and granulosa cells [*Willis et al. 1995*]. Insulin-induced androgen excess occurs via IGF1 receptor activation because insulin acts through IGF1 only when the circulating levels of insulin are extremely high [*Poretsky et al. 1999*]. Steroidogenesis is mainly regulated by LH in theca cells of ovaries via the cAMP-dependent protein kinase (PKA) pathway. Insulin alone was not found to increase cAMP, but it enhanced LH-induced cAMP accumulation in porcine theca cells [*Zhang et al. 2000*]. This accumulation might activate PI3K, MAPK or an alternate pathway for insulin signalling. In human theca cells, insulin requires concomitant cAMP signalling by forskolin to enhance 17α-hydroxylase activity [*Munir et al. 2004*]. This activity is mediated via the PI3K pathway and not via the MAPK pathway [*Munir et al. 2004*], indicating one of the differences between steroidogenic and metabolic pathways because the glucose metabolic pathway does not require concomitant cAMP signalling. The Akt (serine / threonine-specific protein kinase, protein kinase B) activation pathway may be a different insulin-dependent pathway for steroidogenesis [*Diamanti-Kandarakis et al. 2006*⁎⁎]. The altered MAPK pathway with reduced MAPK1/2, extracellular signal-regulated kinase 1-2 (ERK1/2) activity and a parallel increase of CYP17 mRNA expression, and DHT activity might play insulin-independent roles in androgen excess and hyperandrogenism [*Nelson-Degrave et al. 2005*]. Another hypothesis suggests that serine phosphorylation of the β subunits of INR and P450c17 by a “hypothetic” kinase

can contribute to both hyperandrogenemia and hyperinsulinemia [*Bremer et al. 2008*]. The above mentioned serine kinase has not been identified yet. The debate about the hypersensitivity of insulin or the state of the preserved ovarian sensitivity still continues. Poretsky pointed at the high androgen excess of ovarian cells independent of hyperinsulinemia [*Poretsky L. 2006*]. However, cultured theca cells after stimulation by insulin exhibited enhanced androgen production [*Mukherjee et al. 2010*]. Insulin sensitisers were found to decrease androgen levels. Insulin can also facilitate ACTH-stimulated androgen secretion in the adrenal cortex [*Speer G. 2009*]. This activation is also responsible for the hyperandrogenemia observed in IR [*Speer G. 2009, Bremer et al. 2008*]. The higher intraovarian insulin level increases the intraovarian androgen level.

This may halt the maturation of the follicles, increase FSH sensitivity to the granulosa cells and stimulate follicle genesis, contributing to typical polycystic ovarian morphology [*Speer G. 2009*]. Hyperinsulinemia and IR are responsible for the decreased sensitivity and metabolic effectiveness of insulin in different tissues, including in the ovaries. The ovaries may otherwise remain sensitive to insulin and produce androgen [*Mukherjee et al. 2010*]. The pharmacological reduction of insulin levels has been found to improve both hyperinsulinemia and hyperandrogenemia [*Dunaif A. 1997 ,Poretsky et al. 1999, Bremer et al 2008*]. However, the reduction of androgen levels by bilateral oophorectomy and administration of GnRH agonist [*Bremer et al. 2008*] or antiandrogenic compounds [*Dunaif A. 1997*] was reported to be ineffective for IR or hyperinsulinemia in PCOS patients.

Insulin can affect steroid production in granulosa cells, as well. Insulin alone or with FSH augments P450 aromatase activity, stimulates progesterone, and affects only E_2 production in anovulatory PCOS [*Mukherjee et al. 2010*]. In granulosa cells, neither MAPK nor PI3K seemed to be involved in the insulin-mediated pathway of steroidogenesis [*Poretsky et al. 2001*]. Both insulin and LH synergistically up-regulate the transcription of the LDL receptor to facilitate cholesterol uptake [*Sekar et al. 2001*]. Rice and colleagues reported that an abnormal glucose metabolism, with significantly

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impaired insulin, stimulated lactate production in granulosa-lutein cells obtained from women with anovulatory PCOS [*Rice et al. 2005*]. These granulosa cells exhibited normal steroid production in response to physiological doses of insulin. These findings suggest that insulin activity is not only present at the organ level but also in the microenvironment of the ovary [*Diamanti-Kandarakis et al. 2006*⁎⁎⁎]. Granulosa cells seem to be resistant to insulin-mediated glucose metabolism while maintaining the insulin regulation of ovarian steroidogenesis.

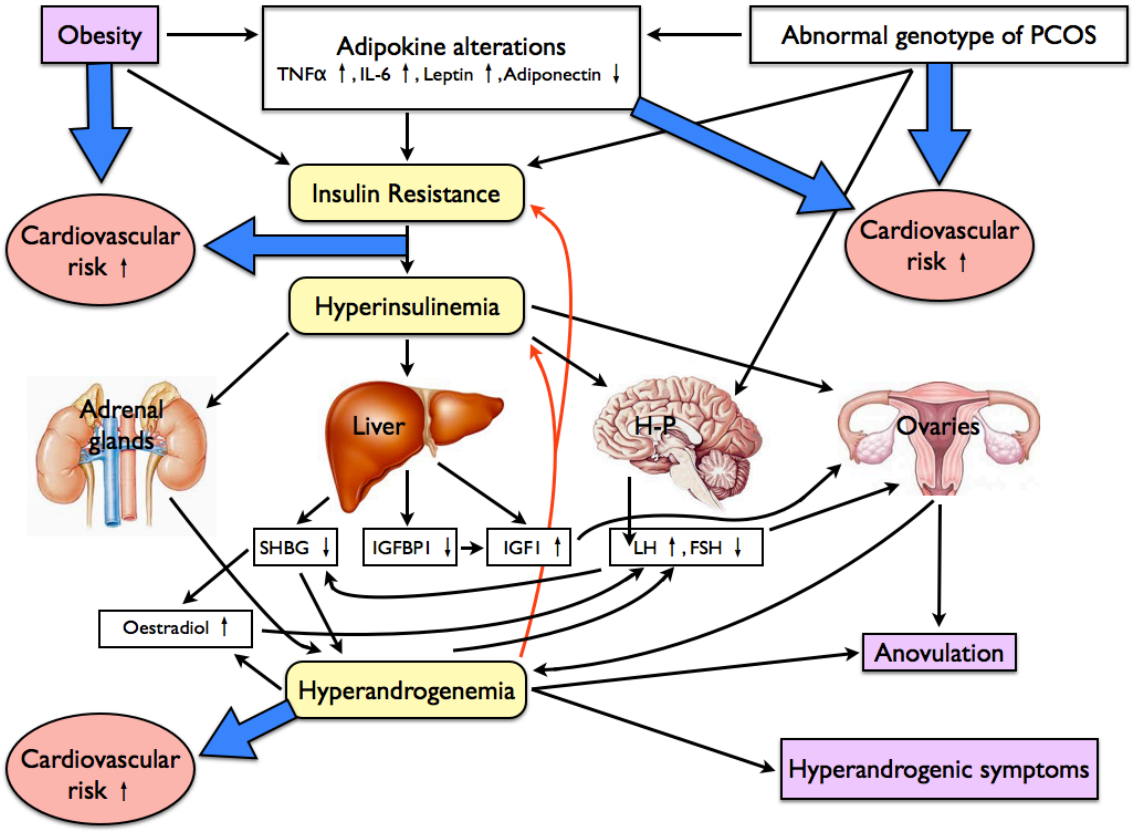

Figure 5.: **The pathophysiology of PCOS.** The black and red arrows show direct effects on altered processes and relationships between biochemical/laboratory changes. Blue arrows indicate the increased cardiometabolic risks. TNFα: Tumour Necrosis Factor α, IL-6: Interleukin-6, SHBG: Sexual Hormone Binding Globulin, IGFBP1: Insulin-like Growth Factor Binding Protein 1, LH: Luteotropic Hormone, FSH: Folliculotropic Hormone, HP: Hypothalamus-pituitary system. Adopted and modified from *Speer G. In: Lakatos P, Speer G. Polycystic Ovary Syndrome. Budapest. Semmelweis Kiadó, 2009. p.31.*

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1.5.5. Vascular alterations in PCOS

In earlier sections, it was mentioned that women with PCOS have a higher risk for CVDs. Obesity, IR or T2DM, hypertension, and dyslipidemia associated with PCOS increase the risk for CVDs. IR and obesity were proposed to have a central role in the development of CVDs [*Meyer et al. 2005, Cussons et al. 2009*]. Although there is a higher incidence of cardiovascular risk factors among PCOS patients, the existence of a significantly higher CVD incidence compared to control patients is debated [*Iftikhar et al. 2012*]. In research yet little is known about cardiovascular alterations of PCOS.

In PCOS, increased arterial stiffness and pulse wave velocity were demonstrated by ultrasound assessments [*Bots et al. 2002*]. The mechanism of these alterations is unclear, but endothelial dysfunction and altered collagen metabolism of the vessel walls may be involved. This is similar to the patterns seen in IR and metabolic syndrome [*Cussons et al. 2009, Orio et al. 2005*]. Human studies have shown both macro- and microvascular dysfunction measured by ultrasound in PCOS, as indicated by the reduction in acetylcholine(ACh)-dependent vasodilation. This impaired response to ACh is similar to that observed in non-insulin-dependent diabetes mellitus. It is proposed to be related to metabolic alterations, especially insulin resistance in PCOS [*Lakhani et al. 2005, Kravarati et al. 2005, Diamanti-Kandarakis et al. 2006*⁎]. Lakhani and Hardiman showed that those women who have a decreased internal carotid artery pulsatility index in PCOS, have a higher cardiovascular risk as well *[Lakhani et al. 2000].* Women with PCOS have a high prevalence of early-onset atherosclerosis, metabolic syndrome and insulin resistance, and they might develop hypertension during their reproductive period [*Dokras et al. 2008, Wild et al. 2012*]. These abnormalities are partly explained by the pharmacological and biomechanical remodelling of resistance arteries. The intima-media thickness of the carotid arteries was confirmed to be greater in PCOS than in age- and BMI-matched controls [*Talbott et al. 2000*]. Similarly, coronary artery calcification was found to be greater in PCOS patients [*Talbott el al. 1995, Christian et al. 2003*]. An endothelial dysfunction is the first step in the formation of atherosclerosis. Uncoupling

of eNOS due to stoichiometric imbalance between l-arginine/asymmetrical dimethylarginine [*Yildizhan et al. 2011*], NADPH and tetrahydrobiopterin impairs NO synthesis in PCOS. Endothelial dysfunction might also increase endothelial cell secretion of endothelin-I and the level of angiotensin [*Cussons et al. 2009*]. The exact molecular mechanisms of the vascular alterations in PCOS are still unclear. Motta et al. hypothesised that the accumulation of reactive oxygen species, toxic oxygen-derived products and nitrogen-derived products might contribute to an uncontrolled lipid peroxidation of the cell membranes [*Motta AB. 2010*]. The stimulation of NADPH oxidase and the uncoupling of mitochondrial oxidative phosphorylation can impair NO synthesis [*Chew et al. 2004*]. An increase in the circulating levels of oxidative stress markers was observed due to the acceleration of lipid metabolism in women with PCOS [*Sabuncu et al. 2001*]. The ApoA/ApoB ratio was described as a sensitive indicator for CVD and atherogenic risk factor in PCOS [*Wild et al. 2012, Yusuf et al. 2004*]. These might explain the cardiovascular disorders often seen in PCOS.

Hyperinsulinemia and IR often associates with PCOS. The independent role of insulin in human vessel relaxation has been demonstrated. Luis A et al. have presented an insulin-mediated direct mechanism of relaxation on renal efferent arterioles in rabbits [*Luis et al. 1993*]. Insulin induced dilation on efferent arterioles was reported to be independent of EDRF/NO and prostaglandins, however the mechanism of insulin induced direct vascular effect remained unclear. Insulin was suggested to affect IP3 release of Ca^{2+} by a cGMP-dependent mechanism that would contribute to its vasodilatory effect [*Saito et al 1993*]. Insulin may also act through NO and Na^{+}-K^{+} ATP-ase in human muscle arterioles [*Steinberg et al. 1994, Scherrer et al. 1994*, *Tack et al. 1996, Yki-Jarvinen et al. 1998*].

In clinical practice, the chronic treatment of PCOS modifies both the reactivity and the mechanical properties of the arteries. Recently, the positive effect of insulin-sensitising therapy on the mechanical and pharmacological responsiveness of arteries has been shown [*Agarwal et al. 2010, Nakka et al. 2011*]. Metformin was shown to minimise

alterations caused by oxidative stress, with elevation of antioxidant concentrations, such as glutathione concentrations, in the ovary and uterus. Metformin also restores TNFα and NOS levels [*Motta AB 2010*]. Metformin, which is a promising therapeutic agent in PCOS, especially in infertility cases, has been shown to decrease this cardiovascular risk [*Meyer et al. 2007*]. Vitamin D may also be used as adjuvant therapy for women with PCOS [*Thys-Jacobs et al. 1999*]. The positive effect of vitamin D therapy on the prevention of general cardiovascular complications has been demonstrated and will be detailed later [*Wong et al. 2010, Przybylsky et al. 2010*]. Low levels of vitamin D_3 confer a smooth muscle cell proliferative effect and increase the levels of elastin fibres, with altered maturation in the aortic wall. [*Tukaj C. 2008*]. In clinical practice, a high-dose of oral contraceptives is rarely administered because of possible increases in cardiovascular risk. Low doses of oral contraceptives likely have no significant effect on cardiovascular system [*Agarwal et al. 2010, Kravariti et al. 2005*].

1.6. Animal models of PCOS

Several animal models have been developed over the last four decades for studying PCOS. Some of them are very similar to human ovulatory and metabolic alterations of PCOS.

1.6.1. Pre- and postnatal models in mammals

It was demonstrated in some mammals model, such as the rhesus monkey and sheep [*Eisner et al. 2000., Dumesic et al. 2002, Recabarren et al. 2005*], that PCOS developed postnatally after prenatal androgen exposure. These models drew attention to the importance of the timing of androgen excess during pregnancy and might explain the linkage between gonadal development and different phenotypes of PCOS. Primates and

other precocial mammals such as the guinea pig, sheep, and rhesus monkey exhibit ovulatory dysfunction when exposed to hyperandrogenism before birth [*Abboth et al. 2008*]. In altricial mammals, prenatal androgen treatment can be ineffective (except excessive DHT treatment) due to their delayed sexual differentiation, neural development and specific placental barrier [*Abboth et al. 2005*]. A delayed puberty and ovulation dysfunction evoked by postnatal androgen treatment was described in dog, piglet, hamster, rat and mice models [*Abboth et al. 2005*]. The key mechanism of anovulation induction is also different in primate and non-primate models. In primates the abnormal function of ovaries plays the primary role, in contrast to non-primates, where the hypothalamic-pituitary system and LH surges seem to induce androgen excess [*Abboth et al. 2005*].

Abhilasha et al. reported an interesting animal model very similar to human PCOS in the Indian bat Scotophilus heathi [*Abhilasha et al. 1997*]. During the period of quiescence, there is an increase in body mass due to the accumulation of adipose tissue, increased levels of androstenedione, reduced glucose tolerance and IR were observed. Parallel to these metabolic alterations, polycystic ovary morphology was identified. S.heathi can be used as an ideal representative PCOS animal model for periods of delayed ovulation [*Abhilasha et al. 1997*].

1.6.2. Hormonally induced rodent models

In early postnatal life, testosterone propionate treatment results in PCOS with persistent anovulation in rats and mice [*Walters et al. 2012*]. Increased ovarian production of androgens and oestrogen due to testosterone propionate, testosterone, and androstenedione administration were also detected to resemble human PCOS [*Weisz et al. 1965.*]. After chronic treatment with testosterone propionate for 35 consecutive days, IR developed in rats [*Beloosesky et al. 2004*]. However, smaller ovaries were detected in

the controls as compared to those in human PCOS [*Roy et al. 1962*]. These models have limited utilisation because androgen can be aromatised to oestrogen E_1 or E_2 [*Walters et al. 2012*]. The non-aromatising DHT treatment can induce PCOS prenatally in rats and mice with irregular oestrus cycles, increased levels of serum LH, and increased frequency of LH pulse secretion. These results suggest that prenatal DHT-treated animals have a defect in the steroidal feedback signalling located in the hypothalamus. In these animals, an impaired glucose tolerance, enlarged adipocytes, normal insulin sensitivity, unchanged BMI and fat mass were detected [*Roland et al. 2010*]. Early postnatal DHT treatment had no effect on ovarian function [*McDonald et al. 1972*]. However, 11-13 weeks of continuous DHT treatment in adolescence (after the 21. postnatal day) contributed to the presence of PCOS ovarian features with similarities to human and metabolic alterations such as IR, increased body fat and weight, elevated leptin, cholesterol level and hypertension [*Manneras et al. 2007, Yanes et al. 2011*]. Chronic DHT treatment of adolescent female rats induces a PCOS-like condition, including the early deterioration of carbohydrate metabolism [*Manneras et al. 2007, Yanes et al. 2011*]. A modified version of this model was used in our study. Postnatal DHEA murine models were reported to have acyclicity, anovulation, polycystic ovaries and hyperandrogenism [*Walters et al. 2012*], similar to human PCOS. The data on the presence of metabolic features, elevated LH levels and ovarian morphology are inconsequential [*Walters et al. 2012*]. Postnatal treatment with oestrogen, such as oestradiol-benzoate, estradiol and oestradiol-valerate, resulted in anovulation and hypertension without the typical endocrine and metabolic traits of PCOS [*Walters et al. 2012*]. Administration of letrozole, which is an aromatase inhibitor, contributed to a secondary endogenous androgen excess [*Manneras et al. 2007*]. Letrosole-treated animals showed acyclicity and anovulation with an ovarian morphology similar to human PCOS. Although the BMI increased, there was no observed change in insulin sensitivity and lipid metabolism. Decreases in E2 levels are a critical distinction from human PCOS and determine the limitations of the model [*Walters et al. 2012*]. Parallel insulin and 0.3 IU hCG daily administration for 22 days was reported to induce endogenous hyperandrogenism in female Wistar rats. Significantly higher androstenedione levels

was demonstrated due to increased activation of insulin induced IRS1-PI3-kinase-Akt pathway in the ovaries. Those animals showed an altered ovarian morphology with numerous fluid-filled cysts resembling to human PCOS and LH induced animal models [*Lima et al. 2006*].

1.6.3. Genetically modified rodent models of PCOS

LH and plasminogen activator inhibitor 1 were earlier proved to associate with the development of PCOS [*Balen A. 2004.*]. The over-expression of luteinizing hormone in transgenic mice (Tg(Cga-LHB/CGB)94Jhn/J) resulted in continuous elevated levels of LH, testosterone and oestrogen levels with polycystic ovaries, infertility and anovulation [*Risma et al. 1995*]. Both hyperinsulinemia and obesity developed similar to human PCOS [*Kero et al. 2003*]. However, the higher incidence of ovarian tumour and multiple corpora lutea showed that an impairment in LH secretion by itself was insufficient to induce PCOS [*Risma et al. 1995, Walters et al. 2012*]. Mice with a transgenic overexpression of plasminogen activator inhibitor-1 (Tg-Serpine1) was presented as a PCOS model with hyperandrogenism, anovulation, infertility and polycystic ovary morphology [*Devin et al. 2007*]. Although this model has been proposed to be useful, data on the hormonal and metabolic alterations are still lacking [*Walters et al. 2012*]. The New Zealand obese (NZO/HlLt) mice and the JCR:LA-cp (cp/cp) rat, which are leptin- (ob/ob) and leptin receptor-deficient (db/db), presented with altered leptin signalling that contributes to severe metabolic disorders such as hyperinsulinemia, IGT, elevated testosterone, and decreased FSH levels [*Walters et al. 2012.*]. In addition, the NZO mice presented with IR, dyslipidemia and hypertension [*Ortlepp et al. 2000*]. These models might be useful for the investigation of aetiology and treatment, especially in relation to metabolic syndrome in PCOS [*Walters et al. 2012*]. Transgenic animals with PCOS traits were discussed earlier in the section for insulin resistance (Table 2.).

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1.7. The possible role of vitamin D_3 in PCOS

Vitamin D is a secosteroid hormone that differs from other steroid hormones and can be obtained mainly from de novo synthesis in the skin. UVB light catalyses the transformation of 7-dehydrocholesterol to pre-vitamin D. After the isomerisation to cholecalciferol, 25-hydroxylase converts to 25-hydroxivitamin D (25(OH)D) in the liver [*Heaney et al. 2003*]. 25(OH)D circulates mainly bound to vitamin D binding protein or albumin [*White P. et al. 2000*]. In the kidney, 1-α-hydroxylase converts 25(OH)D to its active form, calcitriol or $1\alpha,25(OH)_2D$ [*Prentice et al. 2000*].

A daily supplementation of vitamin D (600-800 IU/d) is highly recommended for adults at risk of vitamin D deficiency, such as obese, pregnant and lactating women [*Holick et al. 2011*]. The accompanying metabolic alterations and obesity in PCOS might increase the risk of vitamin D deficiency.

A recent study showed that vitamin D_3 deficiency occurred in approximately 72% of PCOS women. The level of vitamin D_3 was significantly lower in metabolic syndrome [*Wehr et al. 2009*], and supplementation during infancy was observed to protect against T1DM, though the mechanism is unclear [*Zipitis et al.2008*]. A vitamin D_3 deficiency has been reported to be associated with a low insulin sensitivity check index (as a marker for IR) and an elevated level of asymmetric dimethyl-arginine [*Ngo et al. 2011*]. This impairment can partly explain the higher cardiovascular risk resulting from vitamin D deficiency [*Ngo et al. 2011*]. Vitamin D deficiency is strongly determined by the degree of obesity, and genetic variants of vitamin D_3 receptors can enhance the risk of deficiency [*Muscogiuri et al. 2012, Wehr et al. 2011*⁂]. Tare et al. reported a rat vitamin D deficiency model in which altered vascular endothelial and smooth muscle function and elevated blood pressure were observed [*Tare et al. 2011*]. In accordance, increased arterial stiffness and endothelial dysfunction were demonstrated to be associated with vitamin D insufficiency in humans [*Al Mheid et al. 2011*] Several researchers suggest that vitamin D deficiency may contribute to elevated blood pressure and that it has a crucial

role in the development of hypertension [*Judd et al. 2011,Wong et al. 2010*]. Vitamin D might affect the cardiovascular system and blood pressure through the inhibition of renin-angiotensin axis and the regulation of intracellular Ca levels; however, the exact mechanisms are still unclear [*Judd et al. 2011, Motiwalaa et al. 2011*]. Bukoski et al. proposed a direct effect of vitamin D_3 on resistance vessels in 1990, which was demonstrated by Wong et al. [*Bukoski et al. 1990, Wong et al. 2010*].

However, vitamin D_3 supplementation was assigned as an adjuvant therapy in PCOS, and its effectiveness on metabolic symptoms and its possible role need to be further characterised [*Duleba et al. 2012*]. Wehr et al. reported that fifty-seven PCOS women underwent 12 and 24 weeks of therapy with 25-hydroxyvitamin D (20.000 IU weekly). They found a significant decrease in the fasting glucose, triglyceride and oestradiol levels; however, the total cholesterol and LDL levels increased. Menstrual disturbances in a third of these patients improved [*Wehr et al. 2011**]. However, continuous or single dose administrations of vitamin D_3 did not produce changes in testosterone, DEAS or androstenedione levels [*Wehr et al. 2011*, Selimoglu et al. 2010*]. The cardioprotective effects of vitamin D_3 were also studied [*Tare et al. 2011, Ngo et al. 2011*]. However, even though several studies have confirmed the relationship between IR and vitamin D deficiency, the role of vitamin D_3 in glucose metabolism is still under debate, for the resolution of which further studies are needed.

1.8. Animal models related to 1,25-dihydroxyvitamin D3 endocrine system

Hundreds of different animal models have been described in context of the essential functions of vitamin D_3. In the next section the most important animal models were selected in aspect of cardiovascular and diabetic effects of vitamin D_3. It is beyond the power of this work to present all models of vitamin D_3 according to bone metabolism, intestinal and skin anti-carcinogen models, immune, autoimmune, inflammatory and

neurological actions, etc.

1.8.1. Vitamin D_3 deficient models

Tare et al. reported a vitamin D3 deficient rat model, in which females were fed with vitamin D3 deficient chow during their pregnancy and lactation. The offsprings were fed with the same chow as their mothers. They were housed under incandescent (UV free) light [*Tare et al. 2011*]. A significantly higher blood pressure, increased arterial myogenic tone, and halved nitric oxide evoked dilation were demonstrated in mesenteric arteries. In this model early life vitamin D deficiency was suggested as a possible hypertensive factor due to endothelial vasodilator alterations [*Tare et al. 2011*]. Increased renin expression and angiotensin II levels were detected in vitamin D receptor (VDR-/-) null mice and in wild type mice with blocked $1,25(OH)_2D_3$ synthesis by strontium chloride [*Li et al. 2002*]. Hypertension, cardiac hypertrophy and increased water intake were also detected due to the changes in renin-angiotensin system, however salt- and volume-sensing mechanism were intact [*Li et al. 2002*]. These models have been suggested that low levels of vitamin D_3 can contribute to hypertension independently from Ca^{2+} metabolism. VDR null mice show an increased sensitivity to autoimmune diseases and T1DM [*Bouillon et al. 2008*].

1.8.2. Atherosclerotic model

Taura et al. reported an atherosclerotic, normolipemic swine model fed a supplemented diet with 31,250 - 125,000 IU/kg of vitamin D_3 for three months [*Taura et al. 1979*]. Intimal plaques and calcified elastic lesions were shown in coronary arteries with narrowing of the lumens after the overdose of vitamin D_3. The incidence of those lesions was correlate directly to the vitamin D_3 intake [*Taura et al. 1979*].

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1.8.3. Models of low dose chronic vitamin D_3 treatment

Low dose administration of vitamin D_3 was shown to prevent against the progression of the heart failure phenotype by Przybylski et al. In that model 18 ng $1,25(OH)_2D_3$ per 100 g-body weight was given daily sc. for three months to Spontaneously Hypertensive, Heart Failure prone (SHHF) rats. Vitamin D_3 treatment was shown to decrease significantly the incidence of cardiac hypertrophy and dysfunction, while low levels of vitamin D_3 increased the risk in SHHF rats [*Przybylski et al. 2008*]. A modified version of this adaptable vitamin D model was used in our study. Takeda et al. demonstrated an anti-atherosclerotic model on ApoE -/- mice with calcitriol supplementation for 12 weeks. ApoE null mice have an altered lipid profile because the lack of an essential lipid transporter glycoprotein, and rapidly develop atherosclerotic lesions. Low dose vitamin D_3 (20 or 200ng calcitriol orally twice a week) administration was shown to reduce the maturation of dentritic cells and influence regulatory T cell activity compared to control. These changes in the differentiation and function of local immune cells induced by low dose calcitriol may lead to the prevention of atherosclerosis [*Takeda et al. 2010*]. Low dose vitamin D_3 administration was reported to decrease the blood pressure in SHR rats by Wong et al. Pumps loaded with vitamin D_3 derivative were implanted in SHR and WKY rats for six weeks with a daily emission of 10 ng per 100 g-body weight. The chronically treated SHR rats showed a reduced endothelium dependent contraction and a normalised ACh induced relaxation of aortic rings. A reduced expression of COX-1 and decreased level of ROS in the endothelium were suggested to modulate vascular tone [*Wong et al. 2011*].

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2. Aims of the study

In this study it is tried to induce PCOS by 70-day DHT treatment in a modified animal model [*Manneras et al. 2007, Yanes et al. 2011*] for examining early metabolic and functional changes - the earliest detectable lesions.

Our aim is to determine the early effects of DHT treatment on carbohydrate metabolism. We examined the potentially beneficial effects of vitamin D_3.

To identify early mechanical and pharmacological alterations in a morphologically constant skeletal muscle resistance vessel such as the gracilis arteriole in rat in PCOS. We also investigated the effect of low-dose vitamin D_3 treatment on the vascular biomechanical adaptation and the pharmacological responsiveness of the gracilis resistance arterioles.

We aim to clarify the effects of DHT on insulin-dependent vasodilatation on Hyperandrogenic (HAF) and PCOS female rat skeletal muscle resistance arterioles and aortic rings to determine associations of the possible modulatory role of protective doses of vitamin D_3.

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3. Methods and Materials

3.1 Chemicals

Pentobarbital (Nembutal, Phylaxia-Sanofi, Budapest, Hungary) was used for anaesthesia. Following chronic surgical intervention, 20 mg amoxicillin and 4 mg clavulanic acid (Augmentin GlaxoSmithKline, Memphis, US) in 0.2 ml saline was administered intramuscularly to prevent infection. We followed the protocol to induce experimental polycystic ovary syndrome described by Manneras et al. [*Manneras et al. 2007*] Continuous-release pellets that contained 7.5 mg dihydrotestosterone (DHT, Innovative Research of America, Sarasota, Fl, USA) were applied for 70 days. Eighty-three micrograms/day release for 90 days is guaranteed by the manufacturer. We purchased 1,25 $(OH)_2$ D_3 vitamin (Inj. Calcijex, 2 μg/ml) from Abbott Lab., Illinois, USA. The composition of the normal Krebs-Ringer solution used in these *in vitro* studies was as follows (in mmol/L): NaCl 119, KCl 4.7, NaH_2PO4 1.2, $MgSO_4$ 1.17, $NaHCO_3$ 24, $CaCl_2$ 2.5, glucose 5.5 and EDTA 0.034. The Ca^{2+}-free Krebs solution that was used to relax the smooth muscle contained the following (in mmol/L): NaCl 92, KCl 4.7, NaH_2PO_4 1.18, $MgCl_2$ 20, $MgSO_4$ 1.17, $NaHCO_3$ 24, glucose 5.5, EGTA 2 and EDTA 0.025. The temperature of the solution was maintained at 37° C, and it was bubbled with 5% CO_2, 20% O_2, and 75% N_2, which stabilised the pH at 7.4. Human recombinant insulin (Actrapid Penfill 100 IU/ml) was obtained from Novo Nordisk (Copenhagen, Denmark). Norepinephrine (NE), acetylcholine (ACh) and L-N^G-Nitroarginine methyl ester (L-NAME) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA and Budapest, Hungary) and freshly prepared on the day of the experiment.

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3.2 Animals

Thirty adolescent (21- to 28-day-old) female Wistar rats (provided by the Animal Facility at the Semmelweis University in agreement with Charles River Ltd.) that weighed 100-140 g when the study began were used. Twenty of the animals received subcutaneous pellets containing 7.5 mg DHT implanted under the back skin under anaesthesia (Nembutal 45 mg/kg i.p.) in sterile conditions (DHT-treated female groups), which induces polycystic ovary syndrome [*Manneras et al. 2007*] Ten animals underwent sham operations. Ten DHT-treated animals received weekly doses of 120 ng /100 g 1,25 $(OH)_2$ D_3 vitamin (DHT+D3 group) subcutaneously as previously described by Przybylski et al. We applied a weekly dosage instead of a daily administration [*Przybylski et al. 2010*] to reduce stress on the animals. A vehicle was given to the remaining 20 animals. No medical or surgical complications were observed. Conventional rat food and tap water were provided ad libitum. The rat chow used in this study (all animals received this chow): S8106-S011 SMR/M-Z+H, 15mm autoclavable (Spezialdiäten GmbH, Soest, Germany) with crude nutrients (19%), proteins (3.5%), fat (3.6%), fiber (6.5%), calcium (1%), phosphorus (0.7%), sodium (0.2%), magnesium (0.22%), lysine (1.1%), methionine (0.56%); added vitamin D_3 (1000 IU), vitamin A and E (25000 IU and 125mg). The investigation conforms to the Principles of Laboratory Animal Care NIH publication No. 85-23, revised 1985) as well as the Euroconform Hungarian Law on Animal Care (XXVIII/1998), and the study protocol was accepted by the institutional Animal Care Commission (IRB approval: 22.1/2960/003/2009).

3.3 Oral Glucose Tolerance Test (OGTT)

After eight weeks of treatment, an oral glucose tolerance test was performed under short ether narcosis. Blood glucose and plasma insulin were measured following overnight fasting and 120 minutes after an oral glucose load of 0.3g / 100 g body weight given

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through a gauge [*Pan et al. 2009, Marchand et al. 2010*]. Blood glucose was measured by Decont Personal Akucheck (77 Electronics Ltd., Budapest, Hungary). For the insulin measurements, a Rat/Mouse Insulin ELISA assay was used (Millipore Ltd., Billarica, MA, USA). Serum fructose-amine was determined by Roche 930010 test (Roche, Basel, Switzerland).

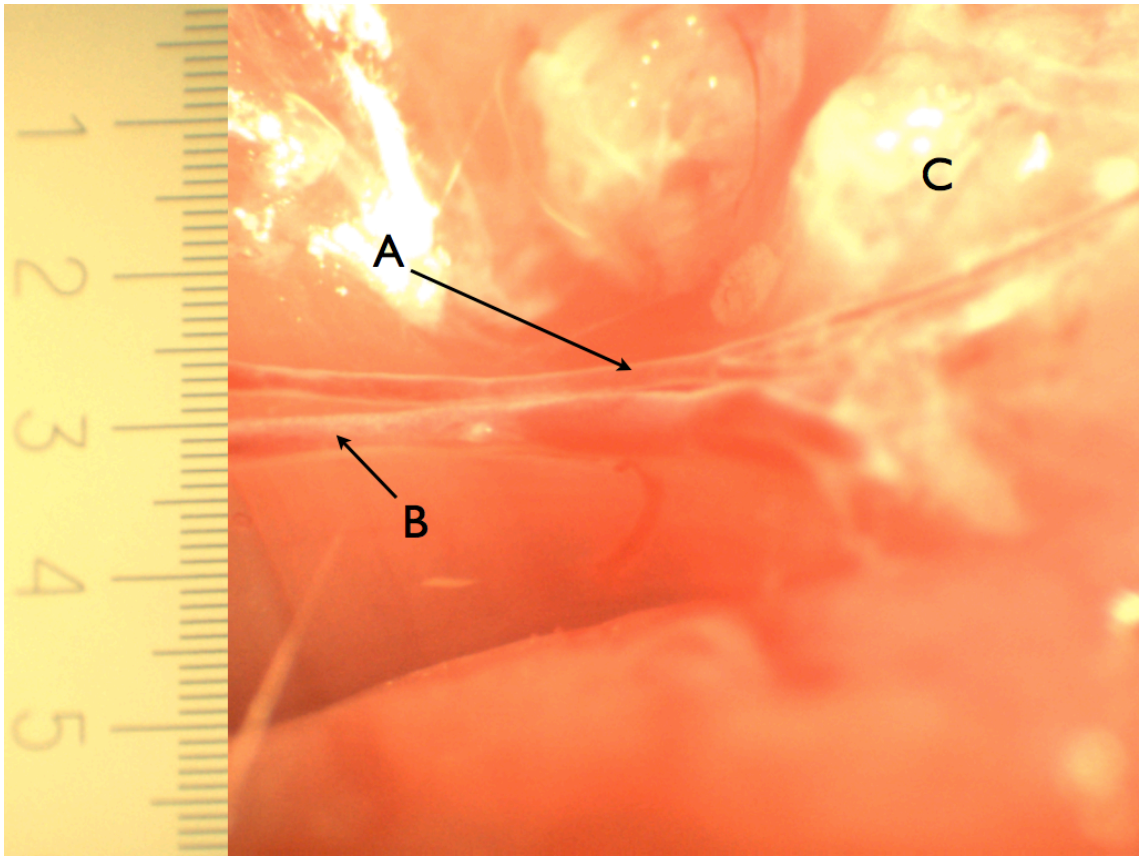

Figure 6. In situ methodical photo of musculocutaneous gracilis vessels (16x magnification). The segments were obtained from the same region of the saphena vascular system. The gracilis vessels can be recognised after lifting up the adductor muscles of rat thigh under the symphisis. This part of the gracilis arterioles usually is a long straight segment without bifurcation, and can be easily isolated. The arrows show the anatomical figures. A: gracilis arteriole passing to the middle part of the gracilis muscle, B: gracilis venule, C: the gracilis muscle. The calibration scale was photographed at the same magnification. Every small calibration means 100 μm and the bigger marks and numbers mean millimetres.

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3.4 In vivo blood pressure measurement

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

3.5. Biomechanics of a Musculocutanous Arteriole (Pressure Arteriography)

Following ten weeks of treatment, the animals were anaesthetised (Nembutal 45 mg/kg i.m.). The segments of gracilis arterioles were obtained from a carefully located point of the iliofemoral region. The adductor muscles, which connect the symphisis to the tuberositas tibiae, were identified first at the inner surface of the thigh. The upper part of these muscles were cross-sectioned right under to the symphisis and a bit far from it. After taking up the cross-sectioned part of the muscles the gracilis arteriole passing to the middle of the gracilis muscle can be well-recognised. Generally, that segment of the gracilis arteriole has no branch and can be removed easily (*Fig.6.*). After opening the mentioned iliofemoral region, the arteriole, which had an in vivo diameter of approximately 150 μm and is the blood supply for the gracilis muscle, was removed and placed into a vessel chamber filled with normal Krebs-Ringer (nKR) solution. It was cannulated at both ends with plastic microcannulas and extended to its in vivo length. Both cannulas were connected to pressure-servo pumps (Living Systems, Burlington, VT), and the arterioles were pressurised under a no-flow condition to 50 mmHg intraluminal pressure (*Figs. 7.A,B and 8.A*).

The outer and the inner diameters of the arterioles were measured by video-microscopic pressure microangiometry (*Figs. 7.A,B and 8.A*). In this setup, the glass-bottomed tissue bath was positioned in the light path of an inverse Leica microscope. A magnified picture of the vessel was formed with the aid of a video camera (Leica DFC320) and Leica QWin software. The digitalised pictures were saved, and off-line measurements of

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the inner and outer diameter were made using Leica QWin image analysis software.

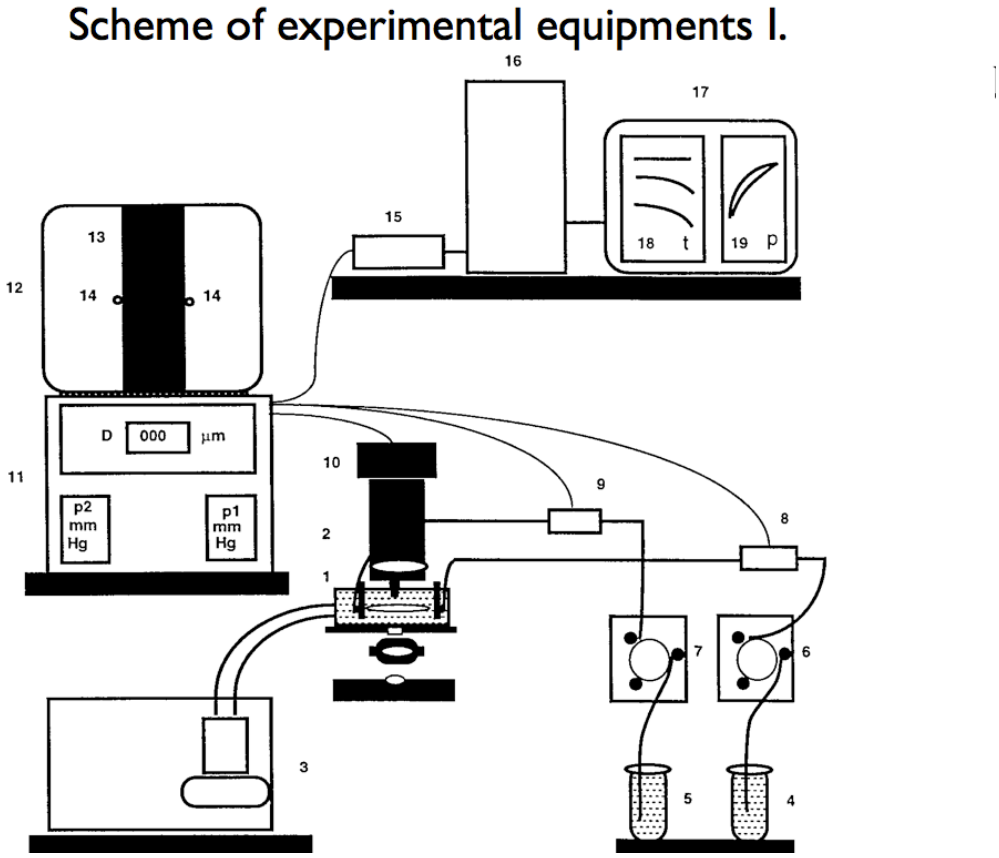

Figure 7. Panel **A**: **Microarteriography of isolated segments of musculocutaneous gracilis arterioles.** 1.Organ-bath with segment of arteriole 2.Microscope 3.Thermostat 4.Fluid inward 5.Fluid outward 6.Inflow servo-pump 7.Outflow servo-pump 8.Inflow manometer 9.Outflow manometer 10.Videocamera 11.Microcomputer 12.Monitor 13.Magnified picture of the segment 14.Points of light following the contour of the segment 15.Analog-digital converter 16.Pentium 17.Computer monitor 18.Time curve of pressures and diameters 19.Pressure-diameter hysteresis loop.

The gracilis arterioles were allowed to equilibrate for 30 minutes at 50 mmHg intraluminal pressure in an oxygenised nKR solution. Following incubation, the pressure was decreased to 10 mmHg and then increased to 100 mmHg in 10-mmHg pressure steps. The steady-state diameter was measured. The diameter was also measured during norepinephrine contraction after a 10-minute incubation period with 10^{-6}M NE.

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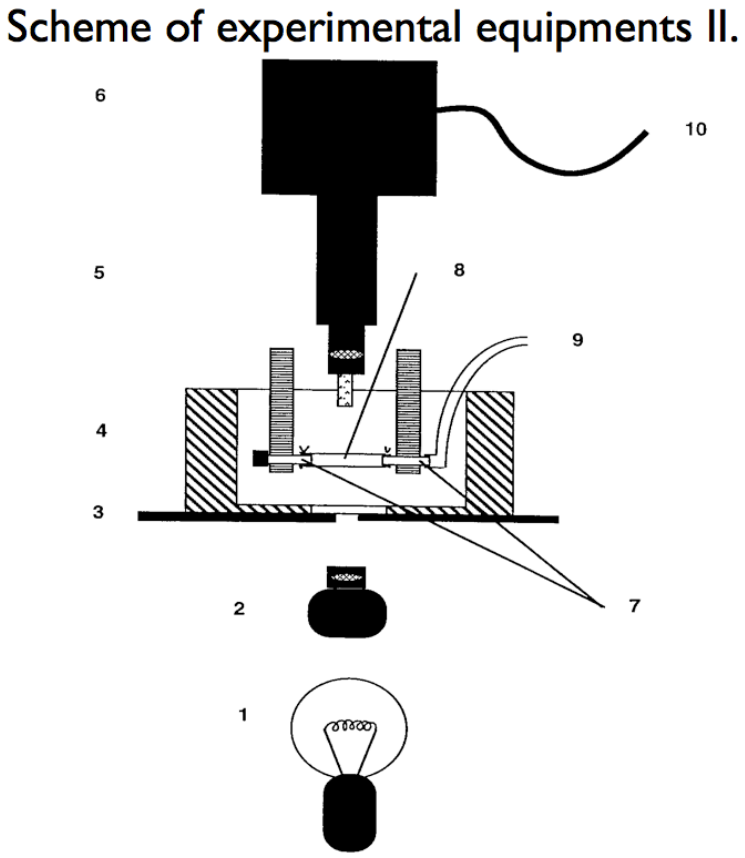

Figure 7. Panel **B**: **Schematic graph of organ-bath with cannulated arteriole segment.** 1.Lamp 2.Condenser of microscope 3.Microscope stage 4.Organ-bath with glass bottom 5.Microscope 6.Videocamera 7.Cannules 8.Segment of arteriole 9.Connection to the servo-pump and manometer 10.Connection to microcomputer and monitors

After recording the NE-diameter (pre-contraction) at 50 mmHg, dose-diameter curves were recorded in the presence of 30, 60, 120, 240 and 600 mU/ml of insulin, leaving an equilibration period of 8-10 minutes for each dose, which allowed a stable diameter to be reached and measured. After rinsing, NE was added to the organ bath in the same dose as previously described. After a 10-minute incubation the pressure-diameter curve was repeated as described above. After recording the NE-curve, at P=50 mmHg, 10^{-6} M ACh was added to the organ bath. Following a 20-minute equilibration period, the outer and inner diameters were measured. Next, 10^{-5} M L-NAME was administered, and the diameters were again measured after reaching equilibrium (25-30 minutes at P=50

mmHg). Finally, the passive diameter was measured in a Ca^{2+}-free Krebs solution. Arteriolar segments were incubated for 20 minutes, and the steady-state, fully relaxed diameter was then measured as described above. The effect of insulin + NE was compared to the NE-induced contraction without insulin and also was compared to full relaxation. We used a micrometre etalon (Wild) for the calibrations.

3.6. Biomechanical calculations

From the originally calibrated pressure-diameter plots, the following geometrical and biomechanical parameters were computed for each intraluminal pressure level [*Várbíró et al. 2010, Mátrai et al. 2010*]. Tangential stress was computed according to the Laplace equation: $\sigma_{tang}= P* r_i /h$, where σ_{tang} is the tangential (circumferential) wall stress, p is the intraluminal pressure, r_i is the inner radius and h is the wall thickness ($h= r_o-r_{i,}$, where r_o is the outer radius). Incremental distensibility was computed as $D_{inc}=\Delta V/ V*\Delta P$ where D_{inc} is the incremental distensibility and ΔV is the change in vessel lumen volume in relation to the initial volume V in response to the pressure change ΔP. The circumferential incremental elastic modulus was computed from the following equation: $E_{inc}= (\Delta P/\Delta r_o)*2r_i^2*r_o/(r_o^2-r_i^2)$, where E_{inc} is the incremental elastic modulus, r_i is the inner radius, r_o is the outer radius, and Δr_o is the change in outer radius in response to intraluminal pressure change of ΔP. Full contraction of the segments was computed as follows: $T_{Full} = 100*(R_{Cafree} - R_{NE})/R_{Cafree}$ (given in %). Myogenic (spontaneous) tone was computed as follows: $T_{nKR} = 100*(R_{Cafree} - R_{nKR})/R_{Cafree}$ (given in %). The NE-induced tone was computed as follows: $T_{NE} = 100*(R_{nKR} - R_{NE})/R_{Cafree}$ (%). ACh-induced relaxation (T_{Ach}) was computed relative to the norepinephrine-induced tone as follows: $(R_{ACh}-R_{NE})/R_{NE}$. The Ca-free vessel radius percentage (compared to the diameter in full relaxation as an absolute reference point) was calculated as follows: $R_{Cafree}-R_{ACh}/R_{Cafree}$. The L-NAME-induced tone ($T_{l\text{-}name}$) was evaluated in comparison with the Ach-relaxed $(R_{ACh}-R_{l\text{-}name})/R_{ACh}$, NE-contracted $(R_{l\text{-}name}-R_{NE})/R_{NE}$, and the absolute (relaxed) $(R_{Cafree}-R_{l\text{-}name})/R_{Cafree}$ radii.

Insulin-induced relaxations of precontracted segments were normalized to the NE-induced precontraction without insulin: $(R_{Ins}-R_{NE})/R_{NE}$. The insulin-induced relaxation of NE-precontracted segments was computed to the relaxation induced in the same segments by the Ca-free medium: $(R_{Ins}-R_{NE})/(R_{Cafree}-R_{NE})$, also. In a similar manner, the ACh-relaxation and the effect of L-NAME were compared with NE-precontraction. Thus, all parameters are independent of absolute vessel diameter.

3.8. Histology

The ovaries and arterioles were fixed in neutral buffered 4% formaldehyde 24 h, placed in 70% ethanol, dehydrated, and embedded in paraffin. The ovaries were serially sectioned at 4 μm; every 7th section (n = 7 per ovary) was mounted on a glass slide and stained with hematoxylin and eosin. For measurements and photographs, the slides were scanned with Pannoramic 250 Scanner (3DHISTECH Ltd., Budapest, Hungary) and analyzed with Pannoramic viewer softver (3DHistech, Hungary). The area of the ovary was magnified (40x) and determined with a calibrated scale tool in the virtual microscope. The area of the largest follicle, the thickness of its follicular wall layer as well as the number of follicles were measured by two persons to avoid duplicate counting. Fig. 8.A and B show microangiometric and histological photos.

3.9. Statistical analysis

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

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uniformly accepted as a significant difference. The data are presented as the mean ± SEM. GraphPad Prism version 5.0d was used as statistical software.

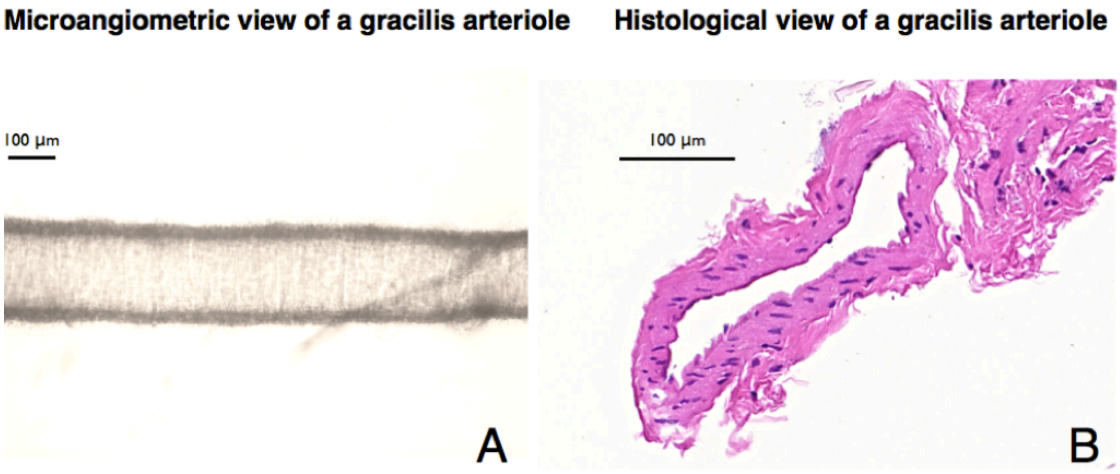

Figure 8. Panel **A**: **Microangimetric view of a gracilis arteriole**. Panel **B: Histological section of a DHT treated arteriole** stained with hematoxilin-eosin. Fixed after pressure arteriography measurement. Applied from experimental study photos.

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4. Results

4.1. Physiological parameters

After ten weeks of DHT treatment blood pressure was measured directly through the carotid artery. The mean arterial pressure was 122 ± 3 mmHg, 123 ± 6 mmHg and 123 ± 4 mmHg for the controls, the DHT-group and the DHT+vitamin D_3-treated group, respectively (non-significant). The body weights at the end of the experiment were 298 ± 8, 354 ± 16 and 353 ± 9 g for the same groups, respectively. The DHT-treated animals had significantly higher body weights than the controls ($p<0.01$), but there was no difference between the two DHT-treated groups.

4.2 Ovarian morphology

We detected a polycystic morphology in the DHT-treated groups and normal ovaries in the controls by histological analysis (Fig. 2.). Multiple premature cysts were detected peripherally without dominant follicles in the DHT treated animals. Follicle diameters were significantly smaller in DHT-treated ovaries compared to control (1609 ± 617 and 2334 ± 451 pixels at 40x magnification, $p<0.05$). Follicles of DHT+D3 ovaries weren't significantly different from the other two groups (2054 ± 442).

4.3. Glucose metabolism

After eight weeks of DHT treatment an oral glucose tolerance test following an overnight fasting was performed. The fasting and 120 min postload blood glucose and insulin values are shown in *Table 3*. The blood glucose levels and the fasting insulin levels were not significantly different among the experimental groups. However,

significant differences were found among the groups for the 120 min postload insulin values. DHT-treated animals had higher plasma insulin levels than the controls, and this difference was eliminated by the D_3 vitamin treatment. Serum fructose amine was similar in all groups and within reference range indicating, that the animals did not develop diabetes or blood glucose elevation.

Table 3.: **The results of an oral glucose tolerance test.** The 120 min postload insulin level in the DHT group was significantly higher than in controls (*, $p<0.01$). Vitamin D treatment normalized the insulin response during OGTT (‡, $p<0.001$). There were no significant differences in other parameters: blood sugar 0', 120', insulin 0' and glycated protein levels.

	Controls	**DHT – treated**	**DHT + Vitamin D treated**
Blood sugar 0'	5.31±0.15 mM/L	5.35±0.24 mM/L	5.18±0.27 mM/L
Blood sugar 120'	6.11±0.11 mM/L	6.36±0.2 mM/L	7.09±0.13 mM/L
Insulin 0'	0.42±0.02 ng/ml	0.42±0.03 ng/mL	0.45±0.04 ng/mL
Insulin 120'	0.71±0.14 ng/mL	1.42±0.33 ng/mL *	0.48±0.07 ng/mL ‡
Glycated protein (Fructoseamine)	157±3 mM/L	151±4 mM/L	156±4 mM/L

4.4 Biomechanical parameters of gracilis arterioles

4.4.1. Arteriole geometry

The relaxed outer radii (measured at 50 mmHg in a Ca^{2+}-free medium) for the different groups were 167 ± 13 µm (control), 193 ± 6 µm (DHT) and 166 ± 10 µm (DHT +

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vitamin D_3). The relaxed inner radii of the gracilis arterioles were 96 ± 3 µm (DHT), 84 ± 6 µm (control), 83 ± 5 µm (DHT+D3). DHT treatment increased both the outer (*Fig. 9.A*) and inner (*Fig. 9.B*) radii, which were diminished by a parallel vitamin D_3 treatment ($p<0.05$ for both comparisons). The wall thicknesses for the different groups in the Ca^{2+}-free medium were as follows: 20 ± 2, 19 ± 1, and 20 ± 1 µm in the control, DHT- and DHT + vitamin D_3-treated groups, respectively (as measured at 50 mmHg, non-significant). In a Ca^{2+}-free solution, the cross section areas for the vessel walls were 12080 ± 1627, 12542 ± 1090, and 11348 ± 660 μm^2 for these groups, which were not significantly different.

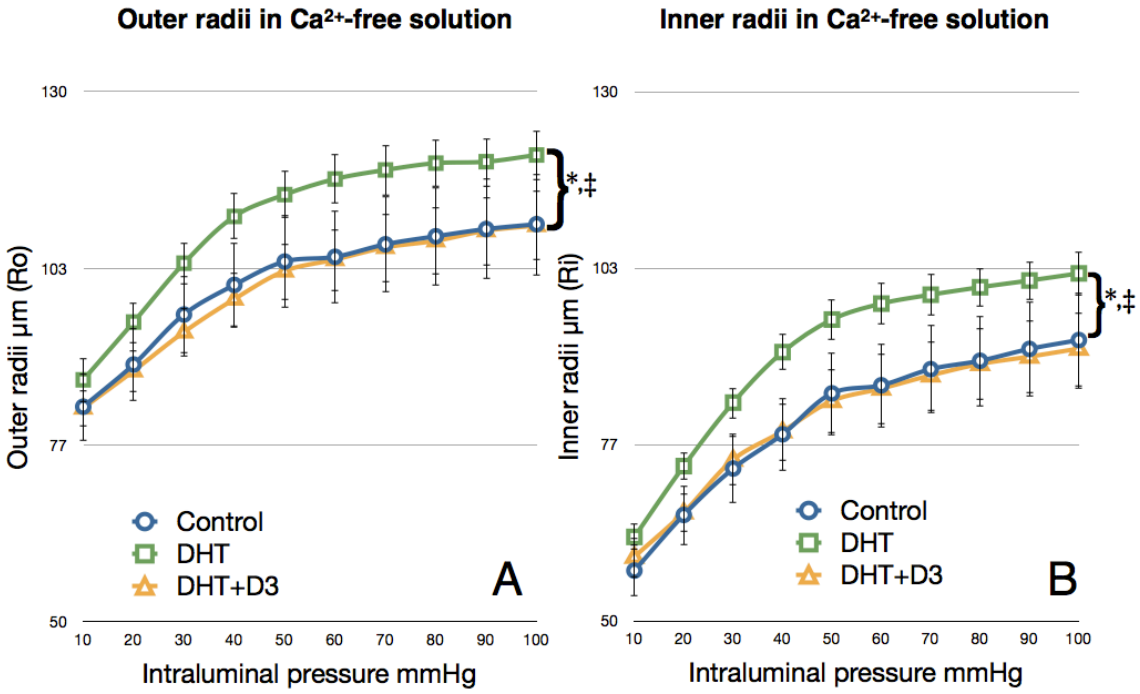

Figure 9. Panel **A** and **B**: **Morphological parameters of the vessels.** Outer radii (Ro) and Inner radii (Ri) in Ca-free solution. The outer and inner radii from Ca-free relaxation at P=50mmHg were not different in the control and DHT+D3. Both radii were smaller than in the arterioles isolated from the DHT-treated animals ($p<0.05$).
*The DHT group is significantly different from the control group. ‡ The DHT+D3 group is significantly different from the DHT group. ($p<0.01$)

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4.4.2. Arteriole elasticity

The mechanical load on the vessel wall was measured, and the tangential wall stress was significantly higher throughout the entire pressure range for the DHT-treated group compared with the other two groups in both a Ca^{2+}-free solution and during norepinephrine contraction (*Fig. 10.A and B*, $p<0.05$). Vitamin D_3 treatment induced a partial reversal of wall stress in the NE-contracted segments and an overcompensation in the Ca^{2+}-free solution (*Fig. 10.*, both for DHT vs. DHT + D3 and the control vs. DHT + D3, $p<0.05$).

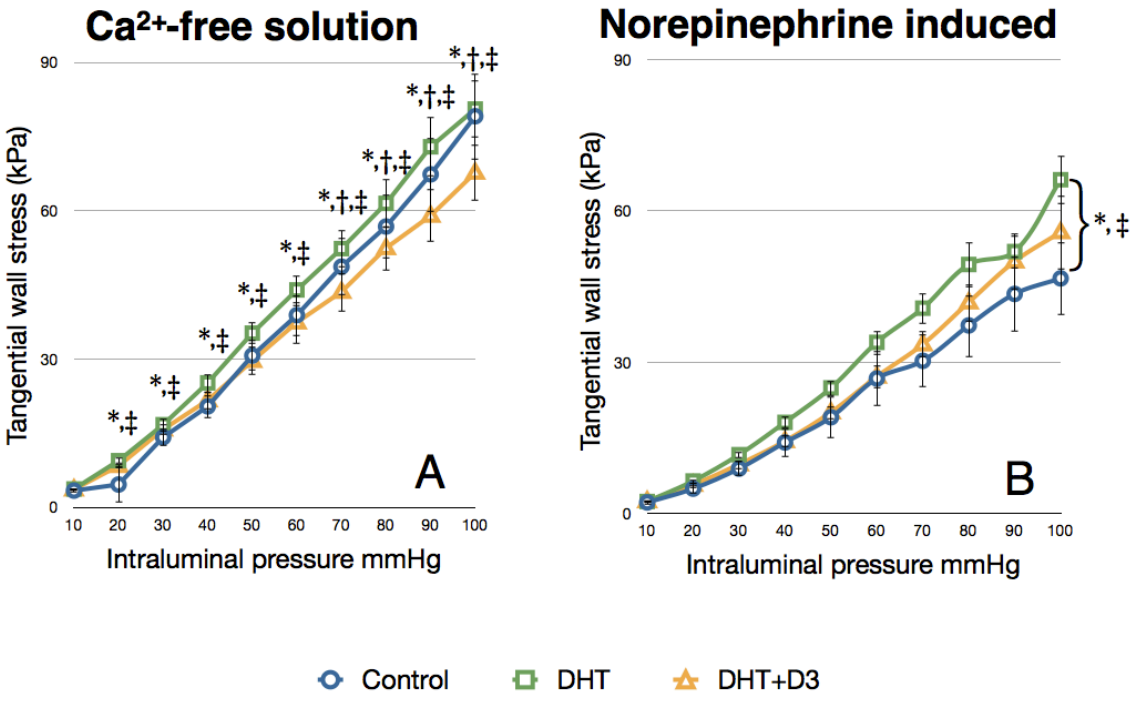

Figure 10. Panel **A**: **Tangential wall stress in the Ca^{2+}-free solution.** The tangential wall stress was significantly greater in the DHT group than in the other two groups throughout the entire pressure range, ($p<0.001$). Between 70-100 mmHg, the stress was significantly lower in the DHT+D3 group ($p<0.001$). Panel **B**: **NE-induced tangential stress.** Tangential wall stress was significantly greater in the DHT group than in the other two groups throughout the entire pressure range, ($p<0.001$).
Values are expressed as the mean ± SEM. * DHT was significantly different from the control. † DHT+D3 was significantly different from the control. ‡ The DHT+D3 group was significantly different from the DHT group.

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Isobaric distensibility was significantly higher throughout the entire pressure range for the DHT group compared with the other two groups in both the Ca^{2+}-free medium and during norepinephrine contraction (*Fig. 11.A and B* $p<0.05$). In response to a parallel treatment with vitamin D_3, relaxed distensibility fully returned to the control in Ca^{2+}-free solution (*Fig. 11.A*). After NE contraction only a partial recovery was detected in DHT+D3 group (*Fig. 11.B*).

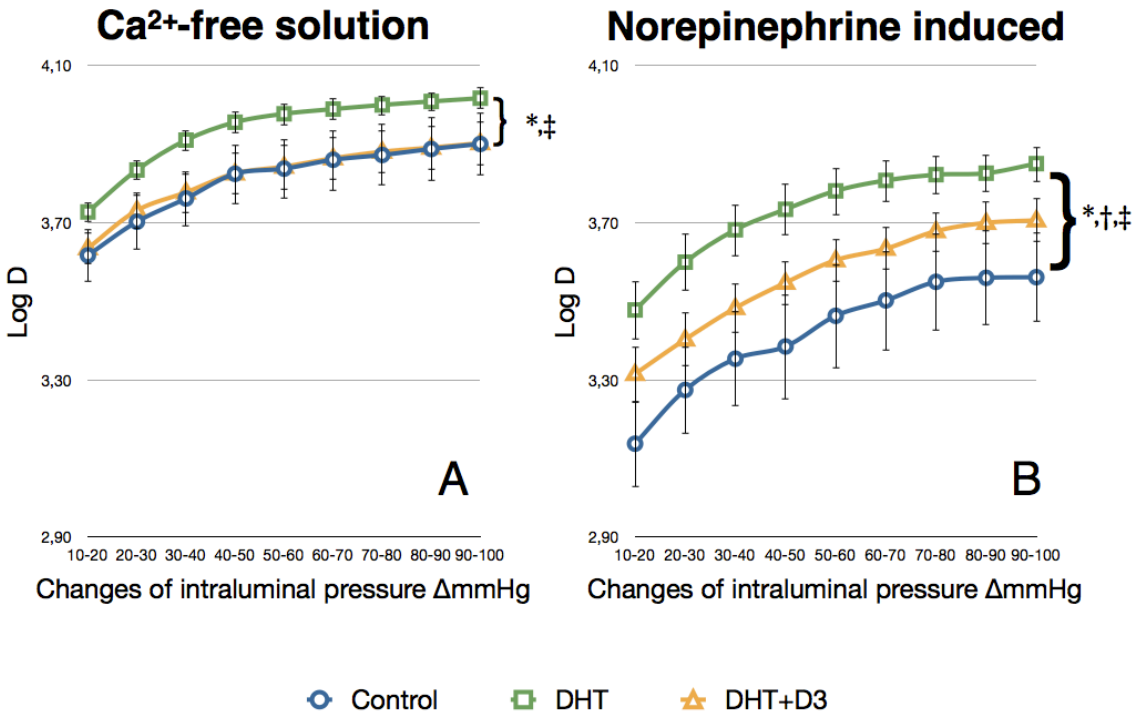

Figure 11. Panel **A**: **Distensibility in a Ca^{2+}-free solution.** Distensibility was significantly greater in the DHT group compared with the other two groups throughout the entire pressure range (10-100 mmHg), ($p<0.001$). Panel **B**: **NE induced distensibility.** Each group was significantly different throughout the entire pressure range ($p<0.001$). Distensibility was greatest in the DHT animals and lowest in the control animals. Values are expressed as the mean ± SEM. * DHT was significantly different from the control. † DHT+D3 was significantly different from the control. ‡ The DHT+D3 group was significantly different from the DHT group.

Wall elastic moduli were not affected by DHT and vitamin D_3 treatment (in a Ca^{2+}-free solution, the log elastic moduli were 2.8 ± 0.14, 2.7 ± 0.16 and 2.4 ± 0.11 log(Pa) in the

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control, DHT- and DHT + vitamin D_3-treated groups, respectively, ns.). There was no difference in the elastic moduli as a function of tangential wall stress (*Fig. 12*., ns.).

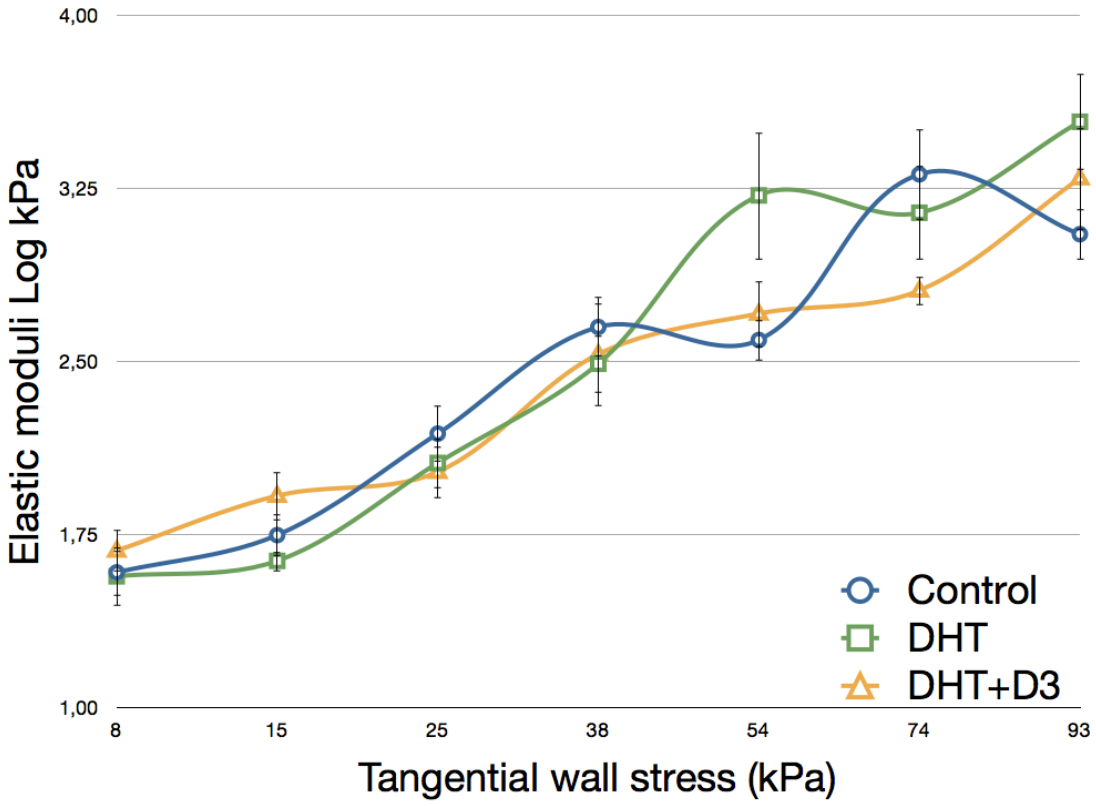

Figure 12.: **Elastic moduli as a function of tangential wall stress in the Ca^{2+}-free solution.** The values for the abscissa show the elastic moduli (logkPa) for the arterioles from the control rats as well as the DHT- and DHT+D3-treated rats. The ordinata shows the tangential stress (kPa). There were no significant differences between the groups. The values are expressed as the mean ± SEM.

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4.5. Pharmacological properties of gracilis arterioles

4.5.1. Arteriolar contractility

DHT treatment significantly reduced NE tone, which was almost fully restored by vitamin D_3 treatment (*Fig. 13.*, $p<0.01$ between the control and DHT, $p<0.05$ for the other comparisons). These segments do maintain substantial myogenic (spontaneous) tone.

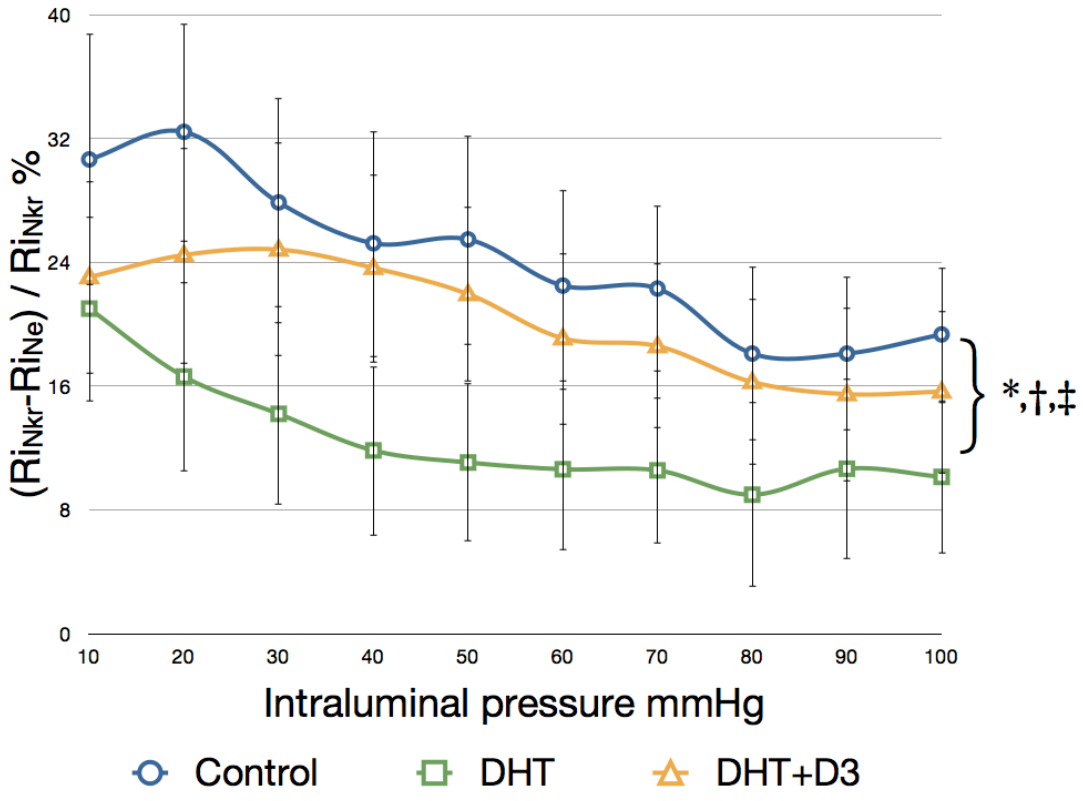

Figure 13.: **NE-induced tone (inner radii, µm) compared with the spontaneous tone in a normal nKR solution.** NE had small effect on the DHT group compared with the other two groups ($p<0.001$). There was no significant difference between the control and DHT+D3 groups.
The values are expressed as the mean ± SEM. * DHT was significantly different from the control. † DHT +D3 was significantly different from the control. ‡ The DHT+D3 group was significantly different from the DHT group.

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The tone was also lower following DHT treatment, and interestingly, co-administration of D_3 vitamin further decreased myogenic tone (*Fig. 14.*, the control and DHT-treated rats, $p<0.01$, as well as the D_3 vitamin-treated and untreated DHT rats, $p<0.05$, were significantly different).

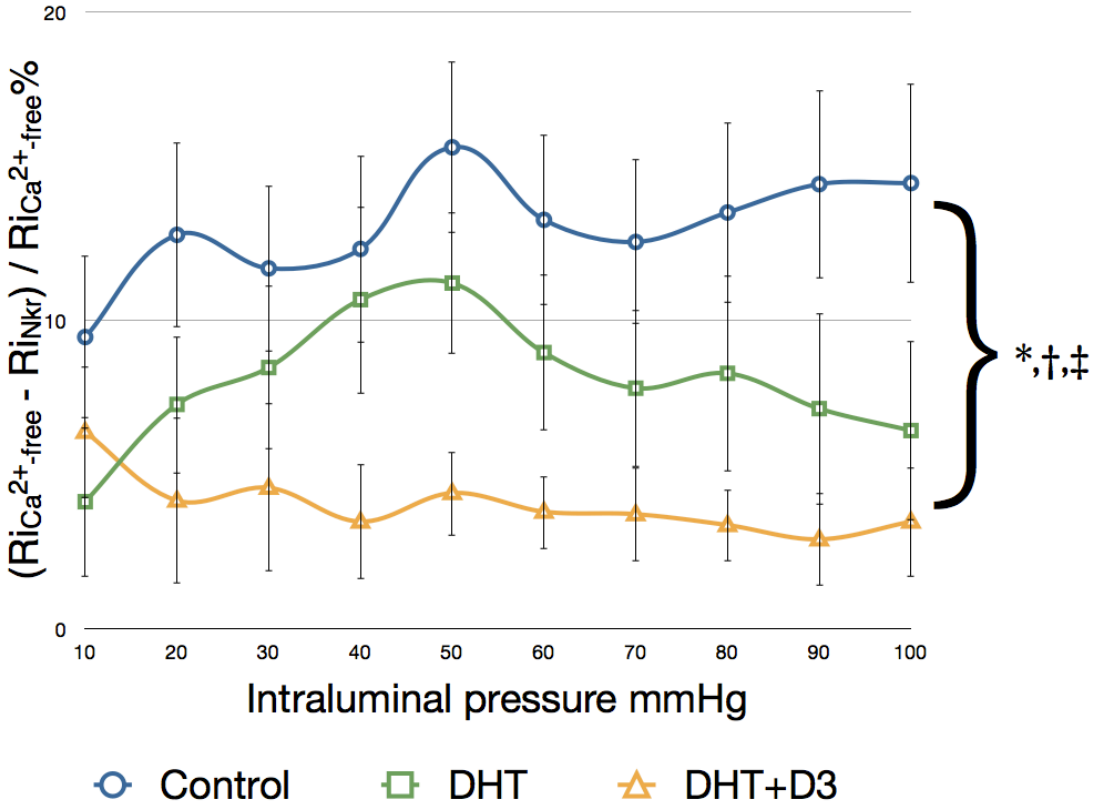

Figure 14.: **The myogenic tone of the arterioles.** Each of the groups were significantly different in the entire pressure range ($p<0.001$). The myogenic tone was the greatest in control, and the lowest in the DHT+D3.
The values are expressed as the mean ± SEM. * DHT was significantly different from the control. † DHT +D3 was significantly different from the control. ‡ The DHT+D3 group was significantly different from the DHT group.

The sum of the two contractions (*Fig. 15.*), which represent the ability of the segments to fully contract (range of total pharmacological adaptation), was significantly lower after DHT treatment and was partially restored by vitamin D treatment (the control and DHT-treated rats, $p<0.01$, as well as the D vitamin-treated and untreated DHT rats, $p<0.05$, were significantly different).

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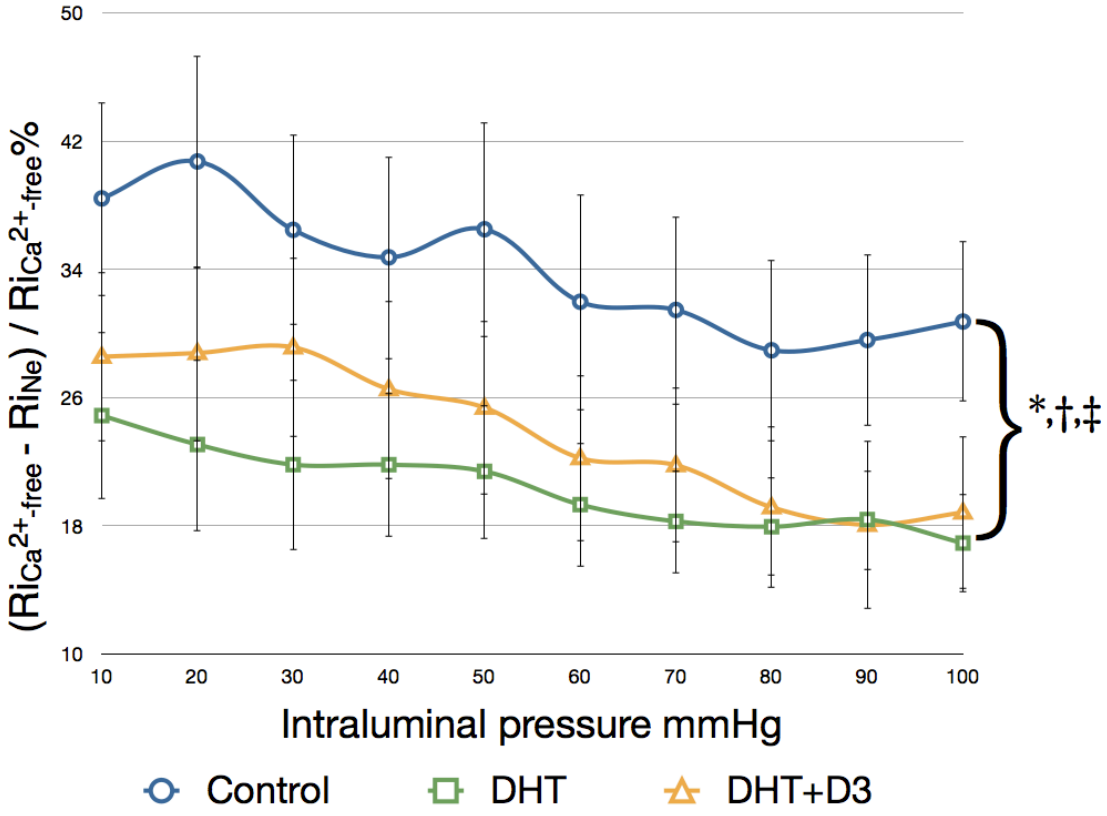

Figure 15.: **Full contraction of arterioles.** Each group was significantly different throughhout the entire pressure range ($p<0.001$). NE had the smallest effect on the DHT group and the greatest effect on the control animals. Vitamin D treatment improved the maximum effect from NE, NE tone in the DHT+D3 group was significantly lowe than the controls.
The values are expressed as the mean ± SEM. * DHT was significantly different from the control. † DHT +D3 was significantly different from the control. ‡ The DHT+D3 group was significantly different from the DHT group.

4.5.2. Endothelial dilation

Segments contracted by NE were relaxed in response to 1 μM acetylcholine, and this relaxation was significantly larger in the control compared with the DHT-treated group (*Fig. 16.A*). ACh dilations were severely diminished in the testosterone-treated female animals. Vitamin D_3 treatment did not compensate this alteration but produced only a nonsignificant trend toward restoring relaxation. (*Fig. 16.A*, $p<0.05$ for both treatments

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comparison with the controls). L-NAME induced substantial contraction in the control segments, which was missing in both DHT-treated groups (*Fig. 16.B*).

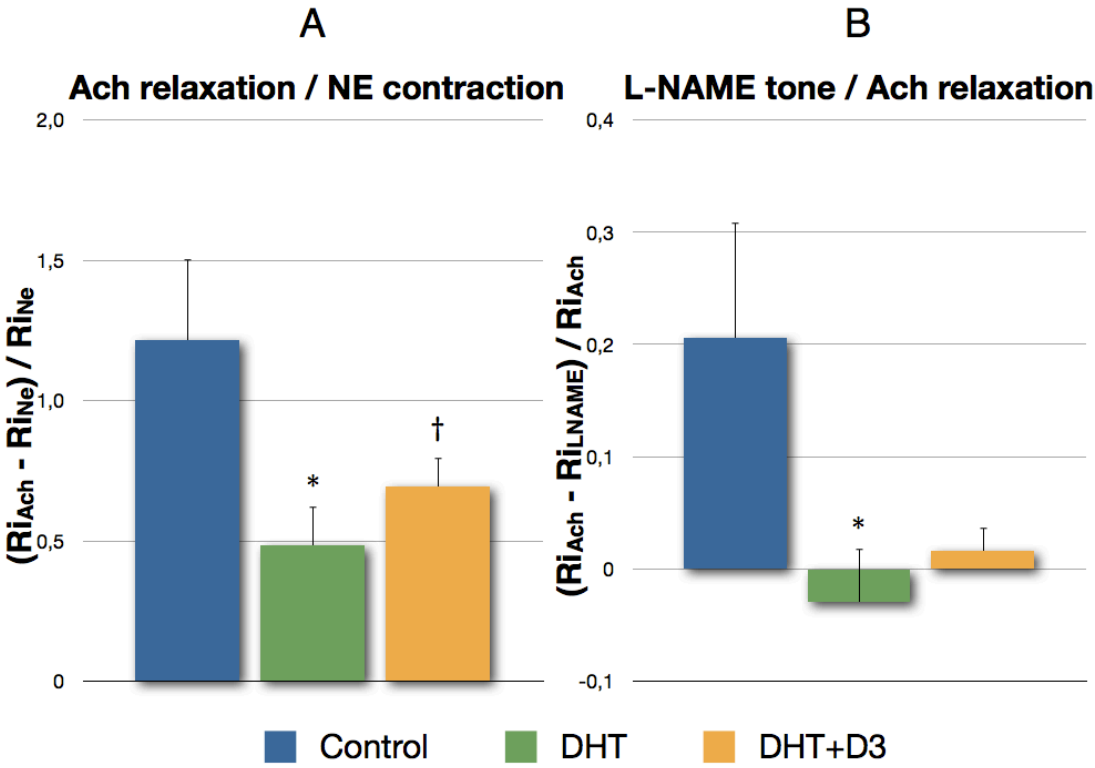

Figure 16. Panel **A**: **ACh-induced relaxation.** The abscissa shows the ratio of ACh-induced relaxation (10^{-6} M) compared to NE-induced contraction (10^{-6} M) for the arterioles from the control rats as well as the DHT- and DHT+D3-treated rats (inner radii, μm). The effectiveness of ACh relaxation was significantly greater in the control ($p<0.05$). There was no difference between the other two groups. Panel **B**: **LNAME induced tone (10^{-5} M) compared with ACh relaxation (10^{-6} M)**. The abscissa shows the ratio of the inner radii for the arterioles from the three experimental groups after ACh relaxation and following NO-blocking by LNAME (%). This ratio was greater for the control group than the DHT group ($p<0.05$).
Values are expressed as the mean ± SEM. * DHT was significantly different from the control. † DHT+D3 was significantly different from the control.

4.6. Insulin-induced vascular relaxation of gracilis arterioles

Insulin administered to NE-precontracted gracilis arteriole segments induced concentration-dependent relaxation in the control animals (*Fig. 17.A and B*). This

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relaxing effect was much reduced in the DHT-treated group ($p<0.001$). The administration of vitamin D_3 partially restored the insulin-induced relaxation ($p<0.001$). Another significant effect of Vitamin D_3 treatment was revealed when we compared the NE-induced contractions of segments before and after in vitro treatment with elevating concentrations of insulin. Typically, NE-induced contractions were more profound after insulin treatment (*Fig. 18.*). This difference was reduced in vitamin D_3-treated animals ($p<0.05$).

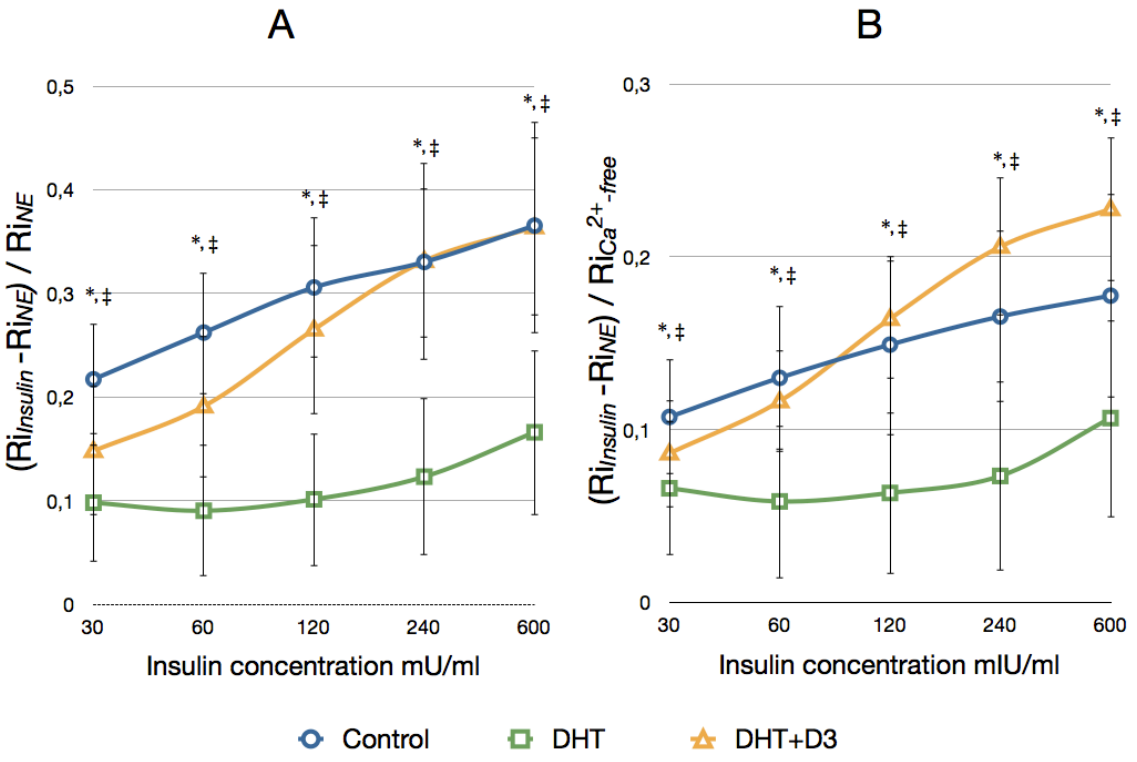

Figure 17. Panel **A**: **The insulin-induced vascular tone (T insulin) as a function of norepinephrine (NE) tone in gracilis arterioles in the three experimental groups.** The values on the abscissa show the inner radii ratios of insulin-induced and norepinephrine-induced tone at 50 mmHg. The x axis shows the different insulin concentrations. Panel **B**: **The insulin-induced vascular tone (T insulin) as a function of Ca-free relaxation in gracilis arterioles in the three experimental groups.** The values on the abscissa show the inner radii ratios of insulin-induced tone in the Ca-free solution compared to the norepinephrine-induced tone at 50 mmHg. The x axis shows different concentrations of insulin levels. The values are expressed as mean ± SEM. *The DHT group is significantly different from the control group. ‡ The DHT+D3 group is significantly different from the DHT group. ($p<0.01$)

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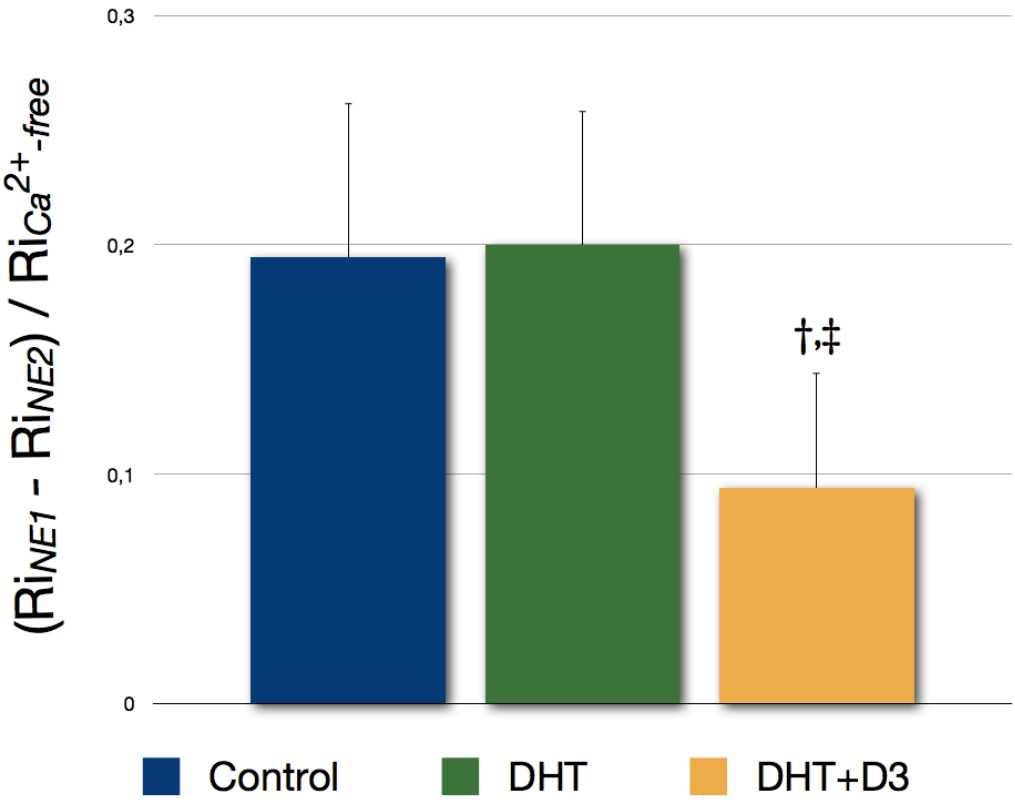

Figure 18.: **The difference between the first and the second norepinephrine induced-contractions after insulin administration compared with the maximal relaxation in Ca-free solution.** The values are expressed as mean ± SEM. † The DHT+D3 group is significantly different from the control. ‡ The DHT+D3 group is significantly different from the DHT group. ($p<0.05$)

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5. Discussion

As a model of human polycystic ovary syndrome, ten weeks of dihydrotestosterone treatment starting during adolescence in female rats [*Manneras et al. 2007, Yanes et al. 2011*] induced significant metabolic and vascular alterations in our studies. Systemic IR without altered glucose metabolism was developed after 70-day DHT treatment. The two hours insulin value of oral glucose tolerance test in the DHT treated animals was nearly two times higher than in controls (*Table 3*.). Chronic DHT treatment of female rats, which is an animal model for human PCOS, induced significant alterations in the biomechanical and pharmacological properties of a skeletal muscle arteriole. We observed an increase in arteriolar diameter (*Fig. 9.A and B*). The relaxed lumen was dilated but was not accompanied by an elevation in wall mass (eutrophic remodelling). This elevated wall stress (*Fig. 10.A and B*) was accompanied by an increase in isobaric distensibility (*Fig. 11.A and B*). The elastic moduli did not change, which demonstrates that passive elastic element remodelling was limited (*Fig. 12.*). There was a substantial alteration in the contractility and spontaneous (myogenic) tone of the segments (*Figs. 13.,14. and 15.*). The additional contraction generated by 1 μM NE was reduced in the DHT-treated segments. Endothelial dilation was diminished in the DHT-treated groups (*Fig. 16*.).

The present study demonstrated that insulin-induced vascular relaxation of arterioles was diminished (*Fig. 17.*). These loss of insulin dependent dilation of arterioles is the vascular form of IR. While increased IR in human PCOS disease and in DHT treated female animals has been described previously [*Yanes et al. 2011*], the alteration of the well-known vasodilator activity of insulin has not been studied.

Vitamin D_3 is an adjuvant therapy for PCOS [*Thys-Jacobs et al. 1999*], and the cardiovascular protective effect of low-dose vitamin D_3 therapy is widely accepted

[*Wong et al. 2010, Przybylsky et al. 2010, Yiu et al. 2011*]. In our study vitamin D_3 therapy was demonstrated to reverse systemic IR in our early PCOS model caused by DHT treatment. Vitamin D_3 supplementation entirely corrected IR (*Table 3.*). Chronic vitamin D_3 treatment restored vascular diameter (*Fig. 9.A and B*). Our experiments demonstrate that vitamin D_3 can reverse morphological remodelling, elevation of wall stress and distensibility in a resistance vessel (*Figs. 10. and 11.*). It improved NE-induced contraction and myogenic tone was further reduced (*Figs. 13. and 14.*). However, loss of endothelial relaxation was unaffected (*Fig. 16.*). It was shown that changes after DHT treatment are partially counteracted by vitamin D_3. Investigation of these basic mechanisms revealed that the first effect from a hyperandrogenic state on musculocutaneous gracilis arterioles is that NO-dependent relaxation is impaired. However, vitamin D_3 treatment has no effect on the NO-dependent abnormalities; other mechanisms are responsible for compensation.

In animals treated with vitamin D_3, the insulin-evoked relaxation returned to the control level in arterioles. In addition, the resistance artery effects of vitamin D_3 treatment, which improves both the alterations associated with PCOS (*Figs. 17.A,B and 18.*) and IR (*Table 3.*), had not been investigated yet.

5.1. Basic physiological and metabolic changes after chronic DHT treatment with or without vitamin D_3 administration

The experimental model used in the present study has been proven to be an adequate rat PCOS model [*Manneras et al. 2007, Yanes et al. 2011*]. It has been shown that an 8 to 12-week DHT treatment identical to the one that we used induces polycystic ovarian syndrome and significantly, approximately threefold, increases androgen levels in female rats [*Manneras et al. 2007*]. Yanes has detected several metabolic abnormalities [*Yanes et al. 2011*]. Despite normal oestradiol levels, DHT-treated animals exhibited oestrus cycle dysfunction. A significant increase in body weight was detected, which

was also noted in the present study. This difference in body weight may be a consequence of an androgen-dependent increase in adiponectin receptor expression and low serum levels of adiponectin [*Tan et al. 2006, Trolle et al. 2010*]. The effect on body weight was not influenced by vitamin D_3 treatment. Based on elevated blood glucose and insulin levels, Yanes hypothesised that IR occurs in HAF rats [*Yanes et al. 2011*]. This hypothesis is supported by our measurements because the oral glucose tolerance test (OGTT) did not show glucose metabolism abnormalities, and no difference was found in the fasting blood insulin levels, but a twofold increase was noted in the 2-hour post-glucose load value compared with the control animals. Considering the facts mentioned above, our experimental model was suitable for studying early, initial abnormalities and vascular damage. Although T2DM had not yet developed, the 2-hour insulin value of the OGTT showed IR. This early glucose metabolism abnormality was fully prevented by vitamin D_3 treatment.

The DHT treatment applied by Yanes resulted in hypertension when administered for a longer period of time (90 days) [*Yanes et al. 2011*]. During our 70-day treatment, no significant differences were found among the blood pressure values of the experimental groups. This result means that the changes detected are directly attributable to either HA or hyperinsulinemia/IR and are independent of blood pressure. In our study the mean arterial blood pressure was measured. After cannulation of carotid artery the systolic and diastolic pressure usually converge to the mean pressure and the pulse pressure decrease. A light narcosis was applied for total analgesia and checked by cornea reflex. This form of narcosis was suitable for our protocol, however higher but still normal blood pressures were detected compared to those during deep narcosis. During the 90-day treatment, Yanes detected an increase in spontaneous proteinuria, morphological renal damage, and elevated TGF alpha, leptin and cholesterol levels, indicating increased oxidative stress and increased NADPH oxidase-4 expression [*Yanes et al. 2011*]. These metabolic abnormalities may influence the pharmacological response of the arteries.

5.2. Vascular effects of DHT treatment

It is well known that vascular biomechanical functions are worse in PCOS. In 2000, Lakhani suggested that internal carotid artery pulsatility index is decreased, and cardiovascular risk is increased in women with PCOS [*Lakhani et al. 2000*]. Lakhani then showed an increased intima-media thickness for the common carotid and femoral arteries in young (under the age of 35) women with PCOS [*Lakhani et al. 2004*]. It is known that in PCOS, large arteries become less elastic and more rigid; the stiffness and intima-media thickness of the common carotid artery increase, and its distensibility decreases [*Dokras A. 2008, Luque-Ramirez et al. 2007⁎⁎, Soares et al. 2009*]. These changes correlate with a hyperandrogenic state rather than obesity [*Luque-Ramirez et al. 2007⁎*]. Calcification of the coronary arteries is enhanced [*Dokras A. 2008*], and a higher brachial-ankle pulse wave velocity is detected [*Sasaki et al. 2011*]; thus, the vicious cycle of atherosclerosis also begins at an earlier age. Therefore, the early development of atherosclerosis and increased cardiovascular risk can be regarded as consequences of the hyperandrogenic state [*Dokras et al. 2008, Luque-Ramirez et al. 2007⁎⁎*]. According to Soares, the early increase of cardiovascular risk, which is independent of other risk factors, is a part of polycystic ovary syndrome [*Soares et al. 2009*].

The direct vascular effects of chronic hyperandrogenism must also be considered. The predominant direct vascular effect of androgens is vasorelaxation. Both DHT and testosterone dose- dependently block the L-type voltage-operated calcium channels, while high concentrations (μM) of testosterone induce direct vasorelaxation because of its Ca-antagonist effect and increased cAMP production in the rat aorta [*Montano et al. 2008*]. A similar but slightly weaker Ca-dependent vasorelaxant effect of oestradiol was noted in rat aorta after phenylephrine precontraction [*Castillo et al. 2006*]. Chronic low dose testosterone administration improved the risk of myocardial ischemia [*Miller et al. 2007*], lipid profile and insulin resistance because of its direct vasoactive and metabolic effects [*Cornoldi et al. 2010*]. Despite progressive atherosclerosis, chronic testosterone treatment enhanced ACh-dependent, endothelium-dependent relaxation in

ovariectomized female monkeys, although it did not influence direct smooth muscle-dependent relaxation elicited by sodium nitroprusside [*Adams et al. 1995*]. Yanes found elevated intrarenal angiotensinogen levels and angiotensin I-converting enzyme (ACE) expression as well as decreased angiotensin II R1 expression in Hyperandrogenic female (HAF) rats [*Yanes et al. 2011*]. Human experiments have shown microvascular dysfunction in PCOS, as indicated by the inhibition of ACh-dependent vasodilation [*Lakhani et al. 2005*]. Endothelial dysfunction and damage has been shown to be enhanced by increased inflammatory and soluble activation markers [*Diamanti-Kandarakis et al. 2006*⁎]. Paraoxonase-1 and flow-mediated dilatation have been shown to be useful predictors of endothelial dysfunction [*Soyman et al. 2011*]. The effects of chronic androgen treatment are currently a subject of debate. On the one hand, these effects are species specific, but on the other hand, distinct effects of the different androgenic compounds probably also exist.

5.3 The metabolic and vascular effects of vitamin D_3

It has been suggested that lack of vitamin D might have influence on high insulin levels found in PCOS, and its substitution has a positive effect on carbohydrate-metabolism [*Ngo et al. 2011,Wehr et al. 2011*⁎⁎]. Vitamin D therapy is also advantageous in manifest T2DM [*Yiu et al. 2011*], by increasing flow-mediated vasodilation in brachial arteries. Pittas described a threefold decrease in risk of T2DM caused by a regimen of 800 IU vitamin D_3 compared to 400 IU daily [*Pittas et al. 2006*].

Wong's results demonstrate that vitamin D_3 has a direct vascular effect—lowering prostanoid-dependent vasoconstriction in SHR to the level of Wistar-Kyoto rats [*Wong et al. 2010.*]. Therefore, a role for vitamin D_3 deficiency has been suggested in the mechanism of hypertension because vitamin D_3 treatment also decreases endothelial dysfunction through weakening of endothelium dependent - 6-keto-PGF1α - vasoconstriction in SHR rats [*Wong et al. 2010, Judd et al. 2012*]. Many authors have

suggested that a low serum level of vitamin D_3 plays a role in the IR observed in PCOS, which may be corrected by vitamin D_3 replacement [*Ngo et al. 2011*]. In T2DM vitamin D_3 improved the flow-mediated vasodilation of the brachial artery [*Yiu et al. 2011*]. The latter study demonstrated the direct vascular effects of vitamin D_3 treatment: the flow-mediated dilation of the brachial artery was significantly diminished in patients with low serum levels of vitamin D_3. Under experimental conditions, a 6-week vitamin D_3 treatment normalised ACh relaxation in SHR rats. However, this effect was not related to intracellular calcium balance, which remained unchanged during the whole treatment; instead, it correlated with decreases in reactive oxygen free radicals and COX-1 expression [*Wong et al. 2010*]. At this time there is no other information available relating to the effect of vitamin D_3 on the vascular prostanoid metabolism. However, Wong described a prostanoid dependent relaxation in contrast with our vasoconstrictor effect. The diversity in the effect of vitamin D_3 may be explained by the differences in the examined vessels and species.

In our study vitamin D_3 treatment was used for 10 weeks, in weekly dosage. Rats received standard rat chow with normal vitamin D_3 content, they were not vitamin D insufficient animals. We used vitamin D_3 as an active treatment. Protective role of vitamin D against cardiovascular diseases are well known: data are available in the literature on the vascular effects of vitamin D_3 in general [*Bukoski et al. 1990, Wong et al. 2010*] and during other pathological conditions, e.g. heart failure [*Przybilsky et al. 2010*]. In light of the above, when interpreting the effects of vitamin D_3 under hyperandrogenic circumstances, one must consider the direct vascular effect of vitamin D_3 and DHT interactions as well as the indirect vascular effects caused by improving IR. The stability of the NE-induced contractions in vitamin D_3-treated rats demonstrates the role of vitamin D_3 in preserving vascular reactivity. According to studies performed on rat aorta, vitamin D_3 plays a role in maintaining vascular reactivity and the vascular effects of sex hormones via creatine kinase activity [*Somjen et al. 2006*]. Increased vascular endothelial growth factor expression may also take part in the chronic vasoprotective effects of vitamin D_3 [*Cardus et al. 2009*].

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5.4. Pharmacological effects on gracilis arterioles

5.4.1. Pharmacological effects of DHT treatment

After assessing endothelium-dependent relaxation, we conclude that the most important vasorelaxation factor in the control group is NO, which is disabled by DHT treatment. Therefore, the decreased spontaneous (myogenic) tone of the DHT-treated segments could not be attributed to NO pathway activation; moreover, this pathway was not affected by additional vitamin D_3 treatment. L-NAME blockade, which is an active vasoconstrictor in the ACh-treated control segments, had no effect on the DHT-treated female animals. The contraction-reducing effect of vitamin D_3 treatment is independent of the NO pathway. However, further investigations are necessary because our results thus far did not provide an exact mechanism for the vitamin D_3 action.

Malkin has shown that acute testosterone treatment induce vasodilation, whereas chronic treatment decreases endothelium-dependent and endothelium-independent vasorelaxation and increases norepinephrine-induced vasoconstriction [*Malkin et al. 2006*]. In orchiectomised rats, thromboxane-dependent increase of the basal arterial tone was observed following testosterone pretreatment. Acute administration of thromboxane did not cause significant vasoconstriction, but the vasodilating effect of thromboxane synthesis and receptor inhibition was stronger on the cerebral arteries of the androgen-treated rats [*Gonzales et al. 2005*]. In case of chronic testosterone exposure, this effect might result in more pronounced vasospasm during cerebral thromboembolism. Gonzales has previously shown a decrease in endothelium-dependent vasodilation independent of COX and NO in response to testosterone in rat cerebral arteries [*Gonzales et al. 2004*].

5.4.2. Pharmacological alterations of arterioles in PCOS

In addition to vascular mechanical damage, significant pharmacological reactivity changes also develop in women with PCOS. Alterations in smooth muscle- and endothelium-dependent relaxation and vasoconstriction have been studied for PCOS and the hyperandrogenic state. Kravariti detected a significant decrease in both endothelium- (flow-mediated) and smooth muscle-dependent (nitrate-mediated) vasodilation in women with PCOS compared with healthy controls [*Kravariti et al. 2005*]. The independent factors for reduced flow were IR, degree of hyperandrogenicity, and cholesterol level. Kravariti concluded that women who have had PCOS since their 20s have obesity-independent endothelial damage and suggested a strict follow-up on this condition to prevent target organ damage [*Kravariti et al. 2005*]. This suggests that women with PCOS are at increased risk for early onset cardiovascular disease and may benefit in particular from measures that improve endothelial function.

Flow-mediated vasodilation has been studied by several different groups, which observed either similar results or did not find any differences between PCOS patients and healthy subjects depending on the population studied and type of assay used [*Soyman et al. 2011, Arikan et al. 2009*]. This apparent contradiction was solved by Cusson, who stated that vascular damage develops gradually. Cusson described the decrease in flow-mediated vasodilation as the earliest detectable abnormality in PCOS; however, he observed normal arterial stiffness [*Cussons et al. 2009*]. It can be concluded that the early, initial changes that presumably develop in the 20s and 30s are the basis for subsequent hypertension and metabolic syndrome [*Dokras A. 2008*].

5.4.3. Pharmacological effects of vitamin D_3 on gracilis arterioles

Our study also investigated the effects of vitamin D_3 treatment on smooth muscle- and endothelium-dependent vasodilation and vasoconstriction of arterioles, which play an important role in target organ damage. It was known before our study that vitamin D_3

analogues influence the vascular effects from sex hormones [*Somjen et al. 2006*]. Chronic vitamin D_3 treatment directly decreased blood pressure and the vasoconstrictor response in isolated arteries from SHR rats. The latter effect was attributed to reduced COX-1 expression, while intracellular Ca-levels remained unchanged [*Wong et al. 2010*]. The parallel structural and functional changes in the vessel wall were demonstrated by Yiu et al. In T2DM, a lack of vitamin D_3 caused endothelial dysfunction, which presented as a decrease in flow-mediated dilation of the brachial artery, and depletion of the circulating endothelial progenitor cells [*Yiu et al. 2011*]. In a recent article lowered endothelium-derived dilation, elevated blood pressure and myogenic tone of mesenteric arteries in vitamin D insufficiency were reported [*Tare et al. 2011*]. Because of unchanged serum calcium concentration those alterations cannot be explained by low Ca^{2+} level. The authors supposed that vitamin D_3 might have a direct effect on cardiovascular system. Vitamin D_3-induced VEGF expression might also improve endothelial dysfunction [*Cardus et al. 2009*]. We have partially proven our hypothesis that vascular reactivity changes in PCOS are affected by chronic vitamin D_3 treatment. Chronic vitamin D_3 treatment resulted in complex changes. In addition to a decrease in the spontaneous and resting vascular tone, the smooth muscle-dependent contraction and total relaxation approached and reached the control level, respectively, compared with the DHT treatment results. Vitamin D_3 treatment prevented arteriolar morphological remodelling and the increase in lumen volume caused by DHT treatment. However, vitamin D_3 treatment did not influence the decreased NO from DHT treatment. Therefore, the relaxation compensation we detected is likely achieved by distinct pathways.

5.4.4. The pharmacological effects of metformin and oral contraceptives as medical treatment in PCOS

The management of PCOS must be suitable to the patient's complaints and future planes. Restoration of regular periods, treatment of hirsutism and acne, restoration of fertility and lowering insulin level must be the most important goals of the therapy in

PCOS. If fertility is not the primary patient's wish, then OCs are highly recommended for the regulation of menstruation. Although insulin sensitisers are not licensed for PCOS treatment, the use of these agents is widely accepted for management of infertility and severe obesity with IR.

The pharmacological effects on vessel reactivity of some products used in PCOS treatment are still under investigation. Meyer noted the importance of accounting for not only the hyperandrogenic symptoms but also the changes in glucose metabolism, stiffness and pulse wave velocity during PCOS treatment; treatment with metformin rather than high dose oral contraceptives has been suggested [*Meyer et al. 2007, Teede et al. 2010*]. Agarwal has shown that metformin decreases the arterial stiffness, aortic and brachial pulse wave velocity, aortic augmentation index and improves endothelium-dependent and independent vascular responses [*Agarwal et al. 2010, Castillo et al. 2006, Orio et al. 2005, Jensterle et al. 2008*]. Increased flow-mediated vasodilation has been considered during insulin sensitizing treatment with metformin or pioglitazone in PCOS women [*Naka et al. 2011*]. Chronic treatment of an other TZD agent - 6-month administration of rosiglitazone - was shown to improve endothelial function (measured by ultrasound flowmetry of brachial artery) similarly to metformin. Based on the mentioned results metformin and TZDs were suggested to reverse atherosclerotic process and reduce risk of CVDs in young PCOS women with mild IR [*Jensterle et al. 2008*].

In PCOS, vascular biomechanical damage is essential and progresses faster than in the general population. The treatment modalities used in PCOS often modify this risk [*Agarwal et al. 2010, Kravariti et al. 2005*]. High-dose OCs might increase cardiovascular risk; metformin has been shown to decrease this risk, whereas low-dose oral contraceptives likely have no significant effect [*Agarwal et al. 2010, Kravariti et al. 2005*]. Oral contraceptives plus metformin combined treatment was shown to increase flow mediated dilatation while OC without metformin didn't change it [*Essah et al. 2011*]. Meendering et al. reported 30μg Ethynil oestradiol combined with 3 mg Drospirenon

(low dose OC) increasing endothelium dependent vasodilation in brachial artery compared with placebo [*Meendering et al. 2010*] Oestradiol administration was presented as an increasing factor of vascular elasticity with a negative association of triglyceride level and previous history of smoking [*Clapauch et al. 2010*].

5.5. Biomechanical and pharmacological changes of gracilis arterioles after DHT treatment and parallel vitamin D_3 administration

Our results show basic changes in vascular biomechanics in response to DHT treatment; the arteriolar walls become more rigid with increased tangential wall stress compared with the controls. However, the tangential wall stress and distensibility differences that were detected even in normotensive animals might be considered prehypertensive abnormalities. The enhanced mechanical load of the arteriolar wall is the local factor for the self-perpetuating, vicious cycle of emerging hypertension. For the decreased spontaneous tone observed in the vitamin D_3-treated group, tangential wall stress is not elevated. This has a local effect against hypertension development. Thus, the mechanical load of the vessels increases. Under passive circumstances, vitamin D_3 treatment fully restores the biomechanical balance of the vessel wall. However, in response to norepinephrine, mechanical load of the vessel wall increases compared with the control group, although it does not reach the level of the DHT-treated group.

As a result of the smooth muscle pharmacological reactivity changes, the total adaptation range for skeletal muscle perfusion significantly decreases in response to DHT treatment. Although vitamin D_3 treatment significantly improves this range, it does not reach the level of the control group. In studying the basis for this phenomenon, an obvious, significant decrease in NE response is noted after DHT treatment. This is almost restored by vitamin D_3 treatment. A decrease of relaxation is detected in response to DHT treatment. This decrease is even more significant when DHT is combined with vitamin D_3. Another interpretation of these findings is that DHT

treatment results in diminished adaptive reserve capacity in both directions. Vitamin D_3 applied with DHT significantly improves pharmacological reactivity but decreases the vessels' spontaneous tone even compared with the control.

5.6. Vascular effects of insulin

5.6.1. Effects on arterioles

In our experiments, vitamin D_3 treatment significantly decreased the vascular IR of arterioles caused by DHT. During NE-induced precontraction, DHT treatment decreased insulin-dependent relaxation, thus producing vascular IR. However, similar relaxation was observed in the control and vitamin D_3-treated groups (*Fig. 17.A and B*). In response to insulin, the NE sensitivity of the vitamin D_3-treated group practically remained unchanged (*Fig. 18.*). In the control and DHT-treated groups, a 20% increase in contractility was observed after rinsing and during the second contraction compared with the first norepinephrine contraction (*Fig. 18.*). No differences were found between the first and second NE contractions in the vitamin D_3-treated group, which suggests that vitamin D_3 treatment improves NE sensitivity. However, the most prominent insulin-dependent relaxation was detected in the vitamin D_3-treated group (*Fig. 16.B*). The vessels were pretreated in the same way and were in the same phase of the protocol. The difference between the first and second NE contraction cannot explained by tachyfilaxis because the incubation with different concentrations of insulin takes longer time than tachyfilaxis lasts. Increased second NE contraction didn't feature vitamin D treated vessels, which shows an interesting difference between vessel reactivity of the groups, and exclude phenomenon tachyfilaxis, also. Insulin has an attenuating effect on smooth muscle cell contractility through iNOS and cGMP [*Kahn et al. 1998*]. As our best knowledge this effect of insulin is not described yet. We suggest that this increase of NE sensitivity may be related to the increased glucose uptake of the vessel smooth muscle cells caused by insulin. Vitamin D also improve utilisation of glucose, which can

explain the lower difference in contractility after insulin pretreatment. The other explanation of this phenomenon might be a rebound effect of insulin antagonism on NE sensitivity. To clarify the exact mechanism further examinations are needed.

Comparing the insulin-dependent relaxation to ACh relaxation, highly significant differences were noted. ACh caused stronger relaxation in the control group than in the two treated groups. Moreover, this difference remained the same or increased during L-NAME blockade. These results suggest that the relaxing effect of insulin is independent from NO in the gracilis arterioles and that vitamin D_3 restores normal vascular tone independently from the NO system. NO-mediated relaxation of insulin in human muscle arterioles [*Steinberg et al. 1994, Scherrer et al. 1994*] and Na^+-K^+ ATP-ase may play independent roles in human vessel relaxation [*Tack et al. 1996, Yki-Jarvinen et al. 1998*] Luis A et al. have presented an NO-independent insulin-mediated direct mechanism of relaxation on renal efferent arterioles in rabbits [*Luis et al. 1993*]. Physical exercise has been found to increase the blood flow stimulated by insulin in skin arterioles by activating K(Ca^{2+}) channels [*Ghafouri et al. 2011*]. Our results demonstrate that DHT treatment probably partially decreases NO-mediated relaxation. During DHT treatment, a difference was noted in ACh relaxation. By blocking the NO pathway, minor contractions were detected, suggesting the local involvement of the prostanoid system [*Wong et al. 2010, Gonzales et al. 2005*] and muscarinic ACh receptors [*Gericke et al. 2011*] in addition to the NO pathway. It has been shown that insulin-dependent relaxation of the large vessels (aorta) and gracilis arterioles are predominantly mediated by NO and EDHF/potassium channels, respectively [*Peuler et al. 1993*]. Our study demonstrated that DHT treatment reduced insulin dependent relaxation of rat aortic rings as well. This loss of insulin dependent dilation is the similar vascular form of IR as was seen in gracilis arterioles. In contrast with systemic IR, the net effect of vitamin D treatment on vascular IR of aorta caused by hyperandrogenic state was not significant.

5.6.2. Altered insulin relaxation of aortic rings

Our research team demonstrated that insulin induced relaxation was significantly lower in DHT treated groups compared to control on aorta rings ($p<0.05$ for control vs DHT and DHT+D3). In contrast with systemic IR, the net effect of vitamin D_3 treatment on vascular insulin resistance caused by hyperandrogenic state was not significant on aortic rings. Similar dissociation of metabolic and vascular insulin resistance has been demonstrated in ageing [*Schulman et al. 2007*].

As suggested by a recent publication [*Kim et al. 2011*], key mechanism of insulin dependent vasorelaxation is activation of the NO pathway. Our results suggest that DHT treatment caused a decline of the insulin dependent relaxation principally through deterioration of NO dependent relaxation, but it interfered with other vasorelaxation mechanism, as among the relaxing effects still remained a little difference after blocking the NO pathway. Partial reversal of DHT induced reduction of NO-dependent vasorelaxation by vitamin D_3 treatment suggests protection of the NO dependent vasorelaxative effects by vitamin D_3 in DHT treated rats.

Taken together, we suggest, that the local effect of vitamin D_3 treatment is a partial restoration of both the NO dependent relaxation and constrictor prostanoid effects. However the lack of vitamin D effects in the insulin-vasorelaxation without pretreatment suggests the involvement of other, unknown effects, such as EDHF, Ca-antagonist or other mechanism.

Although long term systemic IR is accompanied by vessel damage and atherosclerosis, controlling IR alone, did not resolve vascular damage of the aorta caused by the hyperandrogenic state in our model. According to our investigations, the impairment of the aortic vasorelaxation developed mainly as a consequence of hyperandrogenic effect – independently from restoring systemic IR by vitamin D_3. In our study we detected a restoration due to vitamin D_3 administration in insulin induced vasorelaxation in gracilis

arterioles. The contrast between vitamin D_3 effect on relaxation of the aorta and resistance arterioles might be explained by different local domination of dilating endothelial factors. The same type of vasorelaxing mediators are supposed to release in the aorta quantitatively different from the microvessels.

Direct effects of the hyperandrogenic state on blood vessels should be taken into account. The progression of atherosclerosis in the coronary arteries of ovariectomised female monkeys was described in 1995 by Adams et al [*Adams et al. 1995*]. Male sex and long-term testosterone treatment are well-known and independent risk factors of atherosclerotic vascular disease. However, according to Cornoldi's research, restoration of low testosterone level to the normal range reduces the number of ischemic attacks and corrects HOMA-IR in hormone-deprived elderly men. Naturally, this hormone effect is not necessarily true for higher than physiological hormone levels [*Cornoldi et al. 2010*]. In PCOS inflexibility of the big vessels (vascular stiffness) has been established [*Sasaki et al. 2011, Soares et al. 2009*]. All components of the metabolic syndrome - including IR - occur significantly more frequently in PCOS patients compared to the average population [*Dokras A. 2008*]. Regarding to the first steps of this process, a terra incognita can be found: the early mechanisms of blood vessel damage in PCOS. Cussons et al also demonstrated that blood vessel damage developed gradually in PCOS-patients. They have described a decrease in flow mediated dilatation of the brachial artery at normal stiffness [*Cussons et al. 2009*].

Considering vascular effects of vitamin D_3 in HAF, we have to count direct vascular effects of vitamin D_3, interactions with DHT and indirect vascular effects of improving systemic IR. However the latter effect was not dominant on the rat aorta, regarding insulin dependent relaxation.

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6. Conclusions

In this study we used an experimental animal model of PCOS to examine early metabolic and vascular lesions before the development of hypertension. This study investigated the details of the vascular biomechanical and pharmacological changes from vascular adaptation of skeletal muscle arterioles in a PCOS model. Our results show that the hyperandrogenic state resulted in more rigid, less flexible artery walls. These arteries show reduced endothelium- and smooth muscle-dependent vasorelaxation and constriction as well as a complete loss of NO-dependent relaxation compared with the controls. In the relaxed vessels, concurrent vitamin D_3 treatment corrected wall distensibility and mechanics to the control level. However, norepinephrine contraction resulted in slightly less flexible vessels (*Figs. 10. and 11.*). In response to vitamin D_3 treatment, smooth muscle reactivity approached the control, but the spontaneous tone was lower. Vitamin D_3 treatment did not influence the NO-dependent relaxation that was decreased by DHT treatment. However, vitamin D_3 treatment had no effect on the NO-dependent abnormalities; vasorelaxation compensation was achieved by other mechanisms.

Herein, we provide this first data for the effect of vitamin D_3 on the mechanical properties of arteries in PCOS. Vitamin D_3 can be administered as an adjuvant therapy for PCOS [*Thys-Jacobs et al. 1999.*], and the cardiovascular protective effect of low-dose vitamin D_3 therapy is known [*Wong et al. 2010, Przybylsky et al. 2010, Yiu et al. 2011*]. The results from our study show that for PCOS, vitamin D_3 is an adjuvant treatment that might inhibit arterial vascular mechanical impairment and potentiate the vascular protective effect of metformin because of its distinct mechanism.

This is the first study investigating the vascular adaptation and insulin sensitivity of gracilis arterioles in a PCOS model. Our results show a partial NO-dependency of vascular reactivity changes caused by hyperandrogenism. In our experimental model,

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vitamin D_3 restored the systemic insulin response as well as the NO-independent insulin-induced relaxation and norepinephrine sensitivity of the gracilis arterioles. This is the first study demonstrating vascular insulin resistance of the gracilis arterioles and the counteracting effect of vitamin D_3 in an experimental PCOS model.

In our study we were the first to show a decreased insulin dependent relaxing effect on rat aorta. Vitamin D_3 supplementation helped avoiding the elevation of serum insulin level, but did not influence the decrease of insulin dependent relaxation. The diminished relaxation caused by androgenic effect was partly NO dependent. The altogether neutral effect of vitamin D_3 on the rat aorta could be explained by the counterbalance of the local constrictor prostanoids and a moderate improvement of the NO dependent relaxation against effects not studied here. In our animal model we presented a difference between vascular insulin resistance of gracilis arterioles and aorta. Vitamin D_3 administration fully restored vascular insulin resistance in musculocutaneous arterioles but had no effect in the aorta. Further experimental studies are needed to define the different pathways of endothel dependent vasodilatation.

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Summary

Background/Aim Polycystic ovary syndrome (PCOS) affects 4-10% of fertile women. It is already well known that increased insulin resistance and altered metabolic status occur in these patients. PCOS also alters cardiovascular function through various mechanisms. The vascular effects are less investigated. Our aims were to clarify the effects of dihydrotestosterone(DHT)-induced PCOS on carbohydrate metabolism, arteriolar biomechanics, pharmacological reactivity and insulin-induced vasorelaxation in a rat model and the possible modulatory role of vitamin D. **Methods** Female rats were subjected to chronic DHT treatment (subcutaneous pellet, 83μg/100g/day). Some of them obtained vitamin D treatment (120ng/100g/week). Oral glucose tolerance test (OGTT) with insulin level measurements was performed. After ten weeks, arteriolar biomechanics, norepinephrine induced contractility, acetylcholine relaxation and insulin induced dilation were tested in gracilis arterioles by pressure arteriography in control as well as in DHT- and in DHT with vitamin D3-treated animals. **Results** Blood glucose level was normal in all animals, however, this was accompanied by significantly higher 120' insulin levels in DHT treated rats. Insulin elevation was prevented by concomitant vitamin D treatment. DHT treatment increased the morphological diameter, wall stress and distensibility of resistance arterioles, reduced their ability to contract in response to norepinephrine (from 39.5±5.8 to 20.7±2.3%, $p<0.05$), reduced acetylcholine (ACh) induced vasodilation (122.0±2.9 to 48.0±1.4%) and also reduced dilation induced by insulin (at 30 mU/ml from 21.7±5.3 to 9.8±5.6%, $p<0.01$). Concomitant vitamin D treatment lowered the mechanical load of the arterioles, increased the contractile response and resulted in more relaxed vessels. Vitamin D treatment restored insulin relaxation (full recovery at 240 mU/ml). Endothelial dilation tested with acetylcholine was lower after DHT treatment independent of vitamin D supplementation. **Conclusion** Metabolically proven increased insulin resistance of DHT treated female rats associates with reduced ability of the resistance arterioles to relax in response to insulin. The arterioles with more rigid, less flexible walls show reduced endothelium- and smooth muscle-dependent vasorelaxation and constriction with a complete loss of nitric

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oxide(NO)-dependent relaxation compared with controls. This situation caused by hyperandrogenic state is improved in arterioles by parallel chronic vitamin D treatment without affecting NO relaxation. The observed alterations of resistance arteries form an important prehypertensive pathway of vascular damage in PCOS and might explain the observed clinical effectiveness of vitamin D treatment.

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Összefoglalás

Háttér/Célkitűzés: A policisztás petefészek szindróma (PCO) a fertilis korú nők 4-10%-át érinti világszerte. A PCO-szindrómában kialakuló inzulinrezisztencia, metabolikus szindróma, valamint az ezek kapcsán fenyegető súlyos kardiovaszkuláris kockázat jól ismert. A megjelenő érelváltozásokról kevés adat áll rendelkezésre az irodalomban. Korábban kísérletesen igazolták, hogy hosszabb dihidrotesztoszteron (DHT)-kezelés a PCO-szindrómához nagyon hasonló állapotot idéz elő patkányokban. Célunk az volt, hogy vizsgáljuk a krónikus DHT-kezelés kiváltotta PCO-szindróma kiserekre gyakorolt biomechanikai, farmakológiai és inzulin-relaxációs hatását, továbbá a D-vitamin befolyásoló szerepét ezekre a folyamatokra. **Módszer:** Kísérleteinket nőstény patkányokon végeztük, amelyek egy része álműtött volt, másik része napi 83µg dózisban DHT-kezelésben részesült. A DHT-kezelt állatok fele heti 120ng/100g dózisban D-vitamint, másik fele vehiculumot kapott. Nyolc héttel később a cukorterhelés során a szérum inzulinszinteket is mértük. Tíz hetet követően egy nyomás-szervokontroll rendszer segítségével mikroangiometriás vizsgálatokat végeztünk izolált muszkulokután rezisztencia arteriolákon, ahol a biomechanikai tulajdonságokat, noradrenalin-kontrakciót, Ach-relaxációt és az inzulin által kiváltott vazorelaxációt teszteltük a kontroll, a DHT-kezelt és a DHT- és D-vitamin-kezelt csoportban. **Eredmények**: Normál vércukor értékek mellett szignifikánsan emelkedett 120 perces szérum inzulin szinteket mértünk a DHT-kezelt állatokban. Ezt az eltérést a D-vitamin kezelés kivédte. A DHT-kezelés növelte a rezisztencia arteriolák átmérőjét, a tangencionális stresszt és a disztenzibilitást, csökkentette a noradrenalinra jelentkező kontrakció mértékét (39.5±5.8 és 20.7±2.3%, $p<0.05$). Ugyancsak csökkent az Ach (122.0±2.9 és 48.0±1.4%) és az inzulin hatására jelentkező vazodilatáció (30 mU/ml-nél 21.7±5.3 és 9.8±5.6%, $p<0.01$). D-vitamin együttes alkalmazásakor a relaxáltabb alapállapotú arteriolákban csökkent a mechanikai töltőhatás és nőtt a kontrakciós válasz. Az inzulinra jelentkező relaxációt a D-vitamin kezelés a kontrollhoz közeli szintre javította. A DHT kezelést követő Ach-vazorelaxációban bekövetkezett csökkenést a D-vitamin nem változtatta meg. **Következtetések**: A DHT-kezelt állatok igazolt

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inzulinrezisztenciája csökkent inzulin-relaxációs választ eredményezett a kiserekben. A hiperandrogén állapot következtében merevebb és kevésbé nyújtható falú arteriolák simaizom függő kontrakciója és endotélfüggő relaxációja csökkent, miközben az NO hatás teljesen kiesett. D-vitamin alkalmazása javított ezen az eltérésen. Vizsgálataink a metabolikus elváltozások olyan korai állapotában történtek, ahol a PCO szindróma kapcsán a kiserekben kialakuló érkárosodások kezdeti lépéseit figyelhettük meg. A D-vitamin kezeléssel összefüggésben a kiserek szintjén tapasztalható különbségek rávilágítanak a hiperandrogén állapot és az inzulinrezisztencia okozta érkárosodások jelentőségére, és részben magyarázatot adnak a D-vitamin klinikumban is tapasztalt jótékony hatására PCO-szindrómában.

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9. List of Publications

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

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

Sara L, Nadasy GL, Antal P, Monori – Kiss A, Szekeres M, Masszi G, Monos E,Varbiro Sz. *(2012)* Pharmacological reactivity of resistance vessels in a rat PCOS model – vascular effects of parallel vitamin D3 treatment. *Gynecological Endocrinology DOI: 10.3109/09513590.2012.683079. Epub.: 24.05.2012. IF: 1,461 (2010)*

Sara L, Nadasy GL, Antal P, Szekeres M, Monori – Kiss A, Horvath EM, Tokes A, Masszi G, Monos E, Varbiro Sz. Arteriolar biomechanics in a rat PCOS model – effects of parallel vitamin D3 treatment. *Acta Physiologica Hungarica - accepted for publication. IF: 1,226 (2010)*

Submitted manuscript related to the thesis:

Masszi G, Buday A, Novak A, Horvath E.M, Tarszabo R, **Sara L**, Revesz Cs, Benko R, Nadasy GyL, Benyó Z, Hamar P, Varbiro Sz. Altered insulin relaxation of aortic rings in a rodent model of polycystic ovary syndrome.

Other publications:

Sebestyén A, Várbíró S, Deák G, Gimes G, Szabó I, **Sára L**, Paulin F. *(2005)* Difficulties in treating patients with nephrosis syndrome [Nephrosis szindrómás terhesség kezelésének problémái Esetismertetés] *Magyar Nőorvosok Lapja. 68(1): 57-60.*

Sebestyen A, Varbiro S, **Sara L**, Deak G, Kerkovits L, Szabo I, Kiss I, Paulin F. *(2008)* Successful management of pregnancy with nephrotic syndrome due to preexisting membranous glomerulonephritis: A case report. *Fetal Diagnosis and Therapy, 24(3): 186-189. IF:1,184*

Valent S, Németh J, **Sára L**, Gidai J, Tóth P, Schaff Zs, Paulin F, Pajor A. *(2011)* High early uterine vascular resistance values increase the risk of adverse pregnancy outcome independently from placental VEGF and VEGFR1 reactivities *European Journal of Obstetrics Gynecology and Reproductive Biology. 156(2): 165-170. IF: 1,764 (2010)*

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

Pánczél Z, **Sára L**, Tóth P, Hubay M, Keller É, Langmár Z, Pajor A. *(2011)* Spontaneous aortic rupture during pregnancy [Spontán aortaruptura várandósság alatt] *Orvosi Hetilap. 152(23): 929-933.*

Conference proceedings:

Valent S, Tóth P, **Sára L**, Gidai J, Paulin F. Predictive value of the uterine arteries' blood perfusion for preeclamsia. In: *Kurjak A, Chervenak FA: Proceedings of the 7th World Congress of Perinatal Medicine. Zagreb, Croatia 16.09.2005 -19.09.2005* Bologna: Medimond Monduzzi Editore, pp. 427-432. (ISBN:88-7587-179-5)

Mátrai M, **Sára L**, Székács B, Mericli M, Nádasy GyL, Szekeres M, Monos E, Várbíró Sz. *(2009)* Nőstény patkányok intramurális coronáriáinak kontraktilitás- és biomechanikai változásai angiotenzin II indukálta hipertónia hatására. *Hypertonia és Nephrologia. 13(S3): 228. (prize-winner)*

Arora CP, Vari SG, Sijanovic S, Resic J, Kacerovsky M, Flach E, **Sara L**, Issat T, Ceausu I, Lancz K, Pirahova V, Hobel CJ. *(2011)* Preterm Birth, a Neglected Quandary: A Retrospective Data Collection Report from Seven Central and Eastern European Countries. *Reproductive Sciences 18(3): 373A.*

Zinner B, **Sára L**, Pajor A. *(2011)* Risk factors of PTB. Survey based on the retrospective analysis of PTBs at our clinic between 2007 and 2009. *Biopolymers and Cell 27(2): 112.*