Aus dem Charité Centrum für Therapieforschung

Institute für Pharmakologie / Center for Cardiovascular Research (CCR)

Kommissarische Leitung: Prof. Dr. med. Ulrich Kintscher

Habilitationsschrift

Obesity-related cardiovascular and metabolic diseases:
The role of estrogens, estrogen receptors and PPARgamma

Zur Erlangung der Lehrbefähigung

Für das Fach Experimentelle Pharmakologie

Vorgelegt dem Fakultätsrat der Medizinischen Fakultät

Charité -Universitätsmedizin Berlin

von

Dr. Anna Foryst-Ludwig

Geboren am 09.11.1972 in Krakow, Polen

Contents

1   Abbreviations

AF1	N-terminal transactivation domain
AF2	C-terminal second transactivation domain
AKT	Protein Kinase B (PKB)
AMPK	5' AMP-activated protein kinase
ANP	atrial natriuretic peptide
AP2	activator protein 1
ArKO	aromatase-deficient mice
ATGL	adipose triglyceride lipase
beta-MHCH	beta-myosin heavy chain
BMI	body mass index
BNP	brain natriuretic peptide
CaMK	Ca+2-calmodulin dependent kinase
CBP	cAMP-response-element-binding protein CREB-binding protein/p300
COX	cyclooxygenase
CXCL5	C-X-C motif chemokine 5
DPN	diarylpropionitrile
DRIP205	Vitamin D Receptor Interacting Protein
E2	17beta-estradiol
eNOS	endothelial nitric oxide synthase
ERalpha/beta-KO	ERalpha/beta-deficient mice
ERE	estrogen response element
ERK	extracellular-signal-regulated kinases
ERRalpha	estrogen related receptor alpha
ERs	estrogen receptors
ERT	estrogen replacement therapy
ET-1	endothelin 1
FAS	fatty acid synthase
FFAs	free fatty acids
Glut4	glucose transporter 4
GRP30	G-protein-coupled estrogen receptor
GSK-3-beta	glycogen synthase kinase-3-beta
GT	glucose tolerance
HDACs	histone deacetylases
HFD	high fat diet
HIF-1alpha	hypoxia-regulated transcription factor alpha
HMGA1	high-mobility group A1
HRT	hormone replacement therapy
HSL	hormone sensitive lipase
IGF1	insulin-like growth factor 1
IL-1RA	IL-1R antagonists

IL-6	interleukin 6
IR	insulin resistance
IS	insulin sensitivity
LBD	ligand binding domain
LPL	lipoprotein lipase
MCP1	monocytes chemoattractant protein-1
MEK	mitogen-activated protein kinase
MGL	monoacylglycerol lipase
MMP9	matrix metalloproteinase 9
ncmER	non-classical membrane estrogen receptor
NCoR	nuclear receptor repressor
NEFA	nonesterified fatty acids
NHR	nuclear hormone receptor
NO	nitric oxide
OVX - mice	ovariectomized mice
p160/SRC	steroid receptor co-activator
p38	p38 mitogen-activated protein kinases
PCH	physiological cardiac hypertrophy
PGC1	PPARgamma-specific coactivators 1
PPARs	peroxisome proliferator-activated receptors
PPREs	PPAR response elements
PPT	propyl-pyrazole-triol
Raf	rapidly accelerated fibrosarcoma
Ras	rat sarcoma
RIP140	NRIP1, Nuclear receptor interacting protein 1
RXRs	retinoid acid receptors
SERMs	selective estrogen receptor modulators
SHP	small heterodimer partner
SMRT	silencing mediator for RXR and thyroid hormone receptor
SRC1	steroid receptor coactivator 1
T2DM	type 2 diabetes mellitus
T3/T4	thyroid hormones
TAC	transverse aortic constriction
TGs	Triglycerides
TIF2	transcriptional intermediary factor 2
TNFalpha	tumor necrosis factor alpha
TZDs	glitazones /thiazolidinediones
UBC9	ubiquitin-line protein SUMO-1 conjugating enzyme 9
VEGFs	vascular endothelial growth factors
VSMCs	vascular smooth muscle cells
WAT	white adipose tissue
WHO	Word Health Organisation
Wt	wild type

2   Introduction

Obesity and obesity-related metabolic diseases

The incidence of obesity and obesity-related metabolic diseases such as type 2 diabetes mellitus (T2DM) and hypertension is rising persistently (1), even though several efforts have been undertaken to implement lifestyle interventions such as healthy diet and physical activity, likely resulting in body weight reduction (2). Obesity could be defined as a global dilemma: recent data published by Word Health Organisation (WHO) indicates that overweight and obesity are the fifth principal risk for worldwide morbidity and mortality (https://apps.who.int/infobase). Moreover, WHO indicates that obesity attributes to 44% of all diabetes cases, 23% of ischemic heart disease cases and between 7% and 41% of certain cancer cases (such as endometrial, breast, and colon cancer), expressed as % per year.

The main cause of obesity is linked with a persistent imbalance between high calories intake (energy intake) and low calories utilisation (energy expenditure). In a state of body weight stability, energy storage and energy utilisation are balanced: upon increased energy absorption, adipose tissue stores the energy in form of triglycerides (TGs) used also for phospholipid membrane formation and signalling pathways, and upon increased energy demand, for instance during physical training, adipose tissue releases energy in form of free fatty acids (FFAs). Upon increased energy demand, TGs undergo hydrolysis to glycerol and nonesterified fatty acids (NEFA) in a process called lipolysis and subsequent degradation into acetyl units in process of beta oxidation. Lipolysis in white adipose tissue (WAT) is maintained by 3 independent lipases: adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase (MGL). Moreover, lipolytic activity in adipose tissue is tightly regulated by several hormonal and neuronal factors, such as epinephrine, norepinephrine, insulin, ghrelin, sex and growth hormones, and cortisol. Adipose tissue consists of adipocytes, but also preadipocytes, endothelial cells, fibroblasts or immune cells, although only adipocytes are highly specialised cells for FFAs uptake and storage of TGs (3; 4). In obesity, the persistent increase of energy uptake leads to increase in fat mass due to adipocyte hypertrophy and preadipocyte differentiation, and - in consequence - to adipose tissue pathogenesis (5).

The most important parameter contributing to obesity-related morbidity and mortality is not the fat mass per se, but rather its distribution (6; 7). Localisation of the fat tissue in humans depends on the total fat mass. Normally TGs are predominantly stored in subcutaneous adipose tissue, visceral adipose tissue and intra-thoracic fat depots. In situations of sustain positive energy balance FA uptake rises, adipose tissue depots increase, and ectopic fat deposition in liver, pancreas, skeletal muscle and the heart takes place leading to perturbations of theirs metabolic functions (8). Deterioration of the metabolic function of these organs known as lipotoxicity, is proposed as a key mechanism participating in the

development of obesity-induced cardiovascular and metabolic disorders, such as T2DM, hepatic steatosis, hypertension, cardiac hypertrophy or cardiomyopathy and, based on endothelial cell dysfunction, atherosclerosis (9).

The chronic low-grade inflammation of WAT has been implemented as a main etiological factor of obesity-induced adipose tissue pathogenesis (10-13). Moreover, inflammation of adipose tissue is tightly linked with the development of peripheral insulin resistance (IR). The pathophysiological outcome of IR is related to a decline of insulin-mediated glucose uptake in skeletal muscle and adipose tissue, inhibition of insulin mediated glucose storage, enhanced glycogenolysis, and augmented gluconeogenesis in liver, and dysregulation of the adipose tissue lipolysis (14).

The progression of IR is linked with specific adipose tissue remodelling processes, such as hypertrophy of adipocytes and subsequent infiltration of proinflammatory cells, including T-lymphocytes and macrophages into WAT (15-17), as showed in Fig. 1. Although the molecular mechanism regulating the initial low-grade inflammation of adipose tissue reminds elusive, recent data indicates, that enhanced hypertrophic response of adipocytes as result of obesity-induced rapid fat accumulation leads to hypoxia, and local activation of the hypoxia-regulated transcription factor HIF-1alpha (18). In line, Pasarica M and colleagues confirmed reduced adipose tissue oxygenation and decreased angiogenic response, measured in adipose tissue of obese patients, both processes potentially participating in the development of the initial fat tissue inflammation (19).

Several pro-inflammatory cytokines such as interleukin 6 (IL-6), tumor necrosis factor alpha (TNFalpha) and monocytes chemoattractant protein-1 (MCP-1), IL-18 or C-X-C motif chemokine 5 (CXCL5) released from adipose tissue, were demonstrated to mediate adipose tissue inflammation, and to modulate - in a paracrine and systemic manner - the immune response of obese subjects (20). Moreover, obesity-related changes in adipose tissue are linked with subsequent polarization of resident adipose tissue macrophages from an anti-inflammatory, alternatively activated type (M2) to a pro-inflammatory type (M1), resulting in the release of pro-inflammatory cytokines, which in turn promotes recruitment of other pro-inflammatory cells (both M1 macrophages and CD4-positives T lymphocytes) into adipose tissue (21; 22). It is well accepted, that the production and release of anti-inflammatory cytokines, such as IL-4 and IL-10 by M2 macrophages is strongly reduced during the development of obesity (21; 22).

Obesity is associated not only with a proinflammatory response of adipose tissue, but also an alternation in expression and release of several adipocytes-derived proteins (adipocytokines), such as adiponectin, leptin and resistin in addition to IL-6, or TNFalpha. Importantly, obesity-related increase of the plasma levels of leptin and resistin strongly correlates with adipose tissue inflammation, IR and glucose intolerance, observed in both rodent and human studies (17). In contrast, adiponectin plasma levels are extremely decreased in obese subjects, which strongly correlate with decreased insulin sensitivity (IS),

measured in adipose tissue of those patients (23; 24). Adiponectin, when applied to obese rodents, was shown to improve their metabolic outcome. In consonance, adiponectin plasma level seems to show a negative correlation with pro-inflammatory plasma markers in T2DM patients (25). Putative anti-inflammatory properties of adiponectin are linked with its ability to interfere with proinflammatory pathways in-vitro, such as NF-kappaB signalling (discussed by Ouchi, (25)). Importantly, pro-inflammatory cytokines were shown to inhibit the production of adiponectin in-vitro (26). In addition, increased energy expenditure, for instance due to increased physical activity, or reduced caloric intake were shown to diminish inflammatory response of adipose tissue, and to re-establish physiological adipocytokine production (27).
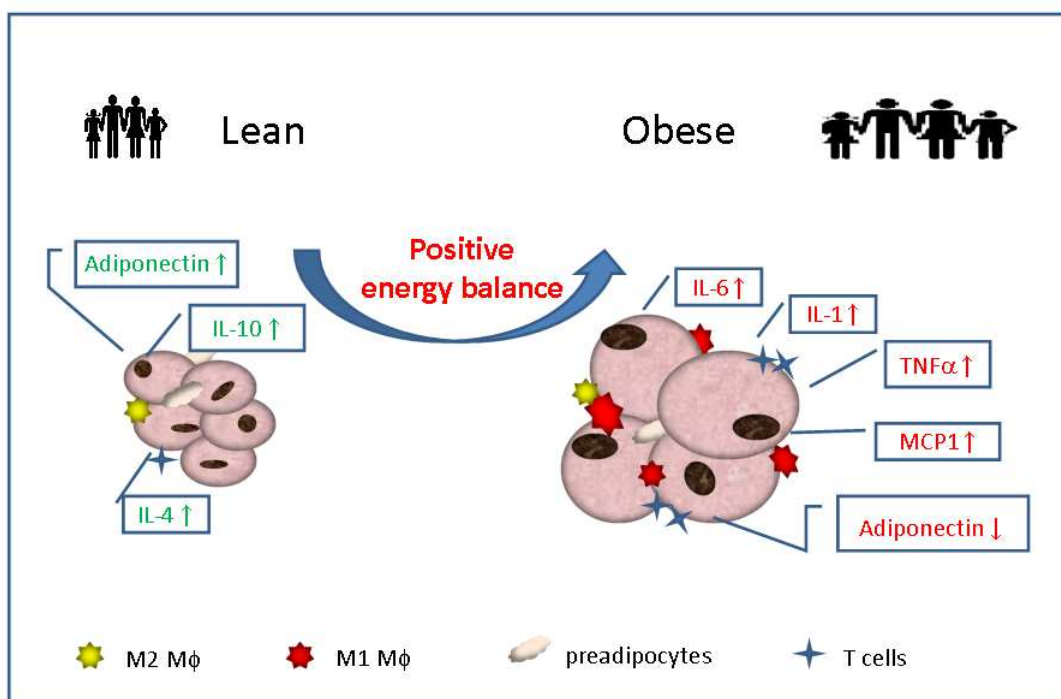

Fig. 1 Obesity-dependent adipose tissue remodelling

Although the direct link between obesity and the development of obesity-related cardiovascular diseases such as atherosclerosis, ischaemic heart disease and heart failure remains elusive (28), several potential mechanisms have been suggested. The first mechanism is based on a direct interaction between adipose tissue and the cardiovascular system, due to the obesity-related aberrant production of adipocytokines, such as adiponectin. Adiponectin was shown to regulate cardiac metabolism, and to diminish obesity-related endothelial dysfunction and the development of atherosclerosis by reducing the expression of endothelial cell adhesion molecules and repressing endothelial cell apoptosis (25). Although the precise molecular mechanism by which adiponectin improves endothelial dysfunction is still not completely understood, data obtained from experiments

performed in obese rats indicate, that adiponectin mediates its beneficial effects by regulating the 5' AMP-activated protein kinase (AMPK) - endothelial nitric oxide synthase (eNOS) pathway (29). Along this line myocardial ischemia-reperfusion injury in rats has been shown to be significantly reduced by adiponectin through eNOS- and cyclooxygenase (COX)-dependent mechanisms (30). In addition, adiponectin shows potent beneficial effects on glucose and fat metabolism, and was demonstrated to regulate systemic blood pressure (25).

Additional mechanisms explaining the putative origin of obesity-related cardiovascular diseases is related to obesity-induced augmented incidence of T2DM, hypertension, and dyslipidemia, that in turn increases risk for cardiovascular diseases (31). Also persistent increase of FAs plasma levels and ectopic fat deposition (lipotoxicity) were recently discussed as further mechanisms inducing obesity-related cardiovascular diseases such as atherosclerosis, ischaemic heart disease and heart failure (8; 9).

In summary, obesity-related changes of adipose tissue metabolism, linked with a low-grade inflammation, dysregulation of adipocytokines production and systemic lipotoxicity participate in the development of obesity-related cardiovascular diseases.

Molecular regulation of adipose tissue metabolism: the role of PPARgamma

The nuclear hormone receptor (NHR) superfamily, consists of several transcription factors and transcriptional regulators, such as peroxisome proliferator-activated receptors (PPARs), thyroid receptors, estrogen receptors (ERs), retinoid acid receptors (RXRs) and others involved in the regulation of metabolism, embryogenesis and whole-body homeostasis (32; 33).

As previously described (34), PPARs regulate transcription of a set of genes determining total carbohydrate and lipid metabolism, beta-cell activity in pancreas and endothelial functions in vasculature (35). All three subtypes of PPARs (PPARalpha, gamma and delta) identified to date were shown to regulate energy and fatty acid oxidation and metabolism (36). PPARgamma is considered as key regulator of glucose and lipid metabolism in adipose tissue and, together with PPARalpha, in the liver (37-40). There are two different isoforms of PPARgamma: ubiquitously expressed PPARgamma1 and adipose tissue specific PPARgamma2. Although the endogenous ligands, specific for PPARgamma are still unknown, some native and modified polyunsaturated FA and prostanoids were proposed as PPARgamma physiological activators (41). Synthetic high-affinity PPARgamma agonists such as glitazones/ thiazolidinediones (TZDs) are used for the treatment of T2DM as potent insulin sensitizers.

PPARgamma shares a common - typical for NHRs - structure, with a N-terminal transactivation domain (AF1), a conserved DNA binding domain, consisting of two Zn-fingers, a ligand binding domain (LBD), and a C-terminal second transactivation domain (AF2), mediating ligand dependent transactivation (42). PPARgamma activity is determined by the

binding of selective PPARgamma agonists within the LBD, analogues to most of the NHRs. In the basal state PPAR forms a heterodimeric receptor complex together with another NHR-member -RXRalpha. In the unstimulated state this complex is localised in the cytoplasm, or - irrespective of the ligand binding status – is bound to the target promoters in a repressed state, within a multiprotein corepressor complex (42). This corepressor complex consists of SMRT (silencing mediator for retinoic acid receptor and thyroid hormone receptor), NCoR (nuclear receptor corepressor) (43), RIP140 (NRIP1, Nuclear receptor interacting protein 1) (44), histone deacetylases (HDACs) family and many other corepressors (45). Most of the NHRs share a set of corepressors, and were shown to interact with other family members and other transcription factors by competitive binding or releasing of specific corepressors or coactivators. Recently published data indicates that the anti-proliferative and anti-inflammatory properties of PPARgamma rely upon its cross-talk with proinflammatory NFkappaB signalling pathway. PPARgamma has been demonstrated to regulate corepressor binding to NFkappaB- target promoters, and by this way inhibits the transcription of proinflammatory cytokines in macrophages and vascular smooth muscle cells (45-47).

Upon ligand-specific activation, the PPAR/RXR complex translocates to the nucleus to bind within target promoters, defined through so-called PPAR response elements (PPREs)(48). Binding of an agonist within the PPAR-LBD causes a LBD-conformational alteration leading to an exchange of corepressors for coactivator proteins. This induces the progression of the PPAR/RXR heterodimer binding to the PPREs, followed by the initiation of transcription. Similar to corepressors, NHRs are believed to share also the pull of coactivators, such as the p160/SRC (steroid receptor co-activator) family of coactivators including steroid receptor coactivator 1 (SRC1), transcriptional intermediary factor 2 (TIF2), and CBP (cAMP-response-element-binding protein CREB-binding protein/p300, DRIP205 (vitamin D Receptor Interacting Protein) and others (49; 50). Putative tissue- and organ specific expression of corepressors and coactivators has been reported enhancing the complexity of NHRs cross-regulation. The corepressor- and coactivator- network, specific for adipose PPARgamma-activation plays a crucial role in the modulation of metabolism and metabolic adaptations of that receptor. By this way, PPAR activity is regulated by co-regulatory proteins, but also by interactions with other NHRs or transcription factors, discussed above, and eventually also by differences within PPRE-sequences determining the frequency of the promoter binding.

In summary, PPARgamma is recognized as a main regulator of glucose and lipid metabolism and synthetic agonists (TZDs) such as pioglitazone and rosiglitazone belong to the oral anti-diabetic drugs family used for the treatment of T2DM as insulin sensitizers since 1999. TZDs were shown to induce preadipocyte differentiation and adipose tissue remodelling, when applied to diabetic subjects. Metabolic action of TZDs was also linked with an increased expression of glucose transporter 4 (Glut4) in skeletal muscle and adipose tissue, leading to an improvement of glucose tolerance (GT) and IS. Moreover, systemic PPARgamma activation was shown to restrain the physiological plasma levels of adipose tissue derived

adipocytokines such as adiponectin, leptin and resistin (51). As discussed above, TZDs display anti-inflammatory properties mainly due to the PPARgamma-mediated inhibitory actions on NFkappaB and activator protein 1 (AP1) signalling. Importantly, PPARgamma ligands, used as oral antidiabetic drugs were shown to improve T2DM-related dyslipidemia (52). Nevertheless, recently published data suggested that rosiglitazone increases cardiovascular risk and cause severe cardiovascular adverse events (myocardial infarction and coronary heart diseases) in diabetic patients, which resulted in its recent withdrawal from the European market in September 2010 (53-56) reviewed by Schernthaner and Chilton (57). Pioglitazone was demonstrated to increase the risk of bladder cancer, and its withdrawal from the European market is already being expected.

In summary, PPARgamma is regarded as a key regulator of the lipid metabolism, influencing systemic insulin sensitivity and glucose utilisation. Synthetic ligands of PPARgamma, such as pioglitazone or rosiglitazone, were commonly used as oral antidiabetic drugs sufficiently improving T2DM-related metabolic outcome. Since the incidence of T2DM is rising persistently, there is a growing need to develop a new set of selective PPARgamma agonists with improved pharmacological properties. Such a development should be based on a careful molecular characterisation of the PPARgamma action in vitro and in vivo.

Estrogen -mediated differences in obesity and metabolic disorders

Estrogens are consider as main regulators of embryogenesis, development and reproduction (58), but were also reported to regulate glucose homeostasis and lipid metabolism, as discussed previously (59). Furthermore, lipogenesis, lipolysis and adipogenesis of adipose tissue seems to be controlled by estrogens (60-62). Estrogens mediate their metabolic action through both estrogen receptors alpha (ERalpha) and beta (ERbeta) both also belonging to the family of NHR. Although the expression pattern of both ERs differ among species, sexes and specific organs, both ERs are expressed in almost all metabolic tissues such as adipose tissue, skeletal muscle, liver and pancreas, as well as in the central nervous system (63; 64). The expression pattern of both ERs seems to be regulated in a sex-specific manner in a number of tissues and organs (65-67), and expression in females differs between pre-/ postmenopausal states (68; 69). Furthermore, a number of distinct ERalpha and ERbeta splicing isoforms were recently identified in rodents, as well as in humans (70).

In addition to 17beta-estradiol (E2), considered as the main endogenous ER-agonists (58), ERs are activated by a variety of synthetic selective estrogen receptor modulators (SERMs), such as the ERalpha-SERMs raloxifene and tamoxifen, ERalpha agonist propyl-pyrazole-triol (PPT) or ERbeta-specific ligand diarylpropionitrile (DPN) (71). ERs display a typical molecular structure like other NHRs (72; 73). Importantly, the first AF1 domain, responsible for ligand independent transactivation, displays only marginal homology between both ERs (approximately 24%). In contrast, DBD- and LBD- domains are quite similar (97% and 56%,

respectively), which may provide an explanation for the similar binding pattern of both ERs within target promoters upon activation (73).

ERs, similar to PPARs, are activated by binding of specific ligands/ agonists within the LBD and subsequent homo- or hetero-dimerization. Unliganded ERs are bound to corepressors, such as SMRT, NCoR or RIP140, and activation and dimerization of ERs leads to the dissociation of corepressors and binding of ER-dimers on specific target promoters, within estrogen response element (ERE) -sequences. Transcriptional activation of ERs depends on the recruitment of coactivators such as p160/SRC (SRC1, TIF2, and SRC3), DRIP205, and many others. Importantly, binding of coactivators or corepressors to the ERs depends on agonists, coactivator/ corepressor availability in the specific tissue and chromatin remodelling processes (74-77). SERMs, such as tamoxifen, display their tissue specificity by recruiting coactivator complexes to ER-dependent promoters in the endometrium, and parallel binding of corepressors to the same promoters in breast cancer cells (74). Moreover, ERs were shown to interfere with the transcriptional activity of AP1 or NF-kappaB, and other NHRs such as PPARs (78; 79).

Taken together, sex-specific differences in obesity and metabolic disorders are known to be regulated in estrogen-dependent manner.

Metabolic action of estrogens on glucose and lipid metabolism

Several recently published studies underline a crucial role of estrogens and ERs in the regulation of glucose- and lipid metabolism (80). Experiments on ovariectomized (OVX) animals indicated, that depletion of estrogens results in an increase of energy uptake, body weight and fat mass (81). Research work performed on OVX-mice and OVX-rats indicated progressive dyslipidemia, impaired GT and impaired insulin-mediated glucose uptake in skeletal muscle of those animals (82; 83). In addition, estrogen receptor (ER)-deficient or aromatase-deficient mice (ArKO) exhibit distinct and gender-specific metabolic phenotypes (61; 78; 84-88). In consonance, estrogen replacement therapy (ERT) was shown to improve systemic IR in those animals, and augmented expression of Glut-4, measured in skeletal muscle. Long term application of estrogens to leptin-deficient, diabetic ob/ob mice improves their metabolic outcome in terms of GT, IS and hepatic lipid accumulation (89). Comparable results were obtained in the studies on ob/ob mice treated with PPT (90).

Also clinical data implements the metabolic role of estrogens: in the postmenopausal state (under estrogens deficiency) women tend to develop visceral obesity, IR and T2DM (91). Postmenopausal women on HRT display reduced incidence of T2DM, lower glucose plasma levels, and improved systemic IS, when compared to placebo-treated control group (92-94).

The beneficial effects of estrogens on glucose- and lipid metabolism are presumably mediated by both ERalpha and ERbeta receptors. The metabolic function of ERalpha was demonstrated in studies, involving ERalpha-deficient mice. ERalpha-KO mice exhibit IR and impaired GT, adipose tissue hyperplasia and hypertrophy (61; 84-86). The metabolic

phenotype of ERalpha-KO is also linked with a very low expression of Glut4 measured in skeletal muscle of those animals (95). Moreover, experiments performed on PPT-treated mice indicated a beneficial function of ERalpha, due to improved metabolic outcome and increased systemic IS, observed in those animals, when compared to vehicle-treated controls (90).

The metabolic role of ERbeta is not entirely understood. Data published by Naaz and colleagues on ERalpha-deficient and OVX mice indicated an enhanced systemic IS and GT, and attenuated adipocytes-specific hypertrophy, when compared with sham operated animals (85). Conversely, ERbeta-deficient mice displayed a similar BW, fat mass, plasma lipid profiling and plasma insulin levels, when compared to control animals (96). When fed with HFD female ERbeta-deficient mice showed averted accumulation of triglycerides and preserved insulin signalling in liver and skeletal muscle, improved whole-body IS and GT (78), which indicated a putative pro-diabetogenic function of ERbeta. In addition, Barros and colleagues (95) demonstrated, that ERbeta plays a suppressive role on Glut-4 expression in skeletal muscle in mice, which also suggests a pro-diabetogenic function of this NHR.

Estrogens regulate adipose tissue metabolism in a sex-specific manner (97; 98). Premenopausal women have a tendency to accumulate more subcutaneous fat whereas men accumulate more visceral fat (93; 94; 99). The incidence of IR and glucose intolerance is higher in men than in women (100; 101). Furthermore, augmented abdominal obesity and IR, reported in postmenopausal women, can be improved by HRT (93). The study from Macotela and colleagues (102) indicated an enhanced lipolytic capacity of adipocytes isolated from female mice, such as insulin-dependent glucose uptake, activation of insulin signalling pathways and expression level of Glut-4, Glut-1 and fatty acid synthase (FAS), when compared to cells isolated form males. In line with these data, OVX-female mice displayed increased food intake and body weight in experiments with pair-feeding protocols (103; 104), demonstrating that E2 inhibits lipogenic gene expression, promotes lipolysis in adipocytes and induces lipid-oxidation in skeletal muscle. Estrogens were shown to induce adipose tissue specific lipolysis due to activation of HSL (105) and to diminish lipogenesis and lipoprotein lipase (LPL) activity (106-108). Moreover, exercise-induced adipose tissue lipolysis is strongly elevated in female mice, when compared to male animals. This can be partially explained by an augmented expression of HSL and/or ATGL in female fat tissue, whereby both lipases seems to be regulated in an estrogen-specific manner (3; 109-111). In addition, the regulation of BW and adipose tissue lipolysis under caloric restriction was shown to be regulated in a sex-specific manner (112). This is in line with human studies, indicating that women exhibit higher adipose tissue-specific lipolytic activity than men under physical training (113).

Estrogens regulate glucose metabolism and glucose uptake not only in adipose tissue, but also in skeletal muscle, liver and in pancreas. As discussed above, both ERs were reported to have divergent effects on the expression of Glut-4 transporters in skeletal muscle (95; 114).

Moreover, a putative insulin-sensitizing effect of ERalpha activation was demonstrated in vivo and in vitro by Breen and colleagues (115; 116). Since glucose homeostasis depends also on hepatic glycogenolysis and gluconeogenesis, the effects of estrogens and ERs on liver metabolism were studied extensively in the few last years. Estrogens were reported to regulate glucose homeostasis and influence cholesterol output in the liver due to activity of ERalpha (86). This is in line with the results published by Zhao and colleagues, showing pronounced IR and diminished endogenous glucose production of ERalpha-deficient mice. In addition, microarray analysis of the hepatic tissue sections isolated from ERalpha-KO and wt mice revealed an ERalpha-dependent upregulation of genes regulating hepatic lipid biosynthesis, and downregulation of genes mediating lipid transport (86).

Estrogens were reported to mediate pancreatic beta cell function. Alonso-Magdalena and colleagues (117) demonstrated, that estradiol augments insulin expression and release of pancreatic beta-cell in an ERalpha dependent manner (117). ERalpha seems to mediate pancreatic beta-cell survival after oxidative stress (118). Moreover, ERbeta-deficient mice were recently reported to display a mild islet hyperplasia and delayed first phase insulin resistance (114).

Taken together, these studies demonstrate that estrogens mediate protective metabolic actions mostly via ERalpha, regulating glucose uptake in adipose tissue and skeletal muscle, increasing lipolysis, and interfering with lipogenesis, glycogenolysis and gluconeogenesis in white adipose tissue, skeletal muscle, liver, and pancreatic beta cells. In addition, ERalpha was demonstrated to regulate food intake and energy expenditure in CNS. In contrast, ERbeta seems to negatively regulate insulin signalling and glucose metabolism interfering with the regular adipose tissue function and downregulating Glut-4 expression in skeletal muscle.

Sex-differences and cardiac hypertrophy

Myocardial hypertrophy is considered as a specific cardiac adaptation to elevated demands for oxygen (O2) (reviewed by Weiner and Baggish (119)). Accordingly, cardiac hypertrophy is characterized by an increase of left ventricular mass (LVM), and an enlargement of ventricular chamber size (120; 121). Depending on the stimulus/ charactereristic of cardiac hypertrophy one could distinguish two forms: physiological hypertrophy, induced by intensive physical training, or pregnancy, and pathological hypertrophy, a result of chronic arterial hypertension, cardiac aortic valve stenosis, myocardial infarction, cardiomyopathy, myocarditis, but also obesity, diabetes, thyroid disorders and many others (122).

The physiological form of cardiac hypertrophy is predominantly linked with a moderate increase of LVM due to enlarged volume of myocytes and the formation of new sarcomers (reviewed by Bernardo BC, (121)). Furthermore, physiological cardiac hypertrophy seems to be reversible, as increased cardiac volume and aerobic fitness, observed in athletes returns back to baseline after the training had been terminated, or in case of pregnancy- after

parturition (123). Numerous cross-sectional studies indicated the cardioprotective role of exercising, as well as augmented life expectancy among ex-athletes (119)). Those studies pointed towards a beneficial role of physical training on cardiovascular fitness and the reduction of major cardiovascular risk factors among physically active individuals (124).

The limited data available from the human studies indicate no obvious sex-specific differences with regards to the cardiac mass in puberty. Afterwards, females seem to preserve their heart mass and myocyte number throughout the whole adult life time (125). Male cardiac tissue is considered to possess a higher hypertrophic potential, when compared to women (reviewed by Bernardo (121)). In contrary, data from Petersen and colleagues (126), obtained from the group of young adult elite athletes and age- and sex-matched sedentary controls, indicated comparable hypertrophic response to training in both sexes, when compared to not-trained controls. Importantly, as discussed previously (127), the cardiac hypertrophic effects observed by the authors were not normalized for the training intensity and/ or quantity, which could potentially mask gender dependent effects. In addition, studies performed by Soto and colleagues (128) indicated higher myocardial glucose and FA uptake and utilization after a prolonged endurance training period in women, when compared to age-matched male participants. An augmented cardiac FA uptake and utilization (129), observed in women in response to exercise points towards sex-specific differences in the development of training-induced physiological cardiac hypertrophy in humans. Interestingly, exercise-induced lipolytic activity of adipose tissue and elevated plasma FFAs measured directly after a training period were strongly induced in women, compared to men (130). In line with those results, women were also reported to respond with a higher lipolytic activity to catecholamine infusion, when compared to men (131), and to show improved ultra-endurance capacity and higher rate of FA-oxidation.

Potential cardioprotective properties of estrogens in the development of cardiac hypertrophy were elucidated in several studies using transgenic mice models and different training systems (forced treadmill training protocols, voluntary cage-wheel running systems, or swimming). Moreover, most of the research work published to date (111; 125; 132-134) implemented sex-specific differences in exercise-induced physiological cardiac hypertrophy, with a more prominent hypertrophic response shown in females than in males.

Although the precise molecular mechanism responsible for the development of physiological cardiac hypertrophy remains elusive, several factors were shown to participate in that process, including thyroid hormones (T3/T4), growth factors such as insulin, vascular endothelial growth factor (VEGFs) and insulin-like growth factor 1 (IGF1) and many others. Downstream targets activated by the development of physiological cardiac hypertrophy are Ca+2-calmodulin dependent kinase (CaMK) (135), AKT kinase (protein Kinase B (PKB)) (132) and the phosphorylated form of glycogen synthase kinase-3-beta (GSK-3-beta) (132; 136). Experiments performed on AKT-deficient mice, confirmed blunted hypertrophic responses in these mice to swimming, but not to pressure-overload-induced pathological cardiac

hypertrophy (137). In addition, other common signalling pathways such as ERK1/2 or p38 mitogen-activated protein kinases (p38) pathways were not regulated upon training (132). Importantly, androgens were shown to stimulate myocardial fibrosis in exercise-induce physiological cardiac hypertrophy and the supplementation of 19-nortestosteron in male rats led to activation of the renin-angiotensin-aldosterone system and increased myocardial collagen synthesis (138). The putative lipolytic effect of testosterone remains controversial (139)(140).

The pathological form of myocardial hypertrophy is linked with a prominent increase of LVM, and subsequent fibrosis and apoptosis of myocardial tissue leading to ventricle remodelling and dilatation proceeding to systolic/ diastolic dysfunction and heart failure. The pathological form of myocardial hypertrophy is strongly related to a metabolic perturbation of cardiac lipid metabolism, leading to a switch from lipid- to glucose-dependent energy production. In addition, pathological cardiac hypertrophy is characterized by the induction and reactivation of fetal gene programming including increased expression of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and beta-myosin heavy chain (beta-MHCH), and activation of myocardial apoptosis and autophagy. Moreover, endothelin 1 (ET-1) and angiotensin II are typical neuro-endocrine hormones initiating this cardiac pathology. In contrast to physiological cardiac hypertrophy, the pathological one is considered as irreversible and maladaptive. The main differences between the physiological and pathological form of cardiac hypertrophy are shown in Fig. 2.

There are well documented sex-differences in the development of pathological hypertrophy and heart failure with better survival rates observed in women when compared to men (141). Furthermore, the recent data obtained from cardiovascular studies performed on pre- and postmenopausal women indicated that premenopausal women seem to be more protected against pathological forms of hypertrophy, heart failure, as well as myocardial infarction and cardiomyopathies when compared to men, and this beneficial effect seems to be strongly abrogated in the postmenopausal state (reviewed by Konhilas, (142)). Nevertheless, HRT including combined estrogen and progesterone therapy has been linked with an increased cardiovascular mortality, such as cardiac infarction or stroke in older postmenopausal women (reviewed by Rossouw (143)). Importantly, increased cardiovascular risk was significantly lower in patients with a short-time perimenopausal application of HRTs.

Moreover, myocardial remodelling observed in women suffering from heart failure seems to differ from that, observed in man with regards to their diastolic function, ventricle size and wall thickness, as well as the rate of fibrosis (144). In line with the human studies, results derived from pressure-overload experiments performed in rodents using a transverse aortic constriction (TAC)-model indicated a protective effect of estrogens on the development of pathological cardiac hypertrophy (145). Moreover, the protective role of estrogens in the development of pathological hypertrophy in mice is well established (146;

147), even though the molecular mechanism involved in those processes is not completely understood. Female sex was linked with an attenuated cardiac remodelling and apoptosis in a TAC model in mice. The attenuated fibroblastic response of female mice was shown to depend on estrogen-mediated activation of the ERalpha/MAP-kinase pathway, leading to the inhibition of matrix metalloproteinase 2 (MMP2) (148). Importantly, male mice subjected to TAC-induced pressure overload were reported to show higher MMP-2 mRNA expression than female mice (149). Those results are confirmed by the studies performed by Westphal C. and collaboratives, demonstrating ERalpha specific anti-fibrotic effects of estrogens and ERalpha-specific SERMs in a TAC-model of myocardial hypertrophy in mice (150). On the other hand, results published by Fliegner and colleagues demonstrated, that although female sex seems to be linked to an attenuated cardiac remodelling and apoptosis in a TAC model in mice, ERbeta seems to be a key mediator of that protective effects (145).
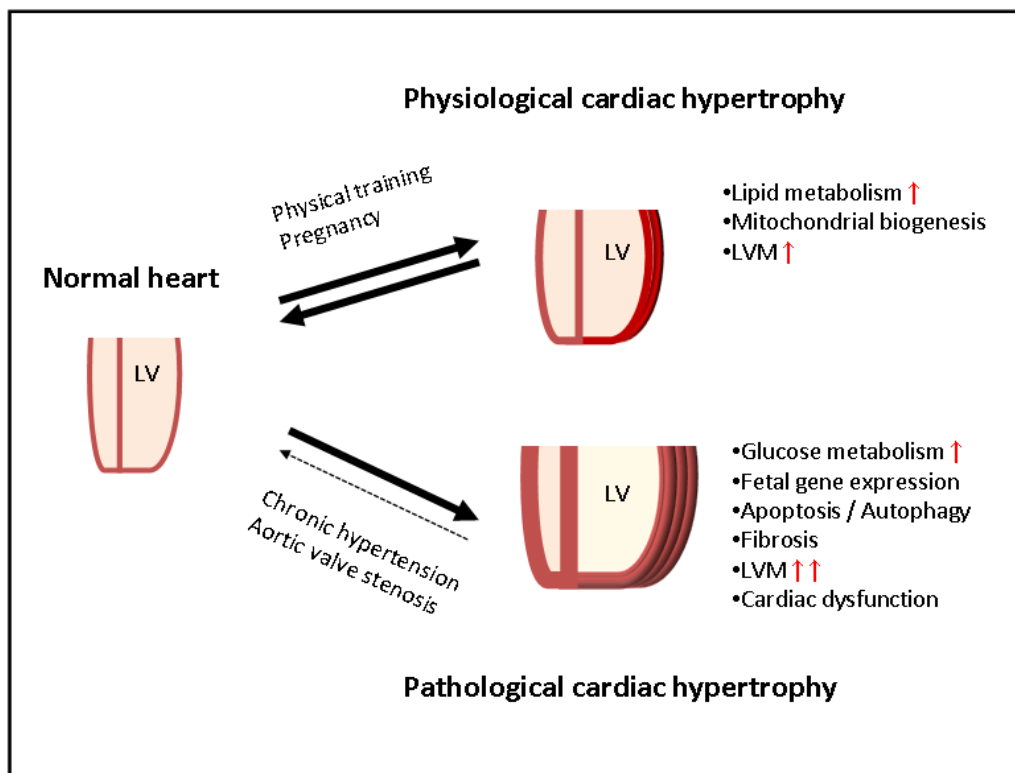

Fig. 2 Physiological and pathological cardiac hypertrophy

3 Objectives

The prevalence of obesity-related metabolic and cardiovascular diseases increase steadily (1), and the better understanding of molecular mechanisms underlying those processes seems to be a first step towards the development of efficient prevention/ treatment strategies.

The present research work was focused on:

- The role of PPARgamma activation in the regulation of obesity-induced inflammation of adipose tissue, systemic insulin sensitivity and glucose homeostasis
- The molecular mechanism of PPARgamma activation in the process of vascular protection/ attenuation of atherosclerosis, mediated by aortic vascular smooth muscle cells
- Metabolic consequences of adipose-tissue specific PPARgamma - ERbeta interaction for obesity-related changes of systemic lipid and glucose metabolism
- Estrogen-dependent regulation of obesity-related pathological cardiac hypertrophy
- Estrogen-dependent regulation of physiological cardiac hypertrophy

This study aims for a better understanding of the molecular mechanisms underlying the development of obesity related cardiovascular and metabolic diseases. In particular, this study implements the role of PPARgamma as a potent anti-inflammatory factor, regulating the metabolic homeostasis of adipose tissue and vasculature. The molecular crosstalk of PPARgamma with other nuclear receptors, such as ERs seems to be an important factor modulating sex-specific differences observed in obesity-mediated metabolic and cardiac diseases.

4   Results

PPARgamma activation attenuates T-lymphocyte-dependent inflammation of adipose tissue and development of insulin resistance in obese mice (15).

Adipose tissue inflammation, mediated by adipocytes and subsequent activated proinflammatory M1 macrophages and T-cells was recently linked with obesity-induced pathological glucose and lipid metabolism. TZDs, such as rosiglitazone are known as effective therapeutic agents for the treatment of T2DM, mostly due to their anti-inflammatory properties, mediated by PPARgamma. Telmisartan, an AT1R- blocker, used for the treatment of hypertension, was previously identified as a partial PPARgamma agonist with putative anti-inflammatory characteristics (151; 152).

The aim of this part of the work was to elucidate if pharmacological activation of PPARgamma in the model of diet-induced obesity would improve adipose tissue inflammation mediated by T-lymphocytes. Our results indicated that activation of PPARgamma by both rosiglitazone and telmisartan resulted in the significant reduction of T-lymphocytes infiltration into adipose tissue, and subsequent recruitment of resident and activated M1- macrophages. Moreover, the anti-inflammatory action of both therapeutic agents was associated with improved metabolic phenotypes (improved IS and GT) of the mice fed with high fat diet, when compared to vehicle-treated control animals.

Foryst-Ludwig A, Hartge M, Clemenz M, Sprang C, Hess K, Marx N, Unger T, Kintscher U: PPARgamma activation attenuates T-lymphocyte-dependent inflammation of adipose tissue and development of insulin resistance in obese mice. *Cardiovasc Diabetol* **9:64, 2010**

URL:
http://dx.doi.org/10.1186/1475-2840-9-64

High-mobility Group A1 Protein: a new coregulator of PPARgamma –mediated transrepression in the vasculature (46).

The beneficial effects of PPARgamma activation in the treatment of obesity-related metabolic and cardiovascular diseases depends on its overall anti-inflammatory properties. The valuable anti-atherosclerotic and anti-proliferative action of PPARgamma in vascular smooth muscle cells (VSMCs) was recently linked with a significant inhibition of MMP9 mediated by that NHR upon activation with TZDs.

We were able to demonstrate, that PPARgamma-mediated transrepression of MMP9 promoter in VSMCs strongly depends on ligand-dependent PPARgamma interaction with HMGA1 (high-mobility group A1), and SUMO-E2 ligase UBC9. Importantly, TZD-dependent formation of the PPARgamma-HMGA1-UBC9 complex facilitates PPARgamma sumoylation, and subsequent binding of SMRT corepressors, which mediate downregulation of MMP9 expression. The importance of HMGA1 for the anti-inflammatory characteristic of PPARgamma in-vivo was demonstrated in the model of arterial wire injury in HMGA1-deficient and control mice. HMGA1-deficient mice were characterized by a complete lack of TZDs-mediated vascular protection through PPARgamma activation when compared to wt littermate animals.

Metabolic actions of estrogen receptor beta (ERbeta) are mediated by a negative crosstalk with PPARgamma (78).

The molecular crosstalk of PPARgamma with other nuclear receptors, such as ERs seems to be an important factor modulating sex-specific differences observed in obesity-mediated metabolic and cardiac diseases.

Our in-vitro experiments indicated that both ERbeta and PPARgamma interact with each other in adipocytes. This interaction led to the significant inhibition of PPARgamma activity and could be reversed by an overexpression of coactivators (SRC1, TIF2) required for activation of both NHRs. Those experiments were supported by in-vivo studies performed on ERbeta-deficient mice fed with HFD. ERbeta-deficient mice exhibited a remarkable metabolic phenotype including an overall increased lipid accumulation, elevated BW and fat mass, with a simultaneous improvement of IS and increased PPARgamma activity in WAT, when compare to wt controls. Consistent with these data, application of PPARgamma anti-sense oligonucleotides reversed the metabolic phenotype of ERbeta-deficient mice, and led to the impairment of glucose tolerance and insulin sensitivity. Altogether our results demonstrated pro-diabetogenic effects of ERbeta resulting from a negative cross-talk with adipose PPARgamma.

Foryst-Ludwig A, Clemenz M, Hohmann S, Hartge M, Sprang C, Frost N, Krikov M, Bhanot S, Barros R, Morani A, Gustafsson JA, Unger T, Kintscher U: Metabolic actions of estrogen receptor beta (ERbeta) are mediated by a negative cross-talk with PPARgamma. *PLoS Genet* **4:e1000108, 2008**

URL:

http://dx.doi.org/10.1371/journal.pgen.1000108

Sexual dimorphism in obesity-mediated left ventricular hypertrophy (153).

In addition to their metabolic functions, estrogen and ERs regulate the development of the mal-adaptive pathological form of hypertrophy induced by HFD feeding in mice.

To investigate putative sex-specific differences in the development of obesity-derived mal-adaptive cardiac hypertrophy, we performed a set of experiments aimed to distinguish differences in cardiac and metabolic phenotype of female and male mice fed with HFD for 25 weeks. Echocardiographic analysis of those mice revealed, that male animals developed more severe left ventricle hypertrophy (LVH), when compared to female littermate mice. Importantly, development of LVH was associated with sexual dimorphic regulation of adiponectin, leptin, and vaspin expression, measured in epicardial fat depot. Intriguingly, vaspin plasma levels were not affected by the sex in our HFD-fed animals. Since epicardial adipose tissue, due to its close localisation to the heart, may potentially regulate the development of cardiac hypertrophy in a paracrine manner, local vaspin production/release could potentially induce hypertrophic responses observed in male animals. Complementary experiments performed on primary murine cardiac fibroblasts supplemented with recombinant vaspin showed its putative pro-fibrotic effects in-vitro likely contributing to LVH development.

Bohm C, Benz V, Clemenz M, Sprang C, Hoft B, Kintscher U, Foryst-Ludwig A: Sexual dimorphism in obesity-mediated left ventricular hypertrophy. *Am J Physiol Heart Circ Physiol* 305:H211-218, 2013

Sex differences in physiological cardiac hypertrophy are associated with exercise-mediated changes in energy substrate availability (111).

The mal-adaptive form of cardiac remodelling induced by HFD-feeding differs from the processes responsible for the development of physiological cardiac hypertrophy induced by physical training. Physiological cardiac hypertrophy mediated by endurance physical training is characterised by a moderate increase of LVM and preserved cardiac integrity/ function.

Since physiological cardiac hypertrophy is regulated in a sex specific manner, and training-induced lipolytic activity of WAT shows dimorphic sex regulation, we investigated if sex-specific differences in training-induced cardiac hypertrophy, linked with lipolytic activity, measured in WAT, potentially regulate cardiac energy substrate availability and utilisation. Female mice exhibited an increased cardiac hypertrophic response to exercise associated with augmented lipolytic activity in WAT, increased expression of ATGL and HSL, and elevated plasma FFA levels measured directly after training. Moreover, female mice showed training-dependent up-regulation of FA utilisation and reduced myocardial glucose uptake (PET analysis). Moreover, also cardiac gene expression profiling revealed augmented expression of genes linked with FA uptake and utilisation in female trained animals, when compared to males. An integral cardiac FA-metabolism seems to be necessary for the cardiac adaptation to physical load, and impaired FA uptake/utilisation is well established as a hallmark of pathological cardiac hypertrophy.

Foryst-Ludwig A, Kreissl MC, Sprang C, Thalke B, Bohm C, Benz V, Gurgen D, Dragun D, Schubert C, Mai K, Stawowy P, Spranger J, Regitz-Zagrosek V, Unger T, Kintscher U: Sex differences in physiological cardiac hypertrophy are associated with exercise-mediated changes in energy substrate availability. *Am J Physiol Heart Circ Physiol* 301:H115-122, 2011

5   Discussion and therapeutic implications

The prevalence of cardiovascular and metabolic (cardiometabolic) diseases increases rapidly, which implies a growing requirement for new and effective therapeutic strategies.

One attractive candidate for pharmacological targeting strategies is PPARgamma. PPARgamma is regarded as a key molecular regulator of energy metabolism and insulin sensitivity, and therapeutic approaches modulating PPARgamma activity are already applied into the clinical treatment regimes of T2DM and obesity-related insulin resistance. In addition to the beneficial effects of PPARgamma activation described in the introduction, activation of that NHR by its full agonist rosiglitazone, led to reduced macrophage and T-lymphocytes infiltration into WAT in a DIO model in mice (manuscript # 1 (15)). Anti-inflammatory actions of PPARgamma were also associated with an improved metabolic phenotype of those animals with regards to glucose tolerance and systemic insulin sensitivity. Importantly, the recruitment of T-lymphocytes seems to be the first and crucial step towards adipose tissue specific inflammation. Moreover, those anti-inflammatory effects, resulting from PPARgamma activation were also mediated by the PPARgamma partial agonist telmisartan, an AT1R- blocker generally used in the clinic for the treatment of hypertension. Telmisartan has been previously demonstrated to improve the metabolic phenotype in a DIO mouse model (151; 152). In this model telmisartan treatment led to a significant reduction of BW and fat mass, which resulted in an improvement of metabolic outcomes of HFD-fed animals, when compared to vehicle treated controls. Furthermore, telmisartan was also reported to mediate beneficial metabolic and antidyslipidemic effects by direct activation of PPARalpha in the liver (154).

Since hypertensive patients are more prone to develop diabetes, and telmisartan is a well-established antihypertensive agent, the potential beneficial cardiometabolic effects of telmisartan were tested in a clinical study, established primary for patients with vascular diseases or high-risk diabetes patients without heart failure (ONTARGET-Study (The Ongoing Telmisartan Alone and in Combination with Ramipril Global Endpoint Trial)) (155). In this double-blind randomized study, patients were assigned to receive an angiotensin-converting–enzyme (ACE)-inhibitor ramipril (10 mg /day) or telmisartan (80 mg /day), or a combination of both drugs (combination therapy). The primary endpoints were: death from cardiovascular causes, myocardial infarction, stroke, or hospitalization for heart failure. This study demonstrated that ramipril and telmisartan were overall equivalent in the tested groups of patients, although the incidence of certain side effects such as cough and angioedema was significantly lower in the telmisartan treated participants. The combination therapy showed no benefit upon mono-therapy, and was generally associated with the occurrence of more adverse effects (155). Although the ONTARGET study was not primarily designed to investigate putative metabolic effects of telmisartan, the incident of new onset of diabetes, used normally to evaluate expected beneficial metabolic effects of therapeutic

agents, was equally reduced in the patient receiving telmisartan, to those receiving ramipril. Notably, the Heart Outcomes Prevention Evaluation study (HOPE study) demonstrated, that when applying ramipril to patients at a high risk of cardiovascular events, this ACE-inhibitor was able to decrease new onset of diabetes by 34%, when compared to placebo-treated patients (156). Since both ramipril and telmisartan were equally effective in diabetes prevention in the ONTARGET study, one could assume some beneficial metabolic effects of telmisartan in a group of patients with high cardiovascular risk. Hereby our previous in-vivo results received from DIO-model in mice were indirectly confirmed by the results from ONTARGET clinical study. However, additional studies are required to identify whether telmisartan´s metabolic actions are mediated solely by PPARgamma, by AT1-receptor blockade, or by both mechanisms.

In addition to its anti-inflammatory properties, activation of PPARgamma seems to be beneficial for vascular protection and VSMCs biology (manuscript #2 (46)). The molecular mechanism responsible for those protective anti-atherosclerotic actions seems to be linked with a ligand-mediated trans-repression of the MMP9 promoter in VSMCs, which is mediated by high-mobility group A1 (HMGA1). HMGA1 seems to act as a key regulator of UBC9-dependent SUMOlyation of PPARgamma. Moreover, HMGA1 was recently shown to interact with other NHRs such as ERalpha, which indicate putative ER-dependent regulation of those processes (157; 158). SUMOlyated PPARgamma mediates a subsequent recruitment of co-repressors, such as SMRT into the NFkappaB-dependent transcriptional complex, inhibiting the transcription of MMP9. The protective vascular action of PPARgamma was also tested in HMGA1-deficient mice in the aortic injury model. Those experiments indicated that PPARgamma activation significantly reduced MMP9-dependent neointima formation in control animals, but not in HMGA1-deficient animals. Hereby we could confirm that the beneficial anti-atherosclerotic properties of PPARgamma activation in VSMCs are specifically mediated by HMGA-1.

The pharmacological approaches, inhibiting specifically VSMCs migration and proliferation are extremely important for patients undergoing coronary angioplasty, or patients with catheterization- induced arterial injury and vascular stenosis. Moreover, effective inhibition of VSMC proliferation and migration is also urgently required during angiographic restenosis, with estimated rate of 21% to 40% after stenting or balloon angioplasty (159; 160). Thus, taking into account severe side effects linked with TZD-based treatment, new therapeutic strategies, based on the development of novel selective PPARgamma modulators, inhibiting MMP9 activity specifically in VSMCs are needed. The development of new PPARgamma agonists with an improved efficacy-side effect profile, and designed to activate and recruit HMGA1 into the PPARgamma complex in VSMCs, should potentially ameliorate PPARgamma-mediated beneficial anti-atherosclerotic actions.

Metabolic action of PPARgamma seems to be negatively regulated by ERbeta (manuscript #3 (78)). Repression of TZD- mediated PPARgamma activity by ERbeta relies on the competitive

requirement of an identical group of coactivators, such as SCR1 or TIF2, essential for the activation of both NHR. The inhibition of PPARgamma activity due to that cross-talk in vivo resulted in an impairment of insulin sensitivity and glucose tolerance under HFD in wt, but not in ERbeta-deficient mice. The putative pro-diabetogenic actions of ERbeta could have important consequences in the development of ERbeta-selective agonists, used for the treatment of postmenopausal osteoporosis or rheumatoid arthritis. Our study indicated potential deleterious effects of ERbeta on glucose and lipid metabolism, which suggests a growing need for the careful metabolic profiling of new compounds selectively activating ERbeta before the final drug approval.

Obesity-related metabolic and cardiovascular diseases seem to be regulated in a sex-specific manner. Both estrogen receptors appear to participate in sex-specific differences, observed in the development of cardiac hypertrophy. Sex-dependent regulation of the pathological form of hypertrophy, induced by HFD-feeding, was associated with an augmented cardiac hypertrophic response of male animals, when compared to females (manuscript #4 (153)). Similar data were published by Fliegner and colleagues (145) showing augmented development of pathological cardiac hypertrophy in male mice in response to cardiac aortic valve stenosis, when compared to females. In contrast, female sex is associated with an increased ability to develop the physiological form of cardiac hypertrophy in response to physical training (manuscript #5 (111)). As both forms of hypertrophy differ in cardiometabolic characteristics, such as FA-uptake and metabolism, fetal gene expression profile, fibrosis and apoptosis, training-induced "protective" remodelling of the pathological form of hypertrophy could represent an attractive therapeutic option. Metabolic re-programming of cardiac metabolism from the pathological – "glucose based", to a physiological state, with predominant cardiac FA-utilisation could be beneficial for the development and progression of mal-adaptive cardiac hypertrophy (129). Thus the identification of new molecular mediators, participating in the development of physiological hypertrophy, could provide some important evidences for the future pharmacotherapy of maladaptive cardiac hypertrophy. It would allow the development of novel therapeutic strategies for the treatment of advanced pathological hypertrophy and heart failure, resulting from a chronic arterial hypertension, cardiac aortic valve stenosis, or obesity.

Since both WAT and skeletal muscle are known to undergo profound metabolic changes during physical training, both of them seem to be the optimal target organs – except of the heart itself - to look for putative molecular mediators, participating in the development of physiological hypertrophy.

We were able to show, that exercise-induced physiological cardiac hypertrophy is linked to augmented lipolysis in WAT (manuscript #5 (111)). Moreover, WAT metabolism seems to be regulated in a sex-specific manner (112). We were also able to demonstrate, that sex-specific differences in the development of physiological hypertrophy are associated with exercise-mediated increase of cardiac FA-utilisation in vivo (manuscript #5 (111)).

Moreover, increased exercise-mediated hypertrophic responses observed in the heart were associated with augmented lipolytic activity in WAT during physical training, and thus elevated plasma FFAs. WAT reacted to endurance training not only with increased rate of lipolysis, resulting in the release of a certain set of FFAs and glycerol, but also with the liberation of a specific set of adipocytokines. Mutual actions of FFAs and adipocytokines could potentially participate in the metabolic adaptations of the cardiomyocytes, discussed above.

Recently, a new set of potential peptide hormones (myokines) were described to modulate adipose tissue metabolism, when released from skeletal muscle upon an exercising (161) (162; 163). Although the role of the novel myokine irisin in human physiology remains elusive (162; 163), study published by Wu and colleagues indicated that when applied to mice irisin protects against DIO, due to a re-programming of adipose tissue (164). Since myokines potentially modulate the lipolytic activity of WAT under exercise, they can be considered as putative mediators determining FA-dependent development of physiological cardiac hypertrophy.

The concept of interorgan communication among WAT, skeletal muscle and the heart seems to be especially attractive for the development of new therapies for severe cardiac hypertrophy and heart failure. The necessity of the new therapeutic approaches is based on the fact that conventional therapy applied to those patients seems to be partially ineffective. Furthermore, an endurance physical training of those patients is not feasible.

In summary, although several efforts were undertaken to implement physical fitness and healthy diet, the persistence of obesity-related metabolic and cardiovascular diseases implies a growing requirement for the development of new and effective therapeutic strategies for cardiometabolic diseases. This work represents a basics research approach, potentially allowing the identification of new molecular mediators of cardiometabolic diseases, such as obesity-derived cardiac hypertrophy or 2TDM. Moreover, taking into account the data on molecular characteristic of PPARgamma and ERbeta interaction presented above, our results indicate an importance of a careful metabolic profiling of co-regulatory proteins involved in the activation of specific NHRs, in the given tissue and under stimulation with a specific ligand. The knowledge of molecular mechanisms involved in the cell type- specific actions of NHR, such as in case of VSMCs and PPARgamma could help to identify novel therapeutic targets for cell-specific interventions, necessary to avoid systemic adverse effects in the future.

The present work indicates a potential role of PPARgamma in the regulation of sex-specific differences in the development of cardiometabolic diseases. Taking into account the current trend towards personalized pharmacotherapy, one could predict the development of a new set of pharmacological agents, activating selectively PPARgamma for the treatment of cardiometabolic diseases specially dedicated to women or men in the near future.

6   Summary

The prevalence of metabolic and obesity-related cardiovascular (cardiometabolic) diseases increases rapidly, which indicates an instant need for novel and effective therapeutic strategies. Our study aimed to investigate the role of selective PPARgamma modulation in a sex-specific manner as a future therapeutic intervention for cardiometabolic diseases.

We identified a crucial basis for sex-specific PPARgamma modulation in the molecular crosstalk of PPARgamma with estrogen receptors (ERs). Our results indicated that PPARgamma and ERbeta physically interact with each other in vitro in adipocytes. In addition, results obtained from a study on ERbeta-deficient mice, fed with high fat diet (HFD), demonstrated pro-diabetogenic effects of ERbeta, resulting from a negative cross-talk with PPARgamma in the adipose tissue.

In addition to a direct crosstalk with ERs, selective PPARgamma modulation may be regulated by competitive cofactor binding. We have identified SUMO-E2 ligase HMGA1 as a new PPARgamma cofactor in vascular smooth muscle cells (VSMCs). Moreover, we were able to demonstrate, that the valuable anti-atherosclerotic and anti-proliferative actions of selective PPARgamma modulation in VSMCs are linked with an inhibition of MMP9 expression by HMGA1 in the process known as transrepression. Interestingly, HMGA1 also interacts with ERs thereby providing an additional mechanism of sexual dimorphic PPARgamma modulation. To further establish these sex differences in PPARgamma modulation, we comprehensively characterized two mouse models for sex-specific cardiac hypertrophy, including one for the development of pathological hypertrophy induced by HFD feeding, and one (physiological hypertrophy model) for endurance training in mice. Both models show strong sexual dimorphisms, in particular, in metabolic processes controlled by PPARgamma, such as lipolysis or adipogenesis. By using those models we are aiming to identify sex-specific PPARgamma modulatory pathways in future experiments.

Taken together, sex-specific PPARgamma modulation likely results from interactions with ERs involving competitive cofactor binding e.g. of HMGA1. Furthermore, central PPARgamma-dependent cardiometabolic processes show a strong sexual dimorphic regulation likely resulting from PPARgamma-ER interactions. More detailed knowledge about the ER-PPARgamma modulatory pathways would help us to develop a sex-tailored, tissue-specific PPARgamma-based pharmacological intervention following the idea of an individualized treatment approach.

7   References

1. Zimmet P, Alberti G, Kaufman F, Tajima N, Silink M, Arslanian S, Wong G, Bennett P, Shaw J, Caprio S: The metabolic syndrome in children and adolescents. *Lancet* 369:2059-2061, 2007

2. Kaila B, Raman M: Obesity: a review of pathogenesis and management strategies. *Can J Gastroenterol* 22:61-68, 2008

3. Zimmermann R, Strauss JG, Haemmerle G, Schoiswohl G, Birner-Gruenberger R, Riederer M, Lass A, Neuberger G, Eisenhaber F, Hermetter A, Zechner R: Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. *Science* 306:1383-1386, 2004

4. Zechner R, Kienesberger PC, Haemmerle G, Zimmermann R, Lass A: Adipose triglyceride lipase and the lipolytic catabolism of cellular fat stores. *J Lipid Res* 50:3-21, 2009

5. Bluher M: Adipose tissue dysfunction in obesity. *Exp Clin Endocrinol Diabetes* 117:241-250, 2009

6. Fox CS, Massaro JM, Hoffmann U, Pou KM, Maurovich-Horvat P, Liu CY, Vasan RS, Murabito JM, Meigs JB, Cupples LA, D'Agostino RB, Sr., O'Donnell CJ: Abdominal visceral and subcutaneous adipose tissue compartments: association with metabolic risk factors in the Framingham Heart Study. *Circulation* 116:39-48, 2007

7. Despres JP, Lemieux I, Bergeron J, Pibarot P, Mathieu P, Larose E, Rodes-Cabau J, Bertrand OF, Poirier P: Abdominal obesity and the metabolic syndrome: contribution to global cardiometabolic risk. *Arterioscler Thromb Vasc Biol* 28:1039-1049, 2008

8. DeFronzo RA: Insulin resistance, lipotoxicity, type 2 diabetes and atherosclerosis: the missing links. The Claude Bernard Lecture 2009. *Diabetologia* 53:1270-1287, 2010

9. Palomer X, Salvado L, Barroso E, Vazquez-Carrera M: An overview of the crosstalk between inflammatory processes and metabolic dysregulation during diabetic cardiomyopathy. *Int J Cardiol*, 2013

10. Hotamisligil GS: Inflammation and metabolic disorders. *Nature* 444:860-867, 2006

11. Olefsky JM, Glass CK: Macrophages, inflammation, and insulin resistance. *Annu Rev Physiol* 72:219-246, 2010

12. Shoelson SE, Lee J, Goldfine AB: Inflammation and insulin resistance. *J Clin Invest* 116:1793-1801, 2006

13. Wellen KE, Hotamisligil GS: Inflammation, stress, and diabetes. *J Clin Invest* 115:1111-1119, 2005

14. Despres JP, Lemieux I: Abdominal obesity and metabolic syndrome. *Nature* 444:881-887, 2006

15. Foryst-Ludwig A, Hartge M, Clemenz M, Sprang C, Hess K, Marx N, Unger T, Kintscher U: PPARgamma activation attenuates T-lymphocyte-dependent inflammation of adipose tissue and development of insulin resistance in obese mice. *Cardiovasc Diabetol* 9:64, 2010

16. Kintscher U, Hartge M, Hess K, Foryst-Ludwig A, Clemenz M, Wabitsch M, Fischer-Posovszky P, Barth TF, Dragun D, Skurk T, Hauner H, Bluher M, Unger T, Wolf AM, Knippschild U, Hombach V, Marx N: T-lymphocyte infiltration in visceral adipose tissue: a primary event in adipose tissue inflammation and the development of obesity-mediated insulin resistance. *Arterioscler Thromb Vasc Biol* 28:1304-1310, 2008

17. Ouchi N, Parker JL, Lugus JJ, Walsh K: Adipokines in inflammation and metabolic disease. *Nat Rev Immunol* 11:85-97, 2011

18. Halberg N, Khan T, Trujillo ME, Wernstedt-Asterholm I, Attie AD, Sherwani S, Wang ZV, Landskroner-Eiger S, Dineen S, Magalang UJ, Brekken RA, Scherer PE: Hypoxia-inducible factor 1alpha induces fibrosis and insulin resistance in white adipose tissue. *Mol Cell Biol* 29:4467-4483, 2009

19. Pasarica M, Sereda OR, Redman LM, Albarado DC, Hymel DT, Roan LE, Rood JC, Burk DH, Smith SR: Reduced adipose tissue oxygenation in human obesity: evidence for rarefaction, macrophage chemotaxis, and inflammation without an angiogenic response. *Diabetes* 58:718-725, 2009

20. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr.: Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 112:1796-1808, 2003

21. Lumeng CN, Bodzin JL, Saltiel AR: Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* 117:175-184, 2007

22. Lumeng CN, Deyoung SM, Bodzin JL, Saltiel AR: Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity. *Diabetes* 56:16-23, 2007

23. Hotta K, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, Iwahashi H, Kuriyama H, Ouchi N, Maeda K, Nishida M, Kihara S, Sakai N, Nakajima T, Hasegawa K, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Hanafusa T, Matsuzawa Y: Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler Thromb Vasc Biol* 20:1595-1599., 2000

24. Ryo M, Nakamura T, Kihara S, Kumada M, Shibazaki S, Takahashi M, Nagai M, Matsuzawa Y, Funahashi T: Adiponectin as a biomarker of the metabolic syndrome. *Circ J* 68:975-981, 2004

25. Ouchi N, Kihara S, Funahashi T, Matsuzawa Y, Walsh K: Obesity, adiponectin and vascular inflammatory disease. *Curr Opin Lipidol* 14:561-566, 2003

26. Fasshauer M, Kralisch S, Klier M, Lossner U, Bluher M, Klein J, Paschke R: Adiponectin gene expression and secretion is inhibited by interleukin-6 in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* 301:1045-1050., 2003

27. Jung SH, Park HS, Kim KS, Choi WH, Ahn CW, Kim BT, Kim SM, Lee SY, Ahn SM, Kim YK, Kim HJ, Kim DJ, Lee KW: Effect of weight loss on some serum cytokines in human obesity: increase in IL-10 after weight loss. *J Nutr Biochem* 19:371-375, 2008

28. Chrysant SG, Chrysant GS: New insights into the true nature of the obesity paradox and the lower cardiovascular risk. *J Am Soc Hypertens* 7:85-94, 2013

29. Deng G, Long Y, Yu YR, Li MR: Adiponectin directly improves endothelial dysfunction in obese rats through the AMPK-eNOS Pathway. *Int J Obes (Lond)* 34:165-171, 2010

30. Shibata R, Sato K, Pimentel DR, Takemura Y, Kihara S, Ohashi K, Funahashi T, Ouchi N, Walsh K: Adiponectin protects against myocardial ischemia-reperfusion injury through AMPK- and COX-2-dependent mechanisms. *Nat Med* 11:1096-1103, 2005

31. Bays HE: Adiposopathy is "sick fat" a cardiovascular disease? *J Am Coll Cardiol* 57:2461-2473, 2011

32. Ferre P: The biology of peroxisome proliferator-activated receptors: relationship with lipid metabolism and insulin sensitivity. *Diabetes* 53 Suppl 1:S43-50, 2004

33. Lonard DM, Lanz RB, O'Malley BW: Nuclear receptor coregulators and human disease. *Endocr Rev* 28:575-587, 2007

34. Benz V, Kintscher U, Foryst-Ludwig A: Sex-specific differences in Type 2 Diabetes Mellitus and dyslipidemia therapy: PPAR agonists. *Handb Exp Pharmacol*:387-410, 2012

35. Tobin JF, Freedman LP: Nuclear receptors as drug targets in metabolic diseases: new approaches to therapy. *Trends Endocrinol Metab* 17:284-290, 2006

36. Reddy JK: Peroxisome proliferators and peroxisome proliferator-activated receptor alpha: biotic and xenobiotic sensing. *Am J Pathol* 164:2305-2321, 2004

37. Brun RP, Tontonoz P, Forman BM, Ellis R, Chen J, Evans RM, Spiegelman BM: Differential activation of adipogenesis by multiple PPAR isoforms. *Genes Dev* 10:974-984, 1996

38. Kliewer SA, Forman BM, Blumberg B, Ong ES, Borgmeyer U, Mangelsdorf DJ, Umesono K, Evans RM: Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc Natl Acad Sci U S A* 91:7355-7359, 1994

39. Picard F, Gehin M, Annicotte J, Rocchi S, Champy MF, O'Malley BW, Chambon P, Auwerx J: SRC-1 and TIF2 control energy balance between white and brown adipose tissues. *Cell* 111:931-941, 2002

40. Semple RK, Chatterjee VK, O'Rahilly S: PPAR gamma and human metabolic disease. *J Clin Invest* 116:581-589, 2006

41. Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM: 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell* 83:803-812, 1995

42. Glass CK: Going nuclear in metabolic and cardiovascular disease. *J Clin Invest* 116:556-560, 2006

43. Jensen JS, Feldt-Rasmussen B, Strandgaard S, Schroll M, Borch-Johnsen K: Arterial hypertension, microalbuminuria, and risk of ischemic heart disease. *Hypertension* 35:898-903, 2000

44. Cavailles V, Dauvois S, L'Horset F, Lopez G, Hoare S, Kushner PJ, Parker MG: Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. *Embo J* 14:3741-3751, 1995

45. Glass CK, Saijo K: Nuclear receptor transrepression pathways that regulate inflammation in macrophages and T cells. *Nat Rev Immunol* 10:365-376, 2010

46. Bloch M, Prock A, Paonessa F, Benz V, Bahr IN, Herbst L, Witt H, Kappert K, Spranger J, Stawowy P, Unger T, Fusco A, Sedding D, Brunetti A, Foryst-Ludwig A, Kintscher U: High-mobility group A1 protein: a new coregulator of peroxisome proliferator-activated receptor-gamma-mediated transrepression in the vasculature. *Circ Res* 110:394-405, 2012

47. Pascual G, Fong AL, Ogawa S, Gamliel A, Li AC, Perissi V, Rose DW, Willson TM, Rosenfeld MG, Glass CK: A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. *Nature* 437:759-763. Epub 2005 Aug 2028., 2005

48. Desvergne B, Wahli W: Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr Rev* 20:649-688, 1999

49. Guan HP, Ishizuka T, Chui PC, Lehrke M, Lazar MA: Corepressors selectively control the transcriptional activity of PPARgamma in adipocytes. *Genes Dev* 19:453-461, 2005

50. McKenna NJ, O'Malley BW: From ligand to response: generating diversity in nuclear receptor coregulator function. *J Steroid Biochem Mol Biol* 74:351-356., 2000

51. Yki-Jarvinen H: Thiazolidinediones. *N Engl J Med* 351:1106-1118, 2004

52. Deeg MA, Tan MH: Pioglitazone versus Rosiglitazone: Effects on Lipids, Lipoproteins, and Apolipoproteins in Head-to-Head Randomized Clinical Studies. *PPAR Res* 2008:520465, 2008

53. Home PD, Pocock SJ, Beck-Nielsen H, Curtis PS, Gomis R, Hanefeld M, Jones NP, Komajda M, McMurray JJ: Rosiglitazone evaluated for cardiovascular outcomes in oral agent combination therapy for type 2 diabetes (RECORD): a multicentre, randomised, open-label trial. *Lancet* 373:2125-2135, 2009

54. Lincoff AM, Wolski K, Nicholls SJ, Nissen SE: Pioglitazone and risk of cardiovascular events in patients with type 2 diabetes mellitus: a meta-analysis of randomized trials. *Jama* 298:1180-1188, 2007

55. Singh S, Loke YK, Furberg CD: Long-term risk of cardiovascular events with rosiglitazone: a meta-analysis. *Jama* 298:1189-1195, 2007

56. Mannucci E, Monami M, Di Bari M, Lamanna C, Gori F, Gensini GF, Marchionni N: Cardiac safety profile of rosiglitazone: a comprehensive meta-analysis of randomized clinical trials. *Int J Cardiol* 143:135-140, 2010

57. Schernthaner G, Chilton RJ: Cardiovascular risk and thiazolidinediones--what do meta-analyses really tell us? *Diabetes Obes Metab* 12:1023-1035, 2010

58. Nilsson S, Gustafsson JA: Biological role of estrogen and estrogen receptors. *Crit Rev Biochem Mol Biol* 37:1-28, 2002

59. Foryst-Ludwig A, Kintscher U: Metabolic impact of estrogen signalling through ERalpha and ERbeta. *J Steroid Biochem Mol Biol* 122:74-81, 2010

60. Cooke PS, Naaz A: Role of estrogens in adipocyte development and function. *Exp Biol Med (Maywood)* 229:1127-1135, 2004

61. Cooke PS, Heine PA, Taylor JA, Lubahn DB: The role of estrogen and estrogen receptor-alpha in male adipose tissue. *Mol Cell Endocrinol* 178:147-154, 2001

62. Bryzgalova G, Lundholm L, Portwood N, Gustafsson JA, Khan A, Efendic S, Dahlman-Wright K: Mechanisms of antidiabetogenic and body weight-lowering effects of estrogen in high-fat diet-fed mice. *Am J Physiol Endocrinol Metab* 295:E904-912, 2008

63. Matthews J, Gustafsson JA: Estrogen signaling: a subtle balance between ER alpha and ER beta. *Mol Interv* 3:281-292, 2003

64. Merchenthaler I, Lane MV, Numan S, Dellovade TL: Distribution of estrogen receptor alpha and beta in the mouse central nervous system: in vivo autoradiographic and immunocytochemical analyses. *J Comp Neurol* 473:270-291, 2004

65. Dieudonne MN, Leneveu MC, Giudicelli Y, Pecquery R: Evidence for functional estrogen receptors alpha and beta in human adipose cells: regional specificities and regulation by estrogens. *Am J Physiol Cell Physiol* 286:C655-661, 2004

66. Sharma PK, Thakur MK: Expression of estrogen receptor (ER) alpha and beta in mouse cerebral cortex: effect of age, sex and gonadal steroids. *Neurobiol Aging* 27:880-887, 2006

67. Batra GS, Hainey L, Freemont AJ, Andrew G, Saunders PT, Hoyland JA, Braidman IP: Evidence for cell-specific changes with age in expression of oestrogen receptor (ER) alpha and beta in bone fractures from men and women. *J Pathol* 200:65-73, 2003

68. Sakaguchi H, Fujimoto J, Aoki I, Tamaya T: Expression of estrogen receptor alpha and beta in myometrium of premenopausal and postmenopausal women. *Steroids* 68:11-19, 2003

69. Bonomo SM, Rigamonti AE, Giunta M, Galimberti D, Guaita A, Gagliano MG, Muller EE, Cella SG: Menopausal transition: a possible risk factor for brain pathologic events. *Neurobiol Aging* 30:71-80, 2009

70. Deroo BJ, Korach KS: Estrogen receptors and human disease. *J Clin Invest* 116:561-570, 2006

71. Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, Tujague M, Strom A, Treuter E, Warner M, Gustafsson JA: Estrogen receptors: how do they signal and what are their targets. *Physiol Rev* 87:905-931, 2007

72. Delaunay F, Pettersson K, Tujague M, Gustafsson JA: Functional differences between the amino-terminal domains of estrogen receptors alpha and beta. *Mol Pharmacol* 58:584-590, 2000

73. Barkhem T, Nilsson S, Gustafsson JA: Molecular mechanisms, physiological consequences and pharmacological implications of estrogen receptor action. *Am J Pharmacogenomics* 4:19-28, 2004

74. Shang Y, Brown M: Molecular determinants for the tissue specificity of SERMs. *Science* 295:2465-2468, 2002

75. Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M: Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell* 103:843-852, 2000

76. Chang EC, Charn TH, Park SH, Helferich WG, Komm B, Katzenellenbogen JA, Katzenellenbogen BS: Estrogen Receptors alpha and beta as determinants of gene expression: influence of ligand, dose, and chromatin binding. *Mol Endocrinol* 22:1032-1043, 2008

77. Okada M, Takezawa S, Mezaki Y, Yamaoka I, Takada I, Kitagawa H, Kato S: Switching of chromatin-remodelling complexes for oestrogen receptor-alpha. *EMBO Rep* 9:563-568, 2008

78. Foryst-Ludwig A, Clemenz M, Hohmann S, Hartge M, Sprang C, Frost N, Krikov M, Bhanot S, Barros R, Morani A, Gustafsson JA, Unger T, Kintscher U: Metabolic actions of estrogen receptor beta (ERbeta) are mediated by a negative cross-talk with PPARgamma. *PLoS Genet* 4:e1000108, 2008

79. Wang X, Kilgore MW: Signal cross-talk between estrogen receptor alpha and beta and the peroxisome proliferator-activated receptor gamma1 in MDA-MB-231 and MCF-7 breast cancer cells. *Mol Cell Endocrinol* 194:123-133, 2002

80. Ropero AB, Alonso-Magdalena P, Quesada I, Nadal A: The role of estrogen receptors in the control of energy and glucose homeostasis. *Steroids* 73:874-879, 2008

81. Laudenslager ML, Wilkinson CW, Carlisle HJ, Hammel HT: Energy balance in ovariectomized rats with and without estrogen replacement. *Am J Physiol* 238:R400-405, 1980

82. Saengsirisuwan V, Pongseeda S, Prasannarong M, Vichaiwong K, Toskulkao C: Modulation of insulin resistance in ovariectomized rats by endurance exercise training and estrogen replacement. *Metabolism* 58:38-47, 2009

83. Riant E, Waget A, Cogo H, Arnal JF, Burcelin R, Gourdy P: Estrogens protect against high-fat diet-induced insulin resistance and glucose intolerance in mice. *Endocrinology* 150:2109-2117, 2009

84. Heine PA, Taylor JA, Iwamoto GA, Lubahn DB, Cooke PS: Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. *Proc Natl Acad Sci U S A* 97:12729-12734, 2000

85. Naaz A, Zakroczymski M, Heine P, Taylor J, Saunders P, Lubahn D, Cooke PS: Effect of ovariectomy on adipose tissue of mice in the absence of estrogen receptor alpha (ERalpha): a potential role for estrogen receptor beta (ERbeta). *Horm Metab Res* 34:758-763, 2002

86. Bryzgalova G, Gao H, Ahren B, Zierath JR, Galuska D, Steiler TL, Dahlman-Wright K, Nilsson S, Gustafsson JA, Efendic S, Khan A: Evidence that oestrogen receptor-alpha plays an important role in the regulation of glucose homeostasis in mice: insulin sensitivity in the liver. *Diabetologia* 49:588-597, 2006

87. Misso ML, Murata Y, Boon WC, Jones ME, Britt KL, Simpson ER: Cellular and molecular characterization of the adipose phenotype of the aromatase-deficient mouse. *Endocrinology* 144:1474-1480, 2003

88. Murata Y, Robertson KM, Jones ME, Simpson ER: Effect of estrogen deficiency in the male: the ArKO mouse model. *Mol Cell Endocrinol* 193:7-12, 2002

89. Gao H, Bryzgalova G, Hedman E, Khan A, Efendic S, Gustafsson JA, Dahlman-Wright K: Long-term administration of estradiol decreases expression of hepatic lipogenic genes and improves insulin sensitivity in ob/ob mice: a possible mechanism is through direct regulation of signal transducer and activator of transcription 3. *Mol Endocrinol* 20:1287-1299, 2006

90. Lundholm L, Bryzgalova G, Gao H, Portwood N, Falt S, Berndt KD, Dicker A, Galuska D, Zierath JR, Gustafsson JA, Efendic S, Dahlman-Wright K, Khan A: The estrogen receptor {alpha}-selective agonist propyl pyrazole triol improves glucose tolerance in ob/ob mice; potential molecular mechanisms. *J Endocrinol* 199:275-286, 2008

91. Louet JF, LeMay C, Mauvais-Jarvis F: Antidiabetic actions of estrogen: insight from human and genetic mouse models. *Curr Atheroscler Rep* 6:180-185, 2004

92. Margolis KL, Bonds DE, Rodabough RJ, Tinker L, Phillips LS, Allen C, Bassford T, Burke G, Torrens J, Howard BV: Effect of oestrogen plus progestin on the incidence of diabetes in postmenopausal women: results from the Women's Health Initiative Hormone Trial. *Diabetologia* 47:1175-1187, 2004

93. Bonds DE, Lasser N, Qi L, Brzyski R, Caan B, Heiss G, Limacher MC, Liu JH, Mason E, Oberman A, O'Sullivan MJ, Phillips LS, Prineas RJ, Tinker L: The effect of conjugated equine oestrogen on diabetes incidence: the Women's Health Initiative randomised trial. *Diabetologia* 49:459-468, 2006

94. Crespo CJ, Smit E, Snelling A, Sempos CT, Andersen RE: Hormone replacement therapy and its relationship to lipid and glucose metabolism in diabetic and nondiabetic postmenopausal women: results from the Third National Health and Nutrition Examination Survey (NHANES III). *Diabetes Care* 25:1675-1680, 2002

95. Barros RP, Machado UF, Warner M, Gustafsson JA: Muscle GLUT4 regulation by estrogen receptors ERbeta and ERalpha. *Proc Natl Acad Sci U S A* 103:1605-1608, 2006

96. Ohlsson C, Hellberg N, Parini P, Vidal O, Bohlooly YM, Rudling M, Lindberg MK, Warner M, Angelin B, Gustafsson JA: Obesity and disturbed lipoprotein profile in estrogen receptor-alpha-deficient male mice. *Biochem Biophys Res Commun* 278:640-645, 2000

97. Barros RP, Machado UF, Gustafsson JA: Estrogen receptors: new players in diabetes mellitus. *Trends Mol Med* 12:425-431, 2006

98. Regitz-Zagrosek V, Lehmkuhl E, Weickert MO: Gender differences in the metabolic syndrome and their role for cardiovascular disease. *Clin Res Cardiol* 95:136-147, 2006

99. Nuutila P, Knuuti MJ, Maki M, Laine H, Ruotsalainen U, Teras M, Haaparanta M, Solin O, Yki-Jarvinen H: Gender and insulin sensitivity in the heart and in skeletal muscles. Studies using positron emission tomography. *Diabetes* 44:31-36, 1995

100. Frias JP, Macaraeg GB, Ofrecio J, Yu JG, Olefsky JM, Kruszynska YT: Decreased susceptibility to fatty acid-induced peripheral tissue insulin resistance in women. *Diabetes* 50:1344-1350, 2001

101. Kuhl J, Hilding A, Ostenson CG, Grill V, Efendic S, Bavenholm P: Characterisation of subjects with early abnormalities of glucose tolerance in the Stockholm Diabetes Prevention Programme: the impact of sex and type 2 diabetes heredity. *Diabetologia* 48:35-40, 2005

102. Macotela Y, Boucher J, Tran TT, Kahn CR: Sex and depot differences in adipocyte insulin sensitivity and glucose metabolism. *Diabetes* 58:803-812, 2009

103. D'Eon TM, Souza SC, Aronovitz M, Obin MS, Fried SK, Greenberg AS: Estrogen regulation of adiposity and fuel partitioning. Evidence of genomic and non-genomic regulation of lipogenic and oxidative pathways. *J Biol Chem* 280:35983-35991, 2005

104. Rogers NH, Perfield JW, 2nd, Strissel KJ, Obin MS, Greenberg AS: Reduced energy expenditure and increased inflammation are early events in the development of ovariectomy-induced obesity. *Endocrinology* 150:2161-2168, 2009

105. Pedersen SB, Kristensen K, Hermann PA, Katzenellenbogen JA, Richelsen B: Estrogen controls lipolysis by up-regulating alpha2A-adrenergic receptors directly in human adipose tissue through the estrogen receptor alpha. Implications for the female fat distribution. *J Clin Endocrinol Metab* 89:1869-1878, 2004

106. Hamosh M, Hamosh P: The effect of estrogen on the lipoprotein lipase activity of rat adipose tissue. *J Clin Invest* 55:1132-1135, 1975

107. Homma H, Kurachi H, Nishio Y, Takeda T, Yamamoto T, Adachi K, Morishige K, Ohmichi M, Matsuzawa Y, Murata Y: Estrogen suppresses transcription of lipoprotein lipase gene. Existence of a unique estrogen response element on the lipoprotein lipase promoter. *J Biol Chem* 275:11404-11411, 2000

108. Price TM, O'Brien SN, Welter BH, George R, Anandjiwala J, Kilgore M: Estrogen regulation of adipose tissue lipoprotein lipase--possible mechanism of body fat distribution. *Am J Obstet Gynecol* 178:101-107, 1998

109. Palin SL, McTernan PG, Anderson LA, Sturdee DW, Barnett AH, Kumar S: 17Beta-estradiol and anti-estrogen ICI:compound 182,780 regulate expression of lipoprotein lipase and hormone-sensitive lipase in isolated subcutaneous abdominal adipocytes. *Metabolism* 52:383-388, 2003

110. Haemmerle G, Zimmermann R, Zechner R: Letting lipids go: hormone-sensitive lipase. *Curr Opin Lipidol* 14:289-297, 2003

111. Foryst-Ludwig A, Kreissl MC, Sprang C, Thalke B, Bohm C, Benz V, Gurgen D, Dragun D, Schubert C, Mai K, Stawowy P, Spranger J, Regitz-Zagrosek V, Unger T, Kintscher U: Sex differences in physiological cardiac hypertrophy are associated with exercise-mediated changes in energy substrate availability. *Am J Physiol Heart Circ Physiol* 301:H115-122, 2011

112. Benz V, Bloch M, Wardat S, Bohm C, Maurer L, Mahmoodzadeh S, Wiedmer P, Spranger J, Foryst-Ludwig A, Kintscher U: Sexual dimorphic regulation of body weight dynamics and adipose tissue lipolysis. *PLoS One* 7:e37794, 2012

113. Ramis JM, Salinas R, Garcia-Sanz JM, Moreiro J, Proenza AM, Llado I: Depot- and gender-related differences in the lipolytic pathway of adipose tissue from severely obese patients. *Cell Physiol Biochem* 17:173-180, 2006

114. Barros RP, Gabbi C, Morani A, Warner M, Gustafsson JA: Participation of ERalpha and ERbeta in glucose homeostasis in skeletal muscle and white adipose tissue. *Am J Physiol Endocrinol Metab* 297:E124-133, 2009

115. Breen DM, Sanli T, Giacca A, Tsiani E: Stimulation of muscle cell glucose uptake by resveratrol through sirtuins and AMPK. *Biochem Biophys Res Commun* 374:117-122, 2008

116. Deng JY, Hsieh PS, Huang JP, Lu LS, Hung LM: Activation of estrogen receptor is crucial for resveratrol-stimulating muscular glucose uptake via both insulin-dependent and -independent pathways. *Diabetes* 57:1814-1823, 2008

117. Alonso-Magdalena P, Ropero AB, Carrera MP, Cederroth CR, Baquie M, Gauthier BR, Nef S, Stefani E, Nadal A: Pancreatic insulin content regulation by the estrogen receptor ER alpha. *PLoS One* 3:e2069, 2008

118. Le May C, Chu K, Hu M, Ortega CS, Simpson ER, Korach KS, Tsai MJ, Mauvais-Jarvis F: Estrogens protect pancreatic beta-cells from apoptosis and prevent insulin-deficient diabetes mellitus in mice. *Proc Natl Acad Sci U S A* 103:9232-9237, 2006

119. Weiner RB, Baggish AL: Exercise-induced cardiac remodeling. *Prog Cardiovasc Dis* 54:380-386, 2012

120. Heineke J, Molkentin JD: Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol* 7:589-600, 2006

121. Bernardo BC, Weeks KL, Pretorius L, McMullen JR: Molecular distinction between physiological and pathological cardiac hypertrophy: experimental findings and therapeutic strategies. *Pharmacol Ther* 128:191-227, 2010

122. Maillet M, van Berlo JH, Molkentin JD: Molecular basis of physiological heart growth: fundamental concepts and new players. *Nat Rev Mol Cell Biol* 14:38-48, 2013

123. Haykowsky MJ: Left ventricular remodelling and the athlete's heart: time to revisit the Morganroth hypothesis. *J Physiol* 589:5915, 2011

124. Carroll JF, Kyser CK: Exercise training in obesity lowers blood pressure independent of weight change. *Med Sci Sports Exerc* 34:596-601, 2002

125. Luczak ED, Leinwand LA: Sex-based cardiac physiology. *Annu Rev Physiol* 71:1-18, 2009

126. Petersen SE, Wiesmann F, Hudsmith LE, Robson MD, Francis JM, Selvanayagam JB, Neubauer S, Channon KM: Functional and structural vascular remodeling in elite rowers assessed by cardiovascular magnetic resonance. *J Am Coll Cardiol* 48:790-797, 2006

127. Foryst-Ludwig A, Kintscher U: Sex differences in exercise-induced cardiac hypertrophy. *Pflugers Arch* 465:731-737, 2013

128. Soto PF, Herrero P, Schechtman KB, Waggoner AD, Baumstark JM, Ehsani AA, Gropler RJ: Exercise training impacts the myocardial metabolism of older individuals in a gender-specific manner. *Am J Physiol Heart Circ Physiol* 295:H842-850, 2008

129. Dorn GW, 2nd: The fuzzy logic of physiological cardiac hypertrophy. *Hypertension* 49:962-970, 2007

130. Mittendorfer B, Horowitz JF, Klein S: Effect of gender on lipid kinetics during endurance exercise of moderate intensity in untrained subjects. *Am J Physiol Endocrinol Metab* 283:E58-65, 2002

131. Horton TJ, Dow S, Armstrong M, Donahoo WT: Greater systemic lipolysis in women compared with men during moderate-dose infusion of epinephrine and/or norepinephrine. *J Appl Physiol* 107:200-210, 2009

132. Konhilas JP, Maass AH, Luckey SW, Stauffer BL, Olson EN, Leinwand LA: Sex modifies exercise and cardiac adaptation in mice. *Am J Physiol Heart Circ Physiol* 287:H2768-2776, 2004

133. Haines CD, Harvey PA, Leinwand LA: Estrogens mediate cardiac hypertrophy in a stimulus-dependent manner. *Endocrinology* 153:4480-4490, 2012

134. De Bono JP, Adlam D, Paterson DJ, Channon KM: Novel quantitative phenotypes of exercise training in mouse models. *Am J Physiol Regul Integr Comp Physiol* 290:R926-934, 2006

135. Passier R, Zeng H, Frey N, Naya FJ, Nicol RL, McKinsey TA, Overbeek P, Richardson JA, Grant SR, Olson EN: CaM kinase signaling induces cardiac hypertrophy and activates the MEF2 transcription factor in vivo. *J Clin Invest* 105:1395-1406, 2000

136. Antos CL, McKinsey TA, Frey N, Kutschke W, McAnally J, Shelton JM, Richardson JA, Hill JA, Olson EN: Activated glycogen synthase-3 beta suppresses cardiac hypertrophy in vivo. *Proc Natl Acad Sci U S A* 99:907-912, 2002

137. DeBosch B, Treskov I, Lupu TS, Weinheimer C, Kovacs A, Courtois M, Muslin AJ: Akt1 is required for physiological cardiac growth. *Circulation* 113:2097-2104, 2006

138. Rocha FL, Carmo EC, Roque FR, Hashimoto NY, Rossoni LV, Frimm C, Aneas I, Negrao CE, Krieger JE, Oliveira EM: Anabolic steroids induce cardiac renin-angiotensin system and impair the beneficial effects of aerobic training in rats. *Am J Physiol Heart Circ Physiol* 293:H3575-3583, 2007

139. Hansen FM, Fahmy N, Nielsen JH: The influence of sexual hormones on lipogenesis and lipolysis in rat fat cells. *Acta Endocrinol (Copenh)* 95:566-570, 1980

140. Dicker A, Ryden M, Naslund E, Muehlen IE, Wiren M, Lafontan M, Arner P: Effect of testosterone on lipolysis in human pre-adipocytes from different fat depots. *Diabetologia* 47:420-428, 2004

141. Levy D, Kenchaiah S, Larson MG, Benjamin EJ, Kupka MJ, Ho KK, Murabito JM, Vasan RS: Long-term trends in the incidence of and survival with heart failure. *N Engl J Med* 347:1397-1402, 2002

142. Konhilas JP: What we know and do not know about sex and cardiac disease. *J Biomed Biotechnol* 2010:562051, 2010

143. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J: Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *JAMA* 288:321-333, 2002

144. Regitz-Zagrosek V, Dworatzek E, Kintscher U, Dragun D: Sex and sex hormone-dependent cardiovascular stress responses. *Hypertension* 61:270-277, 2013

145. Fliegner D, Schubert C, Penkalla A, Witt H, Kararigas G, Dworatzek E, Staub E, Martus P, Ruiz Noppinger P, Kintscher U, Gustafsson JA, Regitz-Zagrosek V: Female sex and estrogen receptor-beta attenuate cardiac remodeling and apoptosis in pressure overload. *Am J Physiol Regul Integr Comp Physiol* 298:R1597-1606, 2010

146. van Eickels M, Grohe C, Cleutjens JP, Janssen BJ, Wellens HJ, Doevendans PA: 17beta-estradiol attenuates the development of pressure-overload hypertrophy. *Circulation* 104:1419-1423, 2001

147. Donaldson C, Eder S, Baker C, Aronovitz MJ, Weiss AD, Hall-Porter M, Wang F, Ackerman A, Karas RH, Molkentin JD, Patten RD: Estrogen attenuates left ventricular and cardiomyocyte hypertrophy by an estrogen receptor-dependent pathway that increases calcineurin degradation. *Circ Res* 104:265-275, 211p following 275, 2009

148. Mahmoodzadeh S, Dworatzek E, Fritschka S, Pham TH, Regitz-Zagrosek V: 17beta-Estradiol inhibits matrix metalloproteinase-2 transcription via MAP kinase in fibroblasts. *Cardiovasc Res* 85:719-728, 2010

149. Witt H, Schubert C, Jaekel J, Fliegner D, Penkalla A, Tiemann K, Stypmann J, Roepcke S, Brokat S, Mahmoodzadeh S, Brozova E, Davidson MM, Ruiz Noppinger P, Grohe C, Regitz-Zagrosek V: Sex-specific pathways in early cardiac response to pressure overload in mice. *J Mol Med (Berl)* 86:1013-1024, 2008

150. Westphal C, Schubert C, Prelle K, Penkalla A, Fliegner D, Petrov G, Regitz-Zagrosek V: Effects of estrogen, an ERalpha agonist and raloxifene on pressure overload induced cardiac hypertrophy. *PLoS One* 7:e50802, 2012

151. Schupp M, Clemenz M, Gineste R, Witt H, Janke J, Helleboid S, Hennuyer N, Ruiz P, Unger T, Staels B, Kintscher U: Molecular characterization of new selective peroxisome proliferator-activated receptor gamma modulators with angiotensin receptor blocking activity. *Diabetes* 54:3442-3452, 2005

152. Schupp M, Janke J, Clasen R, Unger T, Kintscher U: Angiotensin type 1 receptor blockers induce peroxisome proliferator-activated receptor-gamma activity. *Circulation* 109:2054-2057., 2004

153. Bohm C, Benz V, Clemenz M, Sprang C, Hoft B, Kintscher U, Foryst-Ludwig A: Sexual dimorphism in obesity-mediated left ventricular hypertrophy. *Am J Physiol Heart Circ Physiol* 305:H211-218, 2013

154. Clemenz M, Frost N, Schupp M, Caron S, Foryst-Ludwig A, Bohm C, Hartge M, Gust R, Staels B, Unger T, Kintscher U: Liver-specific peroxisome proliferator-activated receptor alpha target gene regulation by the angiotensin type 1 receptor blocker telmisartan. *Diabetes* 57:1405-1413, 2008

155. Yusuf S, Diener HC, Sacco RL, Cotton D, Ounpuu S, Lawton WA, Palesch Y, Martin RH, Albers GW, Bath P, Bornstein N, Chan BP, Chen ST, Cunha L, Dahlof B, De Keyser J, Donnan GA, Estol C, Gorelick P, Gu V, Hermansson K, Hilbrich L, Kaste M, Lu C, Machnig T, Pais P, Roberts R, Skvortsova V, Teal P, Toni D, VanderMaelen C, Voigt T, Weber M, Yoon BW: Telmisartan to prevent recurrent stroke and cardiovascular events. *N Engl J Med* 359:1225-1237, 2008

156. Yusuf S, Sleight P, Pogue J, Bosch J, Davies R, Dagenais G: Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators [see comments] [published errata appear in N Engl J Med 2000 Mar 9;342(10):748 and 2000 May 4;342(18):1376]. *N Engl J Med* 342:145-153, 2000

157. Massaad-Massade L, Navarro S, Krummrei U, Reeves R, Beaune P, Barouki R: HMGA1 enhances the transcriptional activity and binding of the estrogen receptor to its responsive element. *Biochemistry* 41:2760-2768, 2002

158. Massaad-Massade L, Tacine R, Dulauroy S, Reeves R, Barouki R: The functional interaction between HMGA1 and the estrogen receptor requires either the N- or the C-terminal domain of the receptor. *FEBS Lett* 559:89-95, 2004

159. Rodriguez AE: Emerging drugs for coronary restenosis: the role of systemic oral agents the in stent era. *Expert Opin Emerg Drugs* 14:561-576, 2009

160. Farooq V, Gogas BD, Serruys PW: Restenosis: delineating the numerous causes of drug-eluting stent restenosis. *Circ Cardiovasc Interv* 4:195-205, 2011

161. Bostrom P, Wu J, Jedrychowski MP, Korde A, Ye L, Lo JC, Rasbach KA, Bostrom EA, Choi JH, Long JZ, Kajimura S, Zingaretti MC, Vind BF, Tu H, Cinti S, Hojlund K, Gygi SP,

Spiegelman BM: A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. *Nature* 481:463-468, 2012

162. Raschke S, Eckel J: Adipo-myokines: two sides of the same coin--mediators of inflammation and mediators of exercise. *Mediators Inflamm* 2013:320724, 2013

163. Raschke S, Elsen M, Gassenhuber H, Sommerfeld M, Schwahn U, Brockmann B, Jung R, Wisloff U, Tjonna AE, Raastad T, Hallen J, Norheim F, Drevon CA, Romacho T, Eckardt K, Eckel J: Evidence against a Beneficial Effect of Irisin in Humans. *PLoS One* 8:e73680, 2013

164. Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH, Khandekar M, Virtanen KA, Nuutila P, Schaart G, Huang K, Tu H, van Marken Lichtenbelt WD, Hoeks J, Enerback S, Schrauwen P, Spiegelman BM: Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* 150:366-376, 2012

8   Acknowledgements

First of all I would like to thank my family – Sven, Julia and Parents-in-Law Ursula and Werner for the tremendous support, love, understanding und so much more…through all the years.

I would like to express my deep gratitude to my mentor Prof. Ulrich Kintscher for giving me an opportunity to work in his laboratory on several challenging projects. I am greatly thankful for his exceptional scientific supervision, enthusiasm and constant encouragement to develop independent ideas and projects throughout my research work.

I would like to thank Prof. Thomas Unger for the constant interest in my work, fruitful discussions and support through that work.

I own my deepest gratitude to my close colleagues Verena Benz, Mandy Bloch and Markus Clemenz. My sincere thanks also go to Elena Kaschina and Christa Thöne-Reineke for their encouragement and help. I would like to express my deepest thanks and appreciation to Christiane Sprang and Beata Höft. Thank you all so much for the instant support and friendship.

Serdecznie podziekowania przesylam dla Pani Miarndy Schröder za nieustajaca pomoc i oparcie w najrozniejszych malych i duzych problemach. Dziekuje!

I would like to express my gratitude to all members of the "Kintscher Fat Group", and all colleagues form the Institute of Pharmacology for the helpful discussions, great atmosphere and enthusiasm.

This work was almost exclusively supported by the DFG. Thank you.

I would like to dedicate my work "*in memoriam* "my parents Ewa and Juliusz Foryst.

Statement

<div style="text-align:center">

Erklärung

§ 4 Abs 3(k) der HabOMed der Charité

</div>

Hiermit erkläre ich, dass

- weder früher noch gleichzeitig ein Habilitationsverfahren angemeldet noch durchgeführt wurde
- die vorgelegte Habilitationsschrift ohne fremde Hilfe verfasst, die beschriebenen Ergebnisse selbst gewonnen sowie die verwendeten Hilfsmittel, die Zusammenarbeit mit anderen Wissenschaftlern/
Wissenschaftlerinnen und mit technischen Hilfskräften sowie die verwendete Literatur vollständig in der Habilitationsschrift angegeben wurden
- mir die geltende Habilitationsordnung bekannt ist

Datum   Unterschrift