Dissertation

Impact of nitric oxide on thyroidal dysfunctions and related metabolic disorders

submittet by

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STATUTORY DECLARATION

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”.

Graz, 2013-07-04 .....

FOREWORD

This thesis is an extended script of the following original publication on the topic ‘Impact of nitric oxide on thyroidal dysfunctions and related metabolic disorders’:

Ragginer, C; Bernecker. C; Ainoedhofer, H; Pailer, S; Kieslinger, P; Truschnig-Wilders, M; Gruber, HJ. Treatment with the Nitric Oxide Donor SNP Increases Triiodothyronine Levels in Hyper- and Hypothyroid Sprague Dawley Rats. Hormone and Metabolic Research [ahead of print]

The studies for this thesis were performed from 2010 to 2013 at the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz under the supervision of Priv. - Doz. Dr. Hans-Jürgen Gruber.

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ABBREVIATIONS AND DEFINITIONS

ADMA	asymmetric dimethylarginine
Akt/PkB	serin/threonine protein kinase, protein kinase B (PkB)
CaM	calmodulin
cGMP	cyclic guanosine monophosphate
CRP	C-reactive protein
DIT	diiodotyrosine
DIO2	deiodinase type 2
eNOS	endothelial NOS
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GTF	general transcription factors
GTP	guanosine triphosphate
HFD	high-fat diet
HYPER	hyperthyroid
HYPO	hypothyroid
iNOS	inducible NOS
i.p.	intraperitoneal
LDL	low density lipoprotein
mTOR	mammalian target of rapamycin
MAPK	mitogen activated protein kinase
MCT8	monocarboxylate transporter 8
MeS	metabolic syndrome
MIT	monoiodotyrosine
NADPH	nicotinamide adenine dinucleotide phosphate
NIS	sodium-iodide symporter
nNOS	neuronal NOS
NO	nitric oxide
NOS	NO synthase
NOX	serum nitrogen oxides
OATPC1	organic anion transporter polypeptide C1
oxLDL	oxidized low density lipoproteins
ROS	reactive oxygen species

RXR	retinoid-X receptor
SD	Sprague Dawley
sGC	soluble guanylate cyclase
SNP	sodiumnitroprusside
T3	triiodothyronine
T4	thyroxine
TG	triglycerides
TG	thyroglobulin
TPO	thyroperoxidase
THRα	thyroid hormone receptor α
THRβ	thyroid hormone receptor β
TRs	thyroid hormone receptors
TRE	thyroid hormone response element
TRH	thyrotropin-releasing hormone
TRHR	thyrotropin-releasing hormone receptor
TSH	thyroid-stimulating hormone
TSHR	thyroid-stimulating hormone receptor
VSMC	vascular smooth muscle cells

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PUBLISHED CONTRIBUTIONS OF THIS STUDY

Parts of this thesis have been already accepted for publication:

Full papers/articles (Journal)

Ragginer, C; Bernecker. C; Ainoedhofer, H; Pailer, S; Kieslinger, P; Truschnig-Wilders, M; Gruber, HJ. Treatment with the Nitric Oxide Donor SNP Increases Triiodothyronine Levels in Hyper- and Hypothyroid Sprague Dawley Rats. Hormone and Metabolic Research [ahead of print]

Presentations at scientific conferences

Ragginer, C; Bernecker, C; Ainoedhofer, H; Kieslinger, P; Gruber, HJ; Truschnig-Wilders, M. In vivo model demonstrating an association between thyroid-diseases, NO pathway and metabolic dysfunctions. Supplement Wiener Klinische Wochenschrift. 2012; 01(12):11-12.-40. Jahrestagung der Österreichischen Diabetes Gesellschaft; NOV 15-17, 2012; Salzburg, AUSTRIA. [Oral Communication]

Ragginer, C; Bernecker, C; Ainoedhofer, H; Pailer, S; Kieslinger, P; Truschnig-Wilders, M; Gruber, HJ. Thyroidal dysfunctions reduce the bioavailability of nitric oxide which contributes to atherogenesis in Sprague Dawley rats. 80th European Atherosclerosis Society Congress; MAY 25-28, 2012; Milan, ITALY. [Poster]

Ragginer, C; Bernecker, C; Ainoedhofer, H; Pailer, S; Kieslinger, P; Truschnig-Wilders, M; Gruber, HJ. Thyroid dysfunctions have an impact on oxLDL levels in Sprague Dawley rats. Doctoral Day 2012, Medical University Graz ; DEZ 7, 2012; Graz, AUSTRIA. 2012. [Oral Communication]

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ABSTRACT IN GERMAN

Ziel: Stickstoffmonoxid (NO) ist ein pleiotropes, bioaktives Effektormolekül und könnte eine bedeutende Rolle in der Pathophysiologie von thyroidalen Dysfunktionen spielen. Ziel der vorliegenden Studie war es, die Bedeutung von NO in hypothyroiden (HYPO) und hyperthyroiden (HYPER) Sprague Dawley Ratten unter Berücksichtigung möglicher Einflussfaktoren wie Ernährung zu bewerten. Weiters sollten die möglicherweise damit einhergehenden Auswirkungen von Stickstoffoxiden und Reaktiven Sauerstoffspezies auf den Glucose- / Insulinstoffwechsel und Lipidstoffwechsel im Zuge dessen analysiert werden. Darüber hinaus wurden die Auswirkungen des Stickstoffmonoxid-Donors Natrium-Nitroprussid auf den HYPO und HYPER Status untersucht.

Methoden: Hundertsieben weibliche Sprague Dawley Ratten wurden entweder einer Gruppe die herkömmliches Futter erhielt zugeordnet oder einer die eine hochkalorische, fettreiche Diät (HFD) erhielt. Weiters wurden die Tiere in eine Kontrollgruppe, HYPO, HYPER und entsprechende SNP behandelte Gruppe unterteilt. Die Hypothyreose wurde über Propylthiouracilgabe, die Hyperthyreose über Trijodthyroninverabreichung induziert. Nach zwölfwöchiger Behandlung wurden die Serum Stickoxide (NOX), endogenes asymmetrisches Dimethylarginin (ADMA), Serum Trijodthyronin (T3), metabolische Parameter wie Glucose, Insulin, oxidierte Lipoproteine niederer Dichte (oxLDL), Serum Triglyzeride (TG), C-reaktives Protein (CRP), das Körpergewicht sowie die Kalorienaufnahme analysiert.

Resultate: Die HYPO Ratten zeigten verringerte Serum T3 Konzentrationen, die HYPER Ratten erhöhte T3 Levels im Vergleich zur Kontrollgruppe. Die Diät hatte keinen Einfluss auf die T3 Konzentration. Die thyroidalen Dysfunktionen manifestierten sich in Verhaltensänderungen in Bezug auf die Kalorienaufnahme. HYPO und HYPER Status manifestierten sich deutlich in Hinblick auf das Körpergewicht. Serum NOX war in der herkömmlichen Diätgruppe sowohl in der HYPO als auch in der HYPER Gruppe sowie in der Gruppe der HFD HYPER Ratten reduziert. Durch Behandlung der Tiere mit SNP wurde diese Abnahme deutlich kompensiert. Zusätzlich stiegen in diesen Gruppen die T3 Konzentrationen an. Der NO-Synthase Inhibitor ADMA war in der HFD Kontrollgruppe signifikant höher als in der normalen Kontrollgruppe mit

herkömmlicher Diät. ADMA war in beiden HYPO Gruppen erniedrigt und in der HYPER Gruppe mit herkömmlicher Diät erhöht.

Betreffend des Glucose- und Insulinmetabolismus fanden wir signifikant erhöhte Glucose Werte in der HFD HYPO Gruppe, hingegen waren die Serum Insulin Werte signifikant erniedrigt, unabhängig der Diät. Die Hyperthyreose hatte keinen Einfluss auf den Insulin- und Glucosemetabolismus. Die Bestimmung der TG und oxLDL Levels zeigte, dass beide Parameter in den HYPER Ratten erhöht waren. Die Gabe von SNP kehrte diesen Effekt teilweise um. In HYPO Ratten, unabhängig der Diät, waren die Serum TG Levels ähnlich denen der Kontrollgruppen und oxLDL war signifikant erniedrigt. Die Behandlung der normal Diät HYPOs mit SNP kehrte diese Erniedrigung um. Weiters zeigten Regressionsanalysen eine hochsignifikante Assoziation von oxLDL und NOX in der normal Diät Kontrollgruppe auf.

Fazit: Es konnte gezeigt werden, dass eine Assoziation zwischen thyroidalen Dysfunktionen und eingeschränkter Bioverfügbarkeit von NO und damit einhergehender Änderung der Serum ADMA Konzentrationen besteht. Die Gabe des NO-Donors Natrium-Nitroprussid führte zu einer Erhöhung der Serum T3 Konzentrationen. Weiters zeigte sich, dass NO mit metabolischen Risikofaktoren wie oxLDL assoziiert ist. Die vorliegenden Daten zeigen, dass der NO-Pathway in die Pathophysiologie von Hypothyroidismus und Hyperthyroidismus involviert ist. Diese Ergebnisse könnten in Hinblick auf therapeutische Ansätze von klinischer Relevanz sein.

ABSTRACT IN ENGLISH

Objective: Nitric oxide pathway might play a crucial role in the pathophysiology of thyroid dysfunctions. This study aimed to investigate the impact of nitric oxide (NO) on hypothyroid (HYPO) and hyperthyroid (HYPER) Sprague Dawley rats and its associations with metabolic dysfunctions under controlled diet. Furthermore, the effects of the nitric oxide donor sodiumnitroprusside (SNP) on thyroid dysfunctions were also assessed.

Methods: Sprague Dawley rats (n=107) were subdivided into normal diet and high-fat diet (HFD) groups and grouped into controls, HYPO, HYPER and SNP treated groups. Hypothyroidism was induced through propylthiouracil, whereas hyperthyroidism by triiodothyronine (T3). After 12 weeks of T3 treatment, serum nitrogen oxides (NOX), endogenous asymmetric dimethylarginine (ADMA), metabolic parameters like glucose, insulin, oxidized low density lipoproteins (oxLDL) and serum triglycerides (TG), C-reactive protein (CRP), body weight and food intake were analysed.

Results: HYPO rats showed decreased serum T3 levels, HYPER rats increased T3 compared to controls. Diet had no impact on T3. Thyroid dysfunctions were accompanied by changes in calorie intake and body weight. Serum NOX was significantly reduced in normal diet HYPO rats. SNP administration compensated the decrease and markedly increased T3. NO synthase inhibitor ADMA levels were significantly higher in the HFD control group than in the normal diet controls. ADMA was declined in both HYPO groups and increased in normal diet HYPER rats. Regarding the glucose- and insulin metabolism we found significantly increased glucose levels in HFD HYPOs whereas serum insulin was significantly decreased in HYPOs regardless of diet. Hyperthyroidism did not affect the glucose- and insulin metabolism. Next, measurement of TG and oxLDL showed that both parameters were increased in HYPER rats, treatment with SNP partially reversed this effect. In HYPOs, regardless of diet, serum TG levels were similar to controls and oxLDL was significantly decreased. SNP treatment of normal diet HYPOs reversed this decrease. Moreover, regression analyses revealed a highly significant association of oxLDL and NOX in the normal diet controls.

Conclusions: An association of thyroid dysfunctions with reduced bioavailability of NO and alterations of ADMA levels could be established. Treatment with the NO

donor SNP resulted in an increase of serum T3 levels. Moreover, NO is associated with metabolic risk factors like oxLDL. The present results demonstrate that the NO pathway is implicated in thyroid dysfunctions, which may be of clinical relevance.

1. INTRODUCTION

1.1. OVERVIEW ON NITRIC OXIDE (NO)

1.1.1. NITRIC OXIDE PRODUCTION

The pleiotropic and ubiquitous nature of nitric oxide (NO) results in a variety of physiological and pathophysiological effects. NO is a short-lived radical gas molecule that exerts important cell signalling function. It is the smallest known bioactive product of mammalian cells and can be synthesized by most cell types. The vast majority of NO is synthesized enzymatically from the amino acid L-arginine by NO synthases (NOS) through a series of redox reactions with degradation of L-arginine to L-citrulline and NO. NOS activity is dependent upon arginine and oxygen availability as well as upon the presence of sufficient cofactors (nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD)). There are three quite distinct NOS isoforms which are the products of different genes, with different regulation including transcription, translation, post-translation, with different catalytic properties and different localization: endothelial NOS (eNOS, NOS-3), inducible NOS (iNOS, NOS-2) and neuronal NOS (nNOS, NOS-1).

The eNOS and nNOS are constitutive enzymes that are controlled by intracellular Ca^{2+}/calmodulin, whereas the iNOS is inducible at the level of gene transcription and posses a calmodulin (CaM) subunit and is thus Ca^{2+} independent. In terms of enzymatic activity, constitutive NOS rapidly provide short bursts of NO whereas the inducible isoform displays a significant latency period due to transcriptional and translational processing and is permanently active for extended periods of time [4]. Figure 1 illustrates the overall reaction that is catalyzed by the NOS class of enzymes including cofactors.

In addition to the well established NOS/L-arginine signalling pathway, NO is generated nonenzymatically under a variety of physiological and pathophysiological conditions from sources like e.g. L-Arginine, D-Arginine or nitrite [5,6]. Nitrite is a major metabolic product of NO and is found in all cell and tissue types that utilize NO signalling processes [4]. The non-enzymatic production

of NO mainly occurs in tissues under acidic conditions: $e^- + 2H^+ + NO_2^- \rightarrow NO + H_2O$ [7]. Table 1 outlines various possible reactions of NO and reactive nitrogen species.

Table 1: NO chemistry and reactions	
1.	$^{\bullet}NO + O_2^{\bullet-} \rightarrow ONOO^-$
2	$^{\bullet}NO + HbO_2 \rightarrow MetHb + NO_3^-$
3.	$^{\bullet}NO + O_2 \leftrightarrow ONOO^{\bullet}$
4.	$ONOO^{\bullet} + {}^{\bullet}NO \leftrightarrow ONOONO$
5.	$ONOONO \rightarrow 2\ {}^{\bullet}NO_2$
6.	$NO_2 + {}^{\bullet}NO \leftrightarrow N_2O_3$
7.	$N_2O_3 + H_2O \rightarrow 2\ NO_2^- + 2\ H^+$
8.	$ONOO^- + H_3O^+ \leftrightarrow ONOOH + H_2O$
9.	$ONOO^- + CO_2 \leftrightarrow ONOOCO_2^-$
10.	$ONOO^- + Mn(III) \leftrightarrow Mn(III) — (ONOO^-)$
11.	$ONOOH + RS^- \rightarrow RSOH + NO_2^-$
12.	$ONOOH \rightarrow 0.3\ ({}^{\bullet}NO_2 + {}^{\bullet}OH) + 0.7\ (NO_3^- + H^+)$
13.	$ONOOCO_2 \rightarrow 0.35\ ({}^{\bullet}NO_2 + CO_3^{\bullet-}) + 0.65\ (NO_3^- + CO_2)$
14.	$Mn(III) — (ONOO^-) \rightarrow {}^{\bullet}NO_2 + Mn(IV) = O$
15.	$2\ {}^{\bullet}NO_2 \leftrightarrow N_2O_4$
16.	$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^-$
Nitric oxide ($^{\bullet}NO$) reacts with paramagnetic species like other radicals or metal centers. The reaction with dioxygen (O_2; $^{\bullet}NO$ autooxidation) yields to nitrogen dioxide ($^{\bullet}NO_2$). Nitric oxide autoxidation yields to nitrous anhydride (N_2O_3) as intermediate, nitrite (NO_2^-) is the major final product in water.	

Adapted by L.J.Ignarro, Nitric Oxide Biology and Pathobiology, 2nd ed. Elsevier Inc., 2010, pp.28-29 [8]

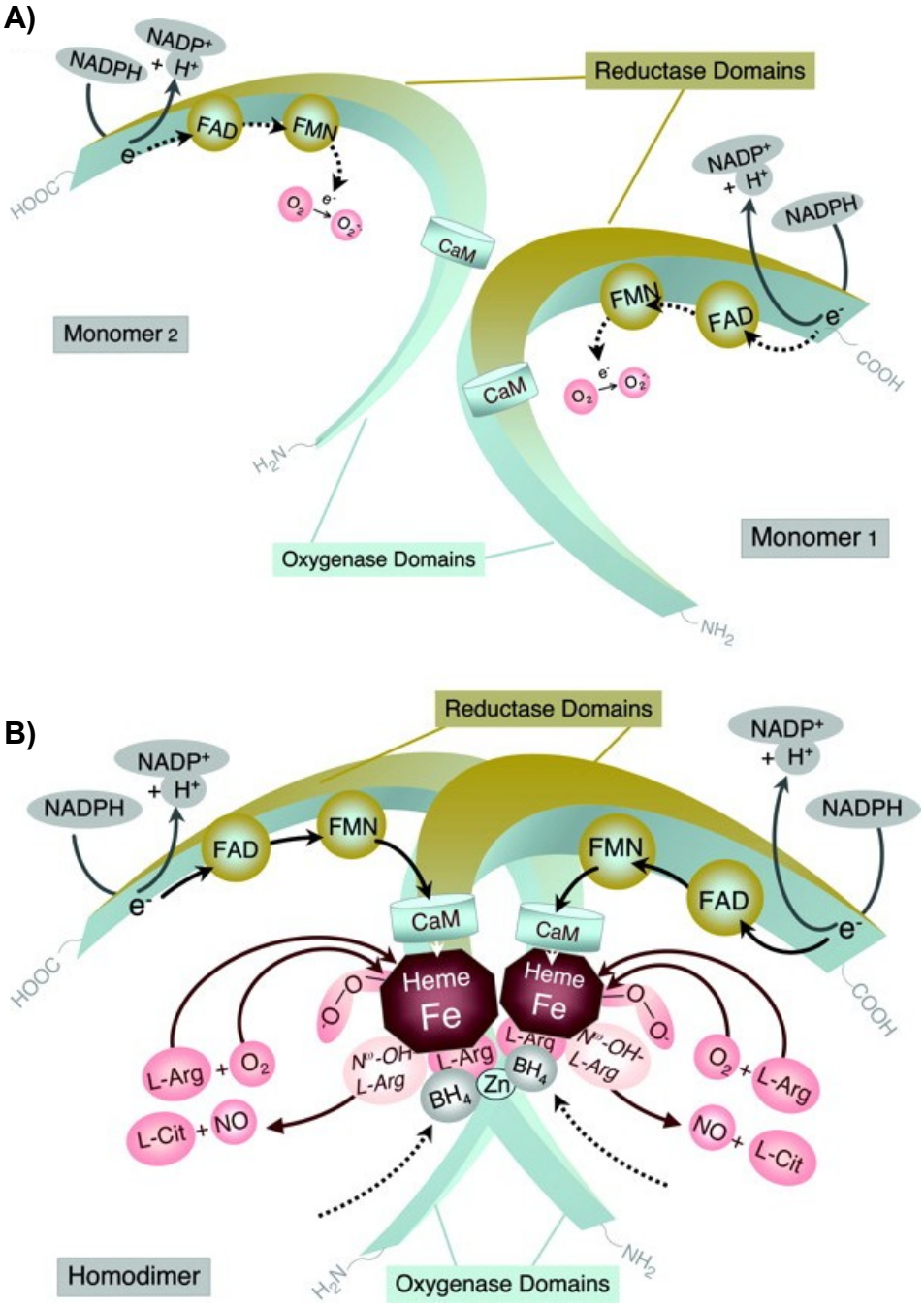

Figure 1: eNOS schematic structure. A) eNOS monomer schematic structure. All NOS isoforms are homodimers, consisting of monomers each holding a reductase- and an oxygenase domain. Reductase domains accomplish electron transfer from NADPH to the FAD and FMN and can reduce oxygen to O_2^-. Reductase domains can bind CaM, as stimulator of electron transfer within the reductase domain. Monomers are unable to bind BH_4 and L-arginine and thus cannot catalyze NO production. B) eNOS dimer schematic structure. Heme is essential for the formation of the active NOS holoenzyme. Heme is involved in enzyme dimerization, the interaction between reductase and oxygenase domains and the interdomain electron transfer from the flavins to the heme of the opposite monomer. In the presence of L-arginine and BH_4, NOS dimers couple their heme and O_2 reduction to the synthesis of NO. L-citrulline is formed as by-product. (NADPH nicotinamide adenine dinucleotide phosphate, FAD flavin adenine dinucleotide, FMN flavin mononucleotide, CaM calmodulin). Adapted from Förstermann et al. 2006 [1].

1.1.2. ENDOTHELIAL NITRIC OXIDE SYNTHASE (eNOS) UNCOUPLING

Uncoupling of the endothelial nitric oxide synthase (eNOS) leads to formation of superoxide anions (O_2^-) instead of nitric oxide (NO). This impairment of NO biosynthesis might be a crucial factor in the pathogenesis of cardiovascular disease [9]. Various studies have shown that eNOS uncoupling is implicated in endothelial dysfunction and associated diseases like diabetes, athersoclerosis, hypertension, vascular thrombosis, and enhanced leucocyte–endothelial cell interactions [10,11].

A prominent cause of NOS uncoupling is oxidative stress which is the imbalance between the activity of endogenous pro- oxidative enzymes and anti-oxidative enzymes in favour of the former. Pro- oxidative enzymes encompass NADPH oxidase, xanthine oxidase or the mitochondrial respiratory chain. Anti-oxidative enzymes comprise superoxide dismutase, glutathione peroxidase, heme oxygenase, thioredoxin peroxidase / peroxiredoxin, catalase and paraoxonase [9,12]. Previous data have identified different mechanisms of NOS uncoupling, all of which were involved in various cardiovascular diseases [13]. It has been shown that phosphorylation of eNOS plays a role in NOS uncoupling however the exact mechanism remains unclear to date and additionally seems to be dependent upon other factors as well [14-18].

Furthermore, NOS uncoupling is caused by the reduction of the essential eNOS cofactor BH_4 [19]. BH_4 is synthesized either de novo via guanosine triphosphate (GTP) to tetrahydrobiopterin via the GTP cyclohydrolase I, 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase enzymes or it is built via a ‘salvage pathway’ with sepiapterin as intermediate. Thus, a lack of GTP as well as down-regulation of GTP cyclohydrolase I constitute limiting factors for BH_4 bioavailability [10].

In addition, prolonged oxidative stress may cause a decline of BH_4 levels and thus propagate NOS uncoupling. Reason therefore is that superoxide (O_2^-) reacts with NO to peroxynitrite ($ONOO^-$) which rapidly oxidizes BH_4 to BH_2, thus destabilizing the NOS dimer, leading to NOS uncoupling [20].

Furthermore, NOS uncoupling can be caused directly by $ONOO^-$ itself destabilizing the NOS dimer due to a zinc release from the zinc-thiolate cluster.
Another NOS uncoupling mechanism might be via NOS s-glutathionylation, a post translational modification that is more common upon oxidative stress [21]. Furthermore, contribution of endogenous asymmetric dimethylarginine (ADMA) in eNOS uncoupling is thinkable [1,22,23]. Figure 2 schematically illustrates the overall process of eNOS uncoupling.
Several approaches to prevent eNOS uncoupling or to further recoupling of eNOS have been performed. In detail, BH_4 supplementation [24-26] as well as folic acid [10,27,28] and vitamin C administration [29,30] have been tested for their clinical efficacy in treating, cardiovascular disease like endothelial dysfunction, atherosclerosis, hypercholesterolemia and diabetes mellitus. To date, the results of these clinical approaches are only in part satisfying, indicating that eNOS uncoupling/recoupling constitute quite complex mechanisms. Consideration of further biochemical determinants of NOS uncoupling, including e.g. cellular concentrations of L-arginine, methylarginines, and oxidized glutathione might be of relevance [1,10,20].

1.1.3. PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL IMPLICATIONS OF NO

NO plays a pivotal role in a variety of physiological processes throughout the whole human body [8]. The soluble guanylate cyclase (sGC) constitutes the principal receptor for NO [31]. Activation of sGC occurs via binding of NO to the heme moiety of the receptor, resulting in increased cyclic guanosine monophosphate (cGMP) levels. In vasculature, NO controls vasodilatation via cGMP mediated relaxation of vascular smooth muscle cells (VSMC) [32,33].
Thus, adequate levels of NO are important to preserve a normal vascular physiology, including e.g. control of vascular tone, blood pressure, hemostasis, attenuation of inflammation, inhibition of platelet adhesion. From two decades of NO research, it has become clear, that the diversity of the implications of NO, which appears to be a dichotomous molecule, is highly dependent upon its concentration. Many pathophysiological conditions underly a NO insufficiency either due to an impaired release (impaired synthesis or impaired availability of bioactive NO) or an enhanced degradation e.g. due to reactive oxygen species (ROS). ROS act as NO scavengers [32]. Independent of its biochemical basis, NO

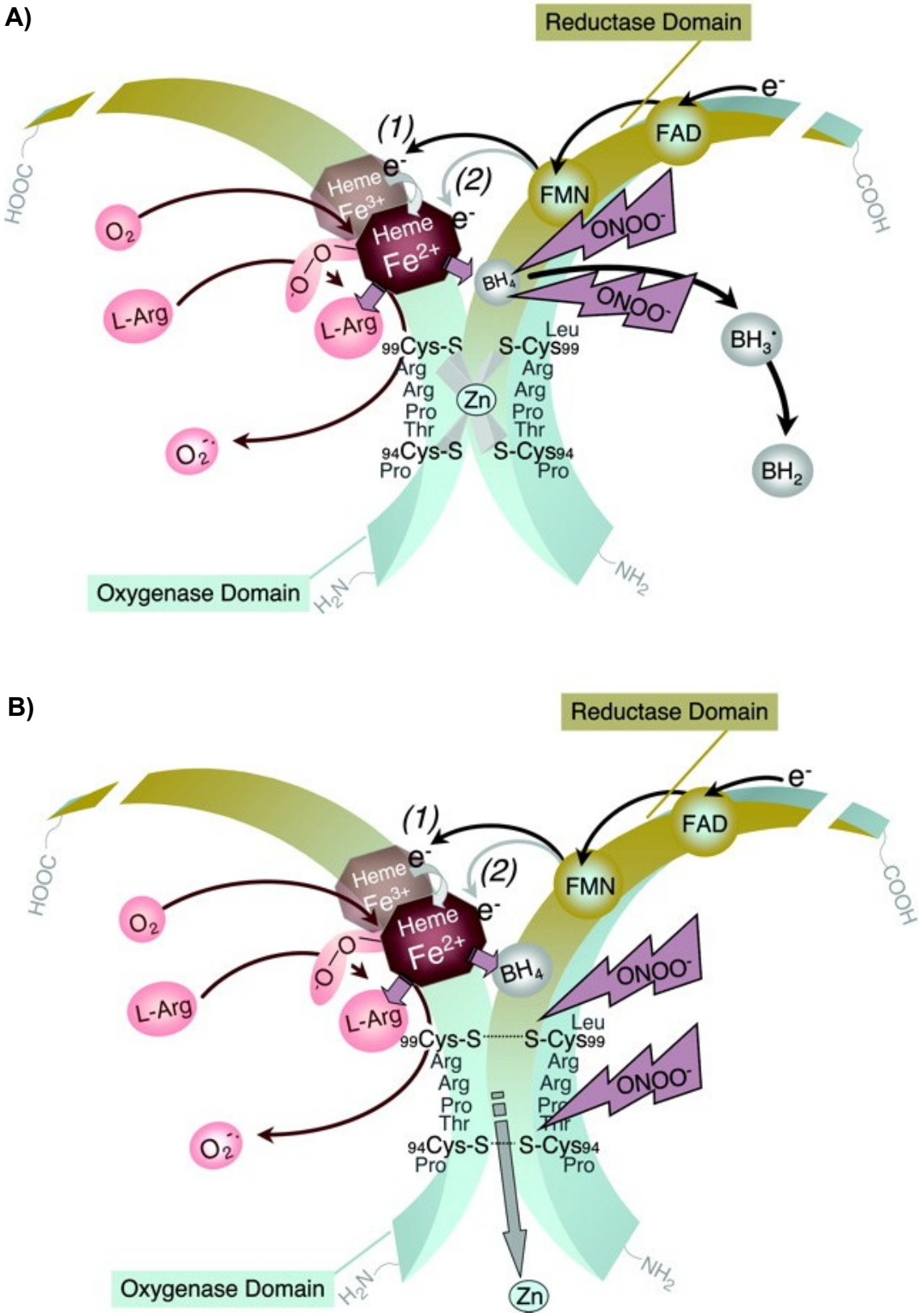

Figure 2: Uncoupling of eNOS. A) Increased formation of $ONOO^-$ leads to oxidation of BH_4 biologically inactive BH_3 radical or BH_2 therby causing uncoupling of NOS. B) Increased formation of $ONOO^-$ may further lead to oxidation of the zinc-thiolate cluster of eNOS, causing loss of zinc and uncoupling of NOS. Adapted from Förstermann et al. 2006 [1].

deprivation limits NO-mediated signal transduction of physiological and protective processes [34]. Endothelial dysfunctions are associated with NO deficiency and are implicated in the underlying pathophysiologies of several diseases [35].

It is substantially delineated that diminishing NO bioavailability causes endothelial dysfunction and vascular damage [35-37]. Several studies have implicated endothelial dysfuntions and a dysfunctional NO-pathway as the most common pathogenic issue in atherosclerosis [38,39].

Furthermore, NO is described to contribute to various pathologic conditions like inflammation, oxidative stress, cardiovascular disease, stroke and metabolic disorders [40-46].

1.2. OVERVIEW ON THYROID HORMONES AND THYROID DISEASES

1.2.1 PRODUCTION AND REGULATION OF THYROID HORMONES

Thyroid hormones play a key role in energy homeostasis, metabolism, growth and other hormone systems. In fact, thyroid hormones are required for the physiological function of nearly all tissues, having important effects on oxygen consumption and metabolic rates [47].

The thyroid hormones thyroxine (T4) and triiodothyronine (T3) are produced and secreted by the follicular cells of the thyroid gland. Thyroid hormone regulation, including the processes of production and secretion, upon the hypothalamus-pituitary-thyroid (HPT) axis, involves two further hormones, the thyrotropin-releasing hormone (TRH) and the thyroid-stimulating hormone (TSH). The HPT-axis constitutes an exquisitely regulated negative feedback control system. Furthermore, somatostatin, steroid hormones and excessively high blood iodide concentrations are implicated in the complex network of thyroid hormone regulation [48-50]. An overview of thyroid hormone production, storage, secretion and regulation is illustrated in Figure 3.

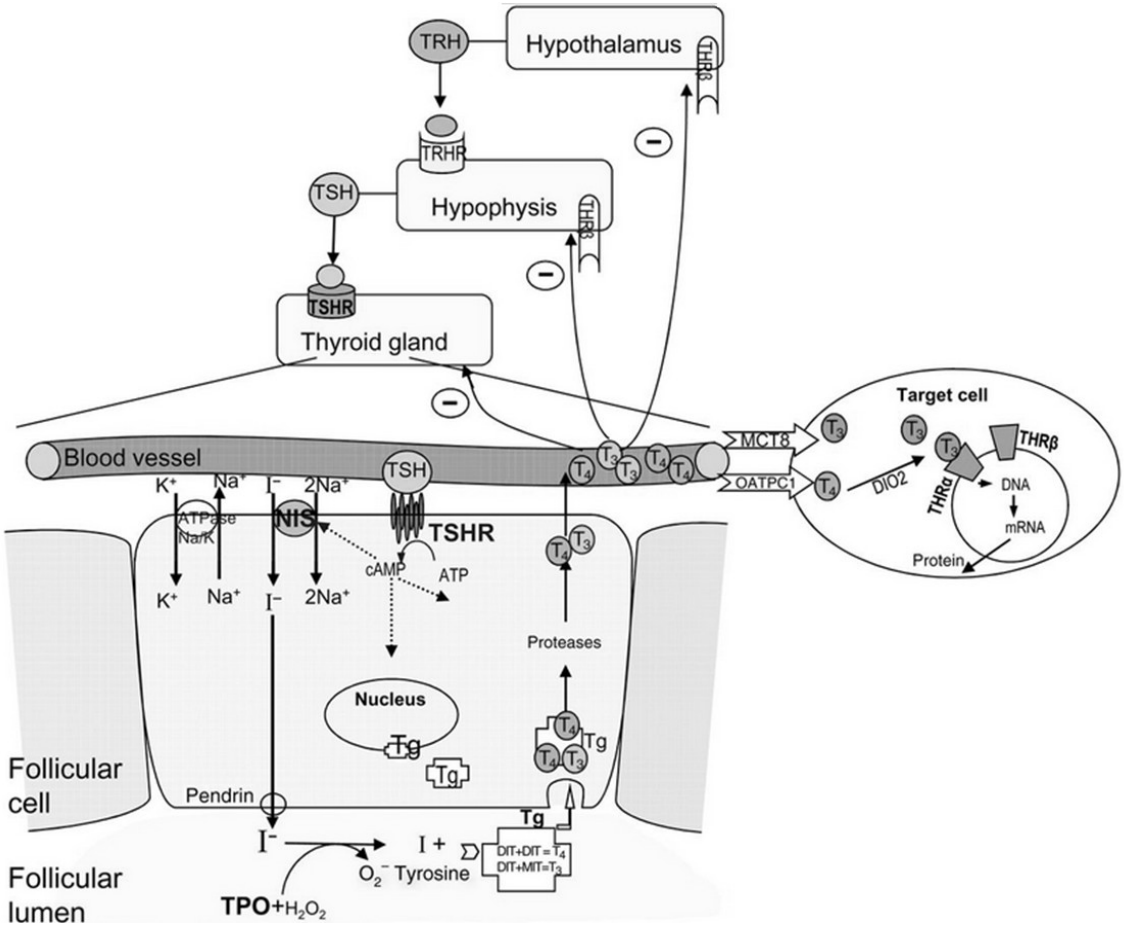

Figure 3: Schematic overview of the thyroid hormone production and regulation via the hypothalamus–pituitary–thyroid axis. (TRH, thyrotropin-releasing hormone; TRHR, thyrotropin-releasing hormone receptor; TSH, thyroid-stimulating hormone; TSHR, thyroid-stimulating hormone receptor; NIS, sodium-iodide symporter; TPO, thyroperoxidase; TG, thyroglobulin; DIT, diiodotyrosine; MIT, monoiodotyrosine; T3, triiodothyronine; T4, thyroxine; MCT8, monocarboxylate transporter 8; OATPC1, organic anion transporter polypeptide C1; DIO2, deiodinase type 2; THRα, thyroid hormone receptor α; THRβ, thyroid hormone receptor β). Adapted from Pastor et al. 2012 [2].

1.2.2. THYROID HORMONE FUNCTION

Thyroid hormone function is either mediated through genomic or non-genomic signal pathways. However, in both cases thyroid hormones have to enter cells to exert their function. In the first case, thyroid hormones bind to thyroid hormone receptors (TRs) in the nucleus. TRs are T3-inducible transcription factors which belong to the nuclear hormone receptor superfamily. The expression of TRs, of both isoforms TRα and TRβ, is tissue-dependent and tissue-development-dependent [2]. TRs regocnize and bind thyroid hormone response elements

(TREs). TREs are located in promoter regions of T3 target genes, causing positive or negative regulation of these genes and subsequently regulate protein synthesis. Furthermore, TRs can form complexes with other nuclear co-regulatory proteins, such as corepressors and coactivators, thus regulating histone acylation and basal transcription. Figure 4 (1) gives an overview on the genomic singnal pathway of thyroid hormones.

Secondly, many important physiological effects of thyroid hormones are mediated through TRs beyond TRE-mediated gene transcription, Figure 4 (2). This is referred to as non-genomic action of thyroid hormones and can be initiated at the plasma membrane, in cytoplasm, and at the mitochondria. Thyroid hormones use various receptors and signalling systems which allow them to regulate multiple cellular functions like e.g. Ca^{2+} entry, intracellular protein trafficking and regulation of protein kinases [51].

One example for a non-gemomic action is the effect of thyroid hormones on the phosphatidylinositol 3-kinase (PI3-kinase)_protein kinase Akt pathway. This pathway is an important regulator of cellular growth, metabolism, and cell survival. TRs activate the PI3-kinase_Akt pathway which leads to a downstream signalling cascade. Results are e.g. the activation of the mammalian target of rapamycin (mTOR) and its substrates or the activation of serine/threonine kinase Akt which again activates the eNOS. This gives reason to speculate that some of the hemodynamic effects of thyroid hormones might be attributed to eNOS activation and synthesis of NO [52]. A further example is the activation of the mitogen activated protein kinase (MAPK) pathway by thyroid hormones. T3 activates the pathway without entering cells, by binding to integrin aVb3. This triggers another signal transduction cascade via phosphorylation of nuclear receptors and can induce angiogenesis and promote cell growth [3,51-54].
Summerizing, thyroid hormones are involved in genomic and non-genomic pathways with noteworthy physiological impacts. Thus, they effect lipid metabolism [55,56], the nervous system [57,58], other endocrine glands [32] and exert calorigenic effects [59].

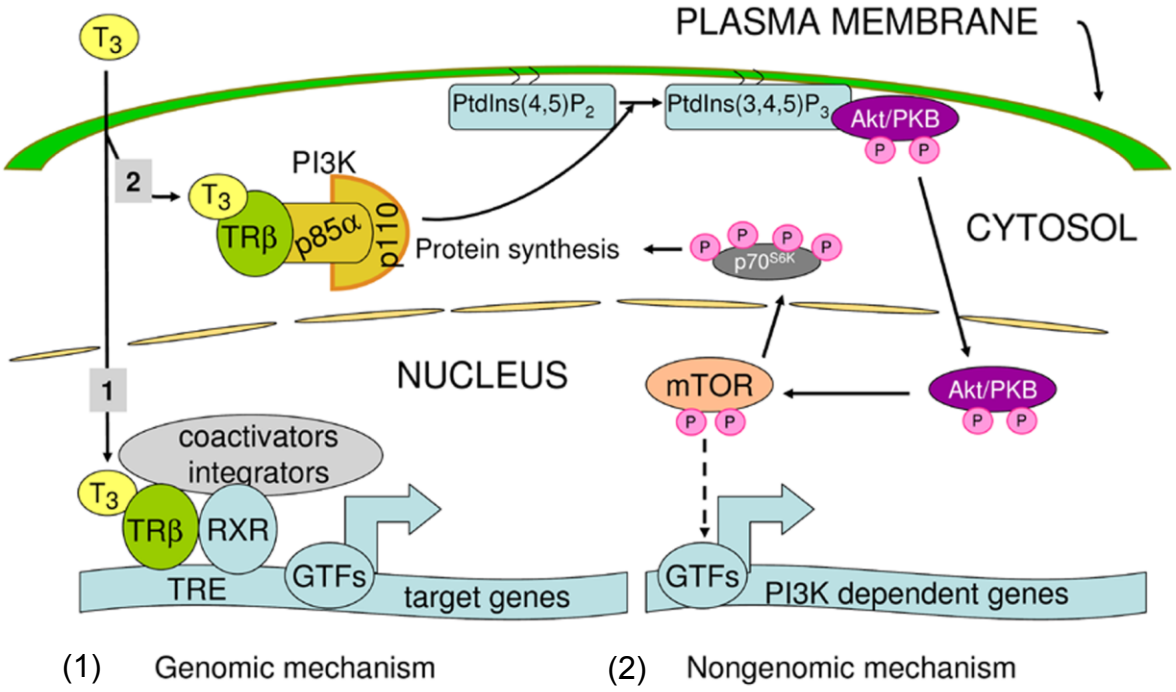

Figure 4: Thyroid hormone (T3) action. (1) Genomic action of thyroid hormones. (2) Non-genomic action of thyroid hormones. (T3, triiodothyronine; TR β, thyroid hormone receptor; GTF: general transcription factors; TRE, thyroid hormone response element; RXR, retinoid-X receptor; Akt/PkB, serin/threonine protein kinase, also known as protein kinase B (PkB)). Adapted from Moeller et al. 2006 [3].

1.2.3. THYROID DISEASES

According to principal classification guidelines, thyroid function is grouped into euthyroidism, hypothyroidism and hyperthyroidism [60]. Thyroid diseases comprise various causes and pathophysiologies, thus a differentiation between direct dysfunctions of the thyroid gland, thyroid dysfunction due to extrathyroidal factors like autoimmune diseases, and thyroid hormone insufficiency including abnormalities of target tissues is useful.

Euthyroidism, under physiological conditions, comprises the normal production of thyroid hormones by the thyroid gland. Euthyroidism, under pathophysiological conditions, specifies diseases like euthyroid goiter, benign and malign thyroid gland tumors as well as acute and subacute thyroiditis, chronic autoimmune thyroiditis or Hashimoto´s disease in the euthyroid phase [61].

Hypothyroidism refers to a pathologic condition that is generally characterized by low circulating levels of the thyroid hormones T3 and T4 as well as elevated levels

of TSH [60]. Hypothyroidism is further subdivided into primary and secondary and tertiary hypothyroidism due to its etiology [60]. Primary hypothyroidism affects the thyroid gland resulting most commonly in Hashimoto's thyroiditis and autoimmune diseases. Secondary hypothyroidism affects the pituitary gland, involving a lack of TSH production necessary for T4 and T3 synthesis. Tertiary hypothyroidism applies for a failure of the hypothalamus to produce TRH [62]. Thus, the disorders of the thyroid hormone production are either due to structural or functional changes of the thyroid gland or malregulation of the HPT axis.

Hyperthyroidism is characterized by pathological high tissue thyroid hormone levels and can be divided into thyroid gland hyperthyroidism, thyrotoxicosis and transient hyperthyroidism [60,63]. The most common cause of hyperthyroidism is Graves's disease (hyperthyroid goiter) [64]. Thyroid gland hyperthyroidism includes diseases like hyperthyroid goiter, Basedow-Graves´ disease, Plummer´s disease, Hashitoxicosis, inborn resistance of the pituitary gland to thyroid hormones and tumors that lead to secretion of TSH [65,66]. Table 2 gives an abridged overview on the classification of thyroid diseases.

Table 2: Short classification of thyroid diseases
I. Diseases characterized by (tissue) euthyroidism
A. Euthyroid goiter
1. Diffuse (chronic), 2. Nodular (chronic), 3. Diffuse (transient)
B. Tumors
1. Benign (single nodule), 2. Malignant: a. Differentiated (papillary and follicular), b. Undifferentiated (anaplastic), c. Medullary
C. Thyroiditis
1. Acute thyroiditis
2. Subacute thyroiditis (De Quervain's) (in the euthyroid phase: polar disease)
3. Chronic autoimmune thyroiditis or Hashimoto's disease (in the euthyroid phase: polar disease)
4. Postpartum and silent thyroiditis (in the euthyroid phase: polar disease)
5. Riedel's thyroiditis
II. Diseases characterized by (tissue) hyperthyroidism
A. With thyroid gland hyperfunction
1. Hyperthyroid goiter with thyroid-associated ophthalmopathy or Basedow-Graves' disease
2. Multinodular hyperthyroid goiter or Plummer's disease
3. Autonomous nodule (hyperthyroid)
4. Rare forms: excessive exogenous iodine, hyperthyroidism due to Hashimoto's disease (Hashitoxicosis), postpartum thyroiditis, pituitary resistance to thyroid hormones, TSH-secreting pituitary adenoma, chorionic gonadotropin-secreting tumor, adenoma or carcinoma of the thyroid
B. Thyrotoxicosis (without thyroid gland hyperfunction)
1. Excessive, exogenous thyroid hormones (thyrotoxicosis factitia and iatrogenic)
2. Postinflammatory or from destruction of the thyroid
3. Amiodarone-induced
C. Transient hyperthyroidism
III. Diseases characterized by (tissue) hypothyroidism
A. With thyroid gland hypofunction
1. Primary hypothyroidism
a. Adult (iatrogenic (surgery, therapy, external radiotherapy), chronic autoimmune thyroiditis (in the hypothyroid phase), Graves' disease (end-stage), diffuse and nodular goiter, iodine deficiency
b. Neonatal congenital (ectopia, agenesis, dyshormonogenesis)
2. Secondary: hypothalamic-pituitary hypothyroidism (or central)
3. Dyshormonogenetic congenital goiter
B. Without hypothyroidism
1. Generalized and peripheral resistance to thyroid hormones (receptor and postreceptor defects)
C. Transient hypothyroidism
IV. Thyroid-associated ophthalmopathy
V. Abnormal thyroid parameters without thyroid diseases (nonthyroidal illness, deficit of TBG, etc.)

According to Monaco 2003 [60]

1.3. THYROID DYSFUNCTIONS AND NO

Hypo- and hyperthyroidism have been described to come along with altered formation of NO in previous animal models [67-69]. In detail, thyroid status modulated the expression of both, endothelial and neuronal NOS isoforms and the response to NO [67]. Moreover, it has been shown in a rat model that production of NO may control the activity of the HPT axis [70]. The endogenous NOS inhibitor asymmetric dimethylarginine (ADMA) might also play a role in these pathophysiological mechanisms. Arikan et al. showed increased ADMA levels in hyper- and hypothyroid patients [71]. Ozcan et al. found elevated ADMA levels in patients with subclinical hypothyroidism and Gu et al. observed increased serum ADMA levels in patients with Graves' disease [72,73].

Findings about an association of thyroid dysfunctions and metabolic disorders like obesity are still in part conflicting. Roos et al. showed an association between subclinical hypothyroidism and hyperlipidemia [74]. Iqbal et al. found a positive correlation between TSH and increased total cholesterol and LDL (low density lipoprotein) cholesterol in subclinical hypothyroid patients [75]. In this regard, investigations on a possible implication of the NO pathway in the development of metabolic disorders which might be associated with thyroid dysfunctions are indicated.

Furthermore, various studies suggest that thyroid dysfunctions may be associated with metabolic dysfunctions such as insulin resistance, type 2 diabetes, hypertension and cardiovascular risk [46,76-83].

2. AIM

We hypothesized that the nitric oxide pathway is implicated in the pathophysiological mechanisms of thyroid dysfunctions and related metabolic disorders. The aim of the present study was to evaluate NO production in juvenile HYPO and HYPER Sprague Dawley (SD) rats under a normal diet or a high-fat diet (HFD). Furthermore, this study evaluated the triiodothyronine (T3) levels as well as the endogenous ADMA. Another objective in this study was to investigate the effect of a NO donor as sodiumnitroprusside (SNP).

Moreover we aimed to assess the potential impact of NO on related metabolic dysfunctions by analysing parameters of the glucose- / insulin-metabolism and lipid metabolism.

3. MATERIALS AND METHODS

3.1. Animals and diet

Female Sprague Dawley (SD) rats (Himberg, Austria), initially weighing 251 ± 21 g, were housed (three per cage) and maintained on a 12 h light : 12 h dark cycle in a temperature- (21 ± 2 C°) and humidity-controlled animal facility. The animals were acclimatized for one week under the same laboratory conditions of photoperiod, humidity and room-temperature. Rats were either assigned to a normal rodent diet group (Altromin, Germany) or a custom designed high-fat diet (HFD) group (Harlan, Austria), (Table 3, Appendix 1, 2). The HFD composition was based on previous studies [84-86]. Food and tap water were provided *ad libitum* to all animals. Experiments and experimental set-up for female SD rats were performed according to the guidelines of the Animal Care and Use Committee of the Ministry of Science and Research, Vienna, Austria. The study was approved by the responsible national ethics committee.

Table 3: Diet composition

	normal rodent diet	high- fat rodent diet (HFD)
metabolizable energy (kcal/g)	2.6	4.7
% kcal from protein	24	12
% kcal from carbohydrate	65	32
% kcal from fat	11	56

3.2. Experimental design and drugs

The rats were randomly allocated into nine groups as shown in Table 4a, 4b. Group 1-5 were fed with normal rodent diet, whereas Group 6-9 were fed with a HFD to induce metabolic stress. HYPO groups were induced via administration of 6-propylthiouracil (0.04 g/100 ml) into tap drinking water over the experimental period as described [67,87]. HYPER groups were treated with 3,3',5-triiodothyronine (T3) (300 µg/kg in 0.50 mM NaOH) via intraperitoneal (i.p.)

injections every 2 days for 12 weeks to induce and maintain hyperthyroidism [67]. The investigation time for all groups lasted for 12 weeks due to a previously approved study design [67]. In a second set, HYPO and HYPER groups were additionally treated with the NO donor sodiumnitroprusside (SNP, 50 µg/rat/injection) via i.p. injections every 2 days over the whole experimental period of 12 weeks. Dose and administration of SNP were selected on the basis of a previous study [88].

Table 4a: Group assignment and treatment of normal diet fed SD rats

Group	1	2	3	4	5
	control (n=10)	HYPO (n=10)	SNP HYPO (n=13)	HYPER (n=10)	SNP HYPER (n=13)
Normal diet (2.6 kcal/g)	+	+	+	+	+
Triiodothyronine (T3)	-	-	-	+	+
Propylthiouracil	-	+	+	-	-
Sodiumnitroprusside (SNP)	-	-	+	-	+

(+) administered; (-) not administered

Table 4b: Group assignment and treatment of high-fat diet fed SD rats

Group	6	7	8	9
	HFD control (n=10)	HFD HYPO (n=10)	HFD HYPER (n=16)	HFD SNP HYPER (n=15)
High-fat diet (HFD)	+	+	+	+
Triiodothyronine	-	-	+	+
Propylthiouracil	-	+	-	-
Sodiumnitroprusside (SNP)	-	-	-	+

(+) administered; (-) not administered

3.3 Blood collection

After consecutive treatment for twelve weeks, blood was obtained via heart puncture after an overnight fast. Rats were anesthetized with isolfluran (Forane,

Abbott, Austria) prior to blood sampling. Blood was collected into evacuated tubes (S-Monovette® Serum-Gel, Sarstedt, Austria) and centrifuged at ambient temperature. Serum samples were aliquoted and stored at -80 °C until analysis.

3.4. Laboratory procedures

Serum triiodothyronine (T3) levels were determined by commercial Rat T3 ELISA (Uscn Life Science Inc., Wuhan, China). Serum nitrogen oxides (NOX) were analyzed with the NO Quantitation kit (Active Motif, Rixensart, Belgium) as recommended by the manufacturer. Serum asymmetric dimethylarginine (ADMA) levels were determined by commercial ADMA ELISA (DLD Diagnostika GmbH, Hamburg, Germany).

Oxidized low-density-lipoprotein (oxLDL) was determined by commercial Rat oxLDL ELISA (Uscn Life Science Inc., Wuhan, China). Serum triglycerides (TG) were measured by commercial Rat TG ELISA Kit (Cusabio, Wuhan, China). C-reactive protein (CRP) was measured with a commercial Rat CRP ELISA (BioVendor GmbH, Heidelberg, Germany). Fasting serum glucose levels were determined via commercial Glucose Assay Kit (Cayman Chemical Company, Michigan, USA). Fasting insulin was measured with an Insulin (Rat) Ultrasensitive ELISA (Hölzel Diagnostika GmbH, Cologne, Germany).

During the course of treatment, body weight measurements were performed three-weekly and food consumption was recorded. The amount of ingested diet was calculated as the difference between the amount of food that was placed in the food bin and the weight of food that remained in the bin. Calorie intake per rat per day was calculated via food consumption per animal per day and the diets' metabolizable energy values as kilocalories per gram (kcal/g).

3.5. Statistical analysis

Data are presented as means ± standard deviations. Continuous variables were compared using Students *t*-test for independent samples or Mann–Whitney *U*-test depending on the distribution of data. Correlations between variables were determined by linear regression analysis according to Pearson (r, Pearson correlation coefficient; *P*, univariate ANOVA). P-values <0.05 were considered statistically significant. Analyses were performed by explorative data analyses using SPSS for Windows (SPSS Inc., Chicago, IL, USA).

4. RESULTS – FINDINGS

Measurement of total free T3 serum levels after twelve weeks of treatment showed that the normal diet HYPO and HFD HYPO group had lower T3 levels than the appropriate controls (Table 5a). The normal diet HYPER and the HFD HYPER group showed higher T3 serum levels compared to controls. HYPO rats of both diet groups exhibited a significantly reduced body weight and a declined calorie intake (Figure 5a/b, Figure 6). All HYPER rats gained weight during the experimental period and showed an increased calorie intake (Figure 5a/b, Figure 6).

a)

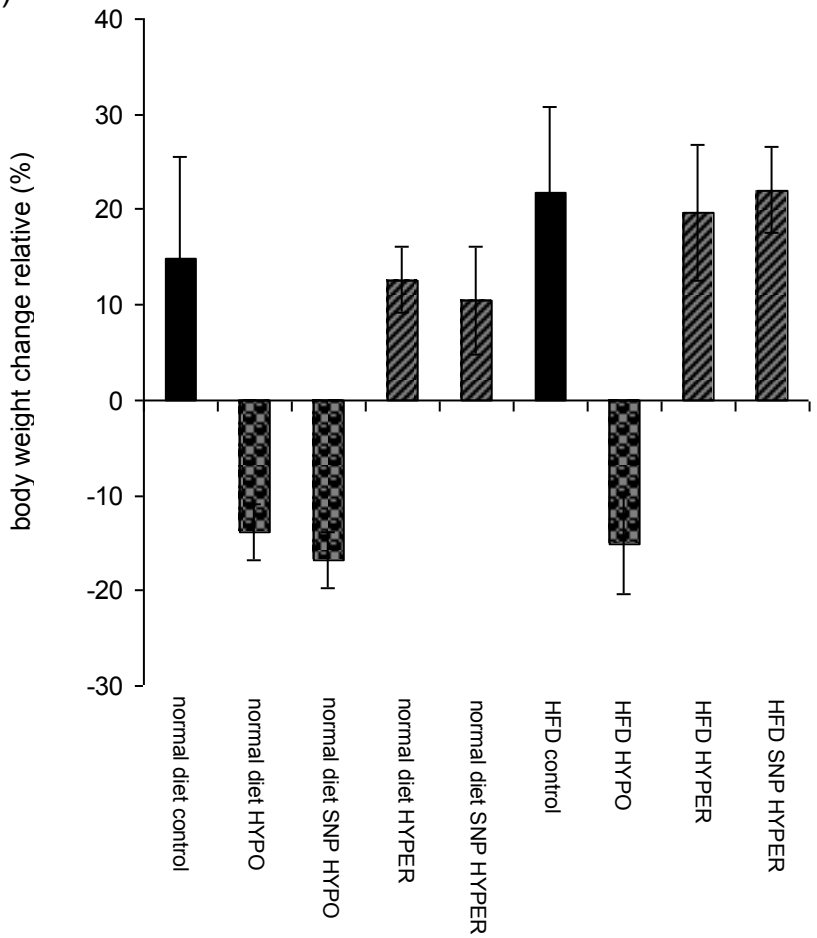

Figure 5a: Effect of thyroid dysfunction on body weight in hypo- and hyperthyroid rats compared to healthy controls during 12 weeks of treatment. a) normal diet (2.6 kcal/g) fed animals. (HFD, high-fat diet; HYPER, hyperthyroid; HYPO, hypothyroid; SNP, sodiumnitroprusside).

b)

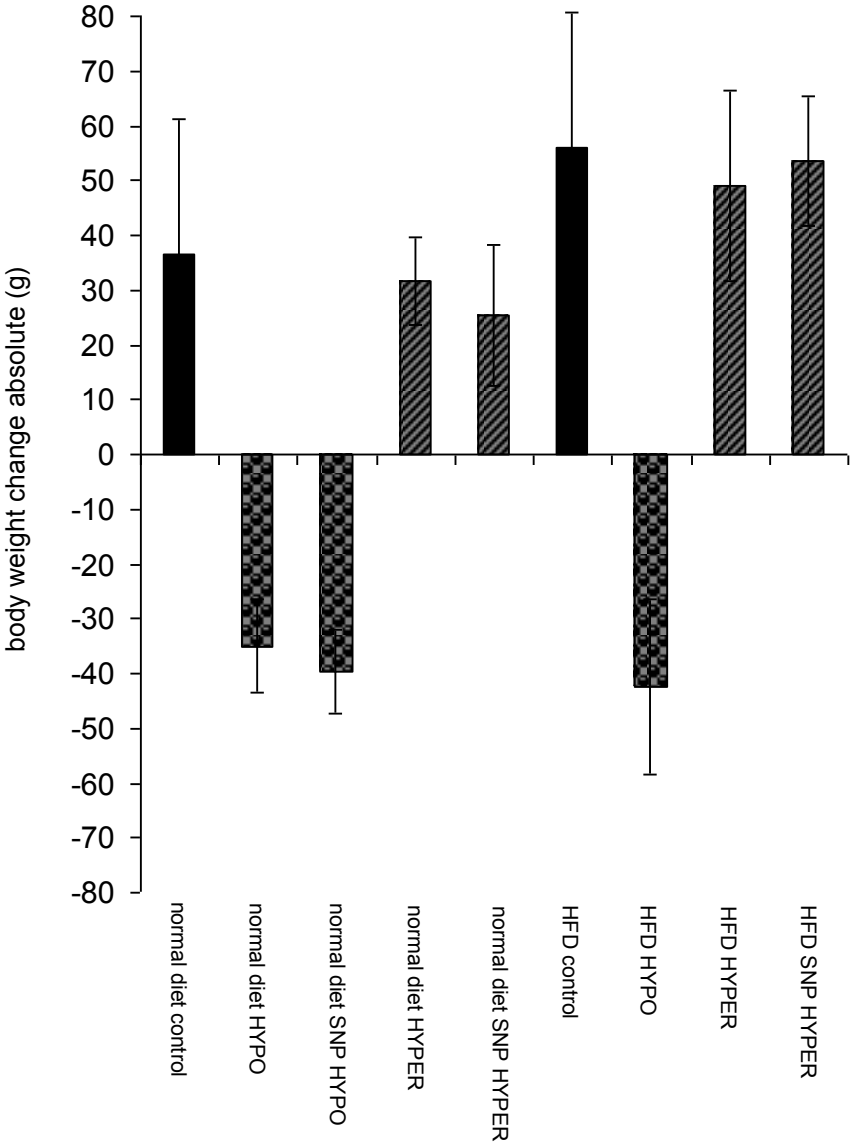

Figure 5b: Effect of thyroid dysfunction on body weight in hypo- and hyperthyroid rats compared to healthy controls during 12 weeks of treatment. b) high fat diet (4.7 kcal/g) fed animals. (HFD, high-fat diet; HYPER, hyperthyroid; HYPO, hypothyroid; SNP, sodiumnitroprusside).

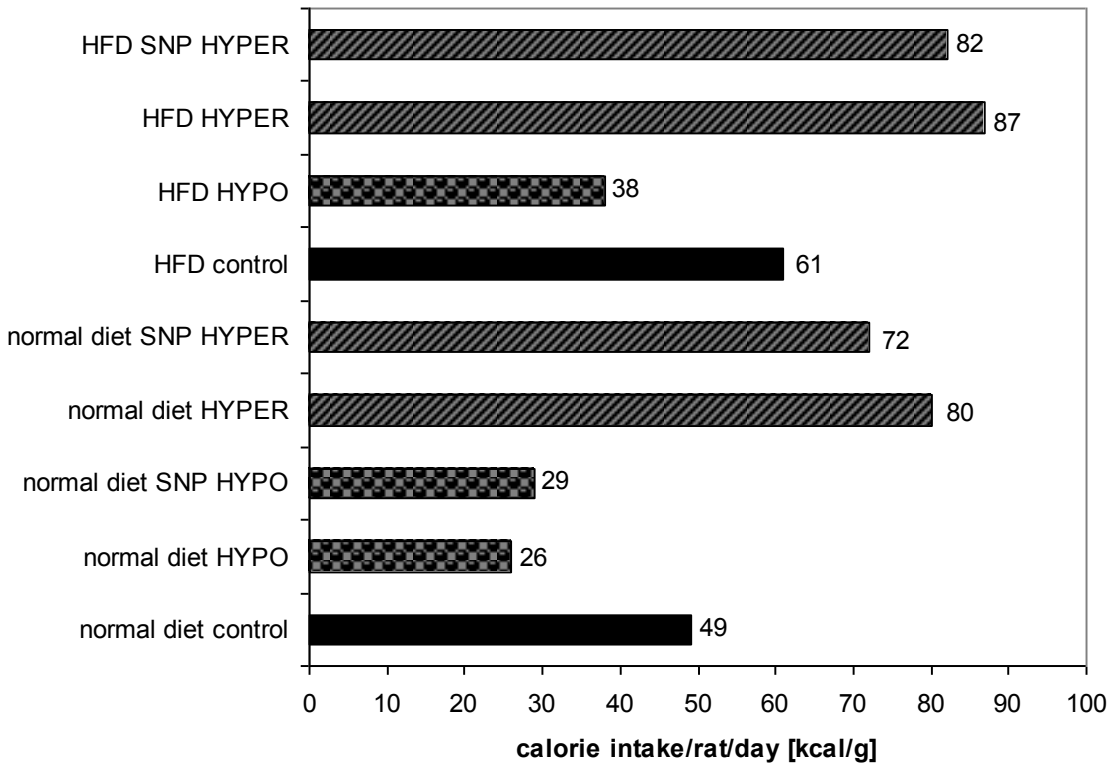

Figure 6: Food consumption during the course of treatment. The amount of ingested diet was calculated as the difference between the amount of food that was placed in the food bin and the weight of food that remained in the bin. Calorie intake per rat per day was calculated via food consumption per animal per day and the diets' metabolizable energy values as kilocalories per gram (kcal/g). (HFD, high-fat diet; HYPER, hyperthyroid; HYPO, hypothyroid; SNP, sodiumnitroprusside).

The analyses of serum NOX revealed significant differences between groups (Figure 7). Normal diet HYPOs and HYPERs had reduced NOX levels compared to the appropriate controls. HFD HYPOs and HFD HYPERs showed no significant difference in NOX levels although NOX levels were lower in the HFD HYPERs compared to HFD controls. Further, we observed that diet did not influence NOX levels as NOX values of normal diet and HFD controls were comparable. In a second experimental set, those thyroid dysfunction groups which showed reduced NOX levels in the first experimental set were treated with the NO donor SNP (see Table 5a).

Table 5a: Characteristics of female SD rats after 12-week treatment

	normal diet group (n=56)					high-fat diet (HFD) group (n=51)			
	control (n=10)	**hypothyroid (n=10)**	**SNP hypothyroid (n=13)**	**hyperthyroid (n=10)**	**SNP hyperthyroid (n=13)**	**control (n=10)**	**hypothyroid (n=10)**	**hyperthyroid (n=16)**	**SNP hyperthyroid (n=15)**
body weight change (g)	36 ± 24	-35 ± 8 ***	-39 ± 7	31 ± 7	25 ± 12	56 ± 24	-42 ± 16***	49 ± 17††	53 ± 11†††
body weight change (%)	14 ± 10	-13 ± 2 ***	-16 ± 3^{++}	12 ± 3	10 ± 5	21 ± 9	-15 ± 5***°°°	19 ± 7	22 ± 4†††
food intake/rat/day (g)	19	10	11	30	27	13	8	18	17
calorie intake/rat/day (kcal)	49	26	29	80	72	61	38	87	82
T3 (pg/ml)	417 ± 85	269 ± 74**	1102 ± 376^{+++}	951 ± 454**	2138 ± 1186^{++}	450 ± 58	446 ± 176††	1708 ± 1447	2642 ± 1730
NOX (µM)	7.8 ± 1.0	5.2 ± 2.1**	7.5 ± 1.6^{+}	6.4 ± 1.9	7.8 ± 1.3	7.9 ± 3.5	8.0 ± 2.1†	7.4 ± 2.3	8.1 ± 1.9
ADMA (µM)	0.56 ± 0.08	0.42 ± 0.04***	0.55 ± 0.07^{+++}	0.66 ± 0.09*	1.19 ± 0.35^{+++}	0.99 ± 0.49°	0.56 ± 0.33*	0.84 ± 0.28	0.66 ± 0.08$^{+\dagger\dagger\dagger}$
oxLDL (ng/ml)	6.1 ± 2.4	2.5 ± 1.3**	4.4 ± 2.9	8.7 ± 3.0*	6.2 ± 0.9^{+}	6.8 ± 1.5	2.0 ± 1.3***	9.5 ± 2.9*	9.3 ± 2.3†††
TG (µg/ml)	1.3 ± 0.2	1.3 ± 0.2	1.5 ± 0.4	1.8 ± 0,2***	1.9 ± 0.6	1.2 ± 0.2	1.4 ± 0.3	1.6 ± 0.6	1.2 ± 0.2$^{+\dagger\dagger\dagger}$
glucose (mg/dl)	102.6 ± 11.9	96.7 ± 21.7	124.4 ± 26.2$^{+\circ}$	93.3 ± 11.5	101.9 ± 13.5	106.1 ± 20.3	128.3 ± 11.7††**	98.9 ± 23.5	91.6 ± 10.3†
insulin (µg/l)	0.44 ± 0.33	0.05 ± 0.05**	0.09 ± 0.06°°	0.22 ± 0.15	0.14 ± 0.13	0.67 ± 0.51	0.18 ± 0.09††**	0.50 ± 0.49	0.26 ± 0.19
CRP (ng/ml)	420 ± 40	147 ± 54***	195 ± 29 $^{+}$ °°°	330 ± 78**	308 ± 82	250 ± 52°°°	114 ± 32	248 ± 51††	227 ± 46††

Data are presented as means ± standard deviations.*p<0.05, **p<0.01, ***p<0.001 compared to appropriate control; $^{+}$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001 compared to appropriate untreated group; °p<0.05, °°p<0.01, °°°p<0.001 compared to normal diet control group; †p <0.05, ††p<0.01, †††p<0.001 compared to appropriate normal diet group; T3, triiodothyronine; NOX, serum nitric oxides; ADMA, asymmetric dimethylarginine; oxLDL, oxidized low density lipoprotein; TG, triglycerides; CRP, C-reactive protein.

Our data show that treatment of thyroid dysfunction groups with SNP compensated decreased NOX levels in the thyroid dysfunction groups and markedly increased T3 levels 2-3 fold (Figure 8).

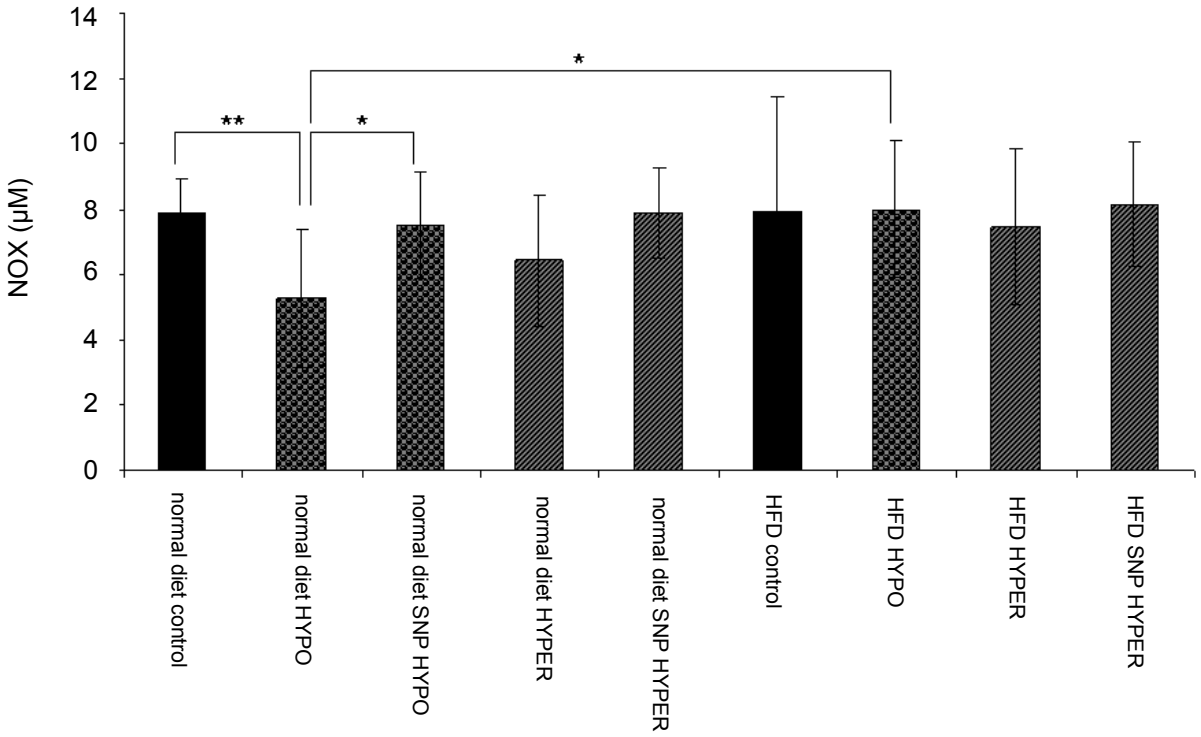

Figure 7: Serum nitrogen oxides (NOX) levels (µM) after 12 weeks of treatment and control subjects. Data are presented as means ± standard deviations. Significant difference: *p<0.05, **p<0.01. (HFD, high-fat diet; HYPER, hyperthyroid; HYPO, hypothyroid; SNP, sodiumnitroprusside).

Next, the analyses of the endogenous NOS inhibitor ADMA revealed that levels were significantly higher in the HFD control group compared to the normal diet control group (p=0.013). Both HYPO groups had significantly decreased ADMA levels. Normal diet HYPOs treated with SNP showed ADMA levels comparable to the control group. In the HYPER groups we observed a significant increase of ADMA in normal diet fed animals, but not in HFD animals. SNP treatment of normal diet HYPERs showed a significant additive effect, whereas in the HFD SNP HYPER group AMDA levels were significantly lower compared to the HFD HYPER group (Table 5a).

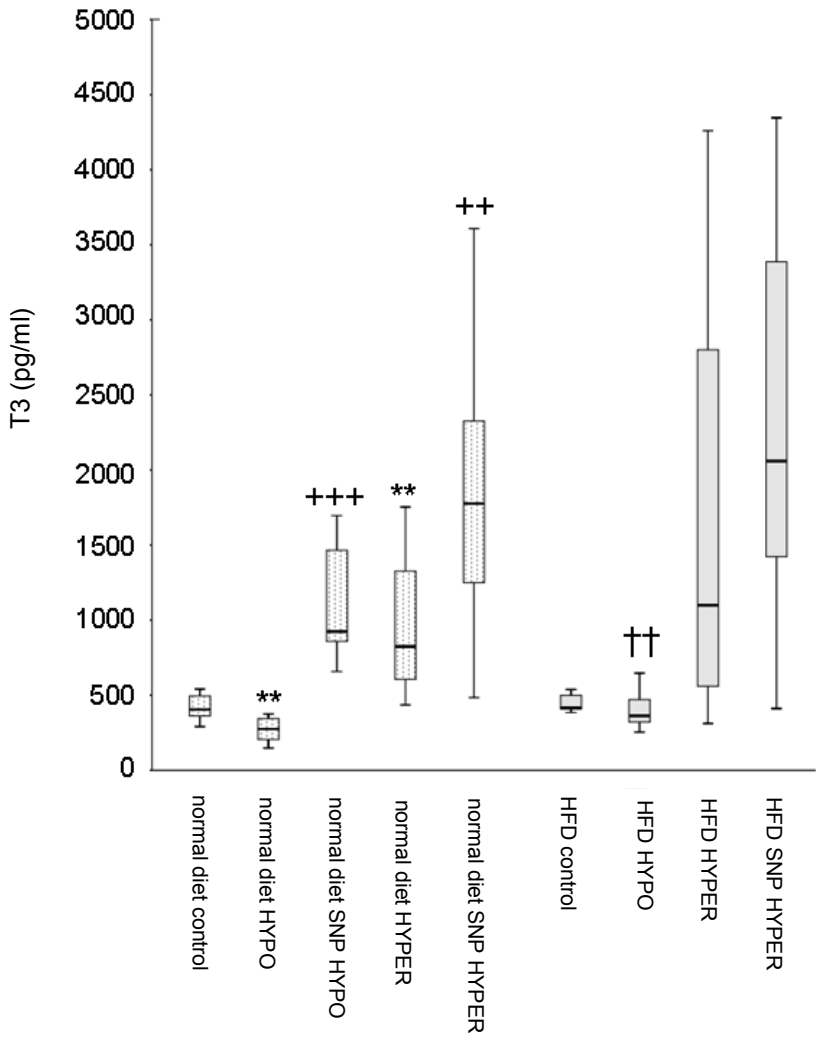

Figure 8: Effects of sodiumnitroprusside (SNP) treatment on serum triiodothyronine (T3) levels of female SD rats. HYPO rats were treated with 6-propylthiouracil, HYPER rats with 3,3',5-triiodothyronine for 12 weeks. Results are expressed as means ± standard deviations (**p<0.01 compared to appropriate control, ++p<0.01, +++p<0.001 compared to appropriate untreated group, ††p<0.01 compared to appropriate normal diet group). (HFD, high-fat diet; HYPER, hyperthyroid; HYPO, hypothyroid; SNP, sodiumnitroprusside).

Correlation analyses did not reach significance analysing NOX and ADMA, NOX and T3 or NOX and changes in body weight in any group.

We further investigated the glucose- and insulin metabolism. Diet did not alter the glucose- and insulin metabolism as normal diet control group and HFD control group exhibited similar glucose- and insulin levels. Thyroid dysfunctions showed no impact

on glucose metabolism except for the HFD HYPO group with a highly significant increase.

The normal diet SNP HYPO group showed elevated glucose levels compared to normal diet HYPOs and controls, the HFD SNP HYPER group decreased levels. Regarding insulin we found significantly decreased insulin levels in HYPOs of both diet groups compared to controls. SNP treatment of HYPOs increased insulin levels but levels were still significantly lowered compared to controls. All HYPER groups had similar levels to controls.

We additionally analyzed C-reactive protein levels. Interestingly, HFD controls showed highly significant decreased CRP levels compared to normal diet controls ($p<0.001$). In normal diet groups, both, the HYPO and HYPER state reduced CRP levels. In the HFD groups only HYPO animals showed a decrease of CRP, HYPER animals showed similar CRP levels as controls. Regression analysis of NOX and CRP revealed a highly significant association of CRP and NOX (P=0.029, r=-0.686) in the HFD control group (Table 5b). SNP treatment increased CRP levels in the normal diet HYPOs. SNP HYPERs showed no effect compared to the untreated HYPER groups.

Regarding the lipid metabolism, we analyzed serum TG and oxLDL levels. The HFD showed no effect on TG levels compared to normal diet groups. The HYPO state did not influence TG levels. Interestingly, we found increased TG levels in HYPER rats compared to the appropriate control group in both diet groups. This effect was reversed in the HFD SNP HYPER group. In normal diet groups SNP treatment did not influence TG levels. Investigations on oxLDL revealed that the HYPO groups had significantly decreased oxLDL levels in both diet groups, whereas the HYPER groups showed a significant increase of oxLDL in both diet groups. SNP treatment of HYPO and HYPER normal diet groups reversed these effects. In the HFD SNP HYPER group SNP treatment showed no effect on oxLDL levels when compared to the untreated HFD HYPER group. Regression analysis of NOX and oxLDL revealed a highly significant association of oxLDL and NOX (P= 0.032, r= 0.675) in the normal diet control group (Table 5b).

Table 5b: Linear regression analyses of NOX in female SD rats		
	r	*P*
oxLDL (ng/ml) of normal diet control group	0.675	0.032
CRP (ng/ml) of high-fat diet control group	-0.686	0.029

r Pearson correlation coefficient; *P* ANOVA; NOX, serum nitric oxides; oxLDL, oxidized low density lipoprotein; CRP, C-reactive protein

5. DISCUSSION AND CONCLUSION

In the present study, serum NOX state in HYPO and HYPER female SD rats under specific diet conditions was investigated. Thyroid dysfunction states were induced as described [67,87] and measurement of serum T3 indicated successful induction of thyroid disorders. Thyroid hormone suppression in female SD rats as a consequence of propylthiouracil treatment has previously been reported and is in line with our observations [89,90]. Findings concerning the effect of age on thyroid dysfunctions and NO are still controversial and need further investigation [69,91-95].

We demonstrated that hypothyroidism was accompanied by loss of body weight and declined calorie intake, whereas HYPER rats gained weight during the experimental period and calorie intake increased compared to controls. These observations are in accordance with previous studies indicating thyroid dysfunction associated growth variations [67,87].

One key finding of this study is that serum NOX were reduced in HYPOs and HYPERs. It must be considered that the reduction of NOX between -20 to -33 % for all treatment groups is limited because of the relatively high standard deviation. Thus, only the reduction of NOX in the normal diet HYPO group was statistically significant. Furthermore, age as well as different thyroid adaption mechanisms might have an impact on NO production and thyroid gland function, affecting hormone production and feedback-regulation, as indicated by previous studies [69,96,97]. Moreover, age-related changes seem to be gender-related, indicating an implication of gonadal hormones in the modulation of thyroid gland function and feedback-regulation [97]. Decreased thyroxine (T4) but unaltered serum thyrotropin (TSH) levels in old rats

have previously been reported [97]. Declined serum T3 in old male rats in contrast to unaltered T3 in female rats might give evidence for a more efficient deiodination of T4 to T3 [97]. This observation is in accordance with a study in humans [96]. Additionally, in females, NO production and cardiovascular function may be female-specific e.g. due to high estrogen levels which are attributed to non-genomic activation of the eNOS [98,99]. Further studies investigating the impact of estrogen and eNOS on NO bioavailabilty are required. However, in the present study we present the NOX average levels during all stages of the ovarian cycle, as the estrous stage lasts for 12 hours every 4-5 days in female rats [99,100].

Our findings are in conformity with previous studies indicating a reduction of NO in thyroid dysfunctions: Data from Virdis et al. indicate that endothelial dysfunction in male HYPO Wistar rats resulted from a reduced NO availability [68]. Furthermore, a study by Vivinanza et al. investigating human endothelial cells indicates that endothelial NO production in the HYPO state might be reduced due to an inhibition of the non-genomic action of thyroid hormone-mediated NO production. The authors further suggest that the reduced NO production might be inhibited due to T3 resistance [101]. In contrast, Liu et al. provide observations showing significantly increased serum NO levels in SD rats with fluoride-induced thyroid goitrogenesis [37]. Our findings are strengthened as it could be determined that the administration of the NO donor SNP compensated the decrease in serum NOX. Notably, the NO donor also significantly influenced the thyroid status as T3 levels were markedly increased in all SNP treated groups. This is the first time that the effect of SNP treatment in rats on thyroid hormone levels was investigated. However, the underlying mechanism governing the T3 increase remains unknown. Further investigations have to be done to clarify the influence of SNP on thyroid hormone production as well as the influence of HFD. Previous studies have shown that NO has an inhibitory effect on iodide uptake and thyroid specific gene expression in thyroid cells indicating no direct stimulatory action of SNP on the thyroid gland [102-105]. Among other possibilities, SNP could affect thyroid hormone transport into cells, thyroid hormone metabolism e.g. deiodination and thyroid hormone transport in blood [102,106,107].

The endogenous NOS inhibitor ADMA was also evaluated in this study because of the fact that ADMA inhibits vascular NO production under pathophysiological conditions [108]. Elevated ADMA levels are described as risk factor for diseases like atherosclerosis, endothelial dysfunction, diabetes mellitus and insulin resistance [109-113]. Previous clinical studies indicate an association of elevated ADMA levels with thyroid dysfunctions [71-73]. To the best of our knowledge, the association of thyroid dysfunctions and ADMA has not been investigated in experimental rat models to date. Our results indicate that thyroid dysfunction related changes in NOX are accompanied by changes in ADMA. Further, comparison of the control groups revealed that the diet has an influence on ADMA levels as ADMA was significantly higher in the HFD controls than in the normal diet controls. SNP treatment strongly influenced ADMA levels, showing an additive effect in normal diet SNP HYPOs and SNP HYPERs and a lowering effect in the HFD SNP HYPER group. Additionally, the results of the present study indicate that the underlying pathophysiological mechanism regarding ADMA in thyroid dysfunctions represents a complex network, as thyroid status, NO-treatment as well as diet have an impact on ADMA levels. As described above, ADMA is implicated in various diseases and one may speculate that the in part intriguing findings, e.g. in the normal diet SNP HYPER group and the HFD SNP HYPER group, may display these complex implications of ADMA.

To determine if thyroid dysfunctions are associated with chronic inflammation we investigated the C-reactive protein after 12 weeks of treatment. We did not find significant changes in CRP between treatment groups and appropriate controls. However, we found a highly significant decrease in the HFD control group compared to the normal diet control group. Our results are at least in part in conflict with clinical studies indicating systemic low grade inflammation in patients with thyroid diseases [114-118]. Differences may be caused by experimental and clinical trial settings. As CRP is an acute phase protein, experimental induction of thyroid dysfunction for 12 weeks in SD rats may therefore influence analyses.

We further investigated the glucose- and insulin metabolism. We found that hypothyroidism might have an impact on the glucose- and insulin metabolism in rats. HFD HYPOs showed significantly increased glucose levels whereas serum insulin was significantly decreased in HYPOs of both diet groups. Treatment of normal diet

HYPOs with SNP led to significantly elevated glucose values compared to normal diet HYPOs and controls. HYPER rats had levels comparable to controls. Anyhow, we have to notice that because of the very high standard deviations of insulin and glucose and the relatively low number of animals in each group, alterations in most groups, especially in HYPERs, did not reach statistical significance. Several studies have strengthened the hypothesis that thyroid dysfunctions affect the glucose- and insulin metabolism although its impact regarding HYPO and HYPER states is not completely assessed to date [76,77,82,119]. Measurements of the blood glucose turnover rate in rats with thyroid dysfunctions have shown that the rate was much faster in HYPER rats and more slowly in HYPO rats than in euthyroid controls. Further, HYPER rats exhibited an enhanced glucose production which might be due to gluconeogenesis, whereas glucose production of HYPO rats was suppressed [119]. The HYPO state in mature rats may further lead to a decrease in the responsiveness of glucose metabolism to insulin in adipocytes and skeletal muscles [82]. This is in line with investigations on the effect of insulin on the glucose disposal which revealed that in soleus muscles of rats, insulin-stimulated rates of glucose utilization were decreased in HYPO and increased in HYPER rats. Also prostaglandins might be involved in the changes in sensitivity of glucose utilization to insulin [46,76]. Furthermore, a clinical trial study by Maratou et al. on insulin resistance in patients with clinical and subclinical hypothyroidism showed that hypothyroidism increases the risk for insulin resistance and associated disorders like cardiovascular disease [78]. Taken together, the present observations substantiate associations between thyroid dysfunctions and insulin resistance which are quite complex, partly due to the complex mechanisms of glucose homeostasis.

Regarding the lipid metabolism, we determined TG and oxLDL serum levels. Measurements revealed that both parameters were increased in HYPER rats, treatment with the NO donor SNP partially reversed this effect. This indicates an implication of NO in the development of symptoms of the metabolic syndrome which are known to be associated with thyroid dysfunctions [83,115,120-127]. In HYPO rats, regardless of diet, serum TG levels were similar to controls and oxLDL was significantly decreased compared to controls. SNP treatment of normal diet HYPOs reversed this decrease. In accordance to that, regression analyses revealed a highly significant association of oxLDL and NOX in the normal diet controls. In contrast to

our data, previous studies by Dory et al., investigating male HYPO SD rats, showed that decreased circulating thyroid hormone concentrations were accompanied by reduced plasma TG concentrations. The authors stated that alterations may be due to a reduction of TG secretion into circulation and a declined removal from circulation [128,129]. Regarding the oxidation of lipoproteins it was proposed that patients with overt hypothyroidism show elevated circulating oxLDL levels in comparison to mild thyroid failure and controls [130]. Diekman et al. stated that patients' HYPO state might not only be associated with a quantitative increase of plasma LDL particles, but also with an increase of the oxidizability of LDL [131]. It is supposed that the oxidizabilty of LDL in HYPO patients might depend on the degree of hypothyroidism and associated changes in lipid profile, meaning elevated cholesterol and TG levels are factors for increased LDL oxidation [132]. Summarizing, thyroid dysfunctions, notably the HYPO state, seem to be strongly associated with lipid metabolism.

In conclusion, the present study demonstrates that NOX levels are significantly decreased in hypothyroid SD rats. This decrease could be compensated via treatment with the NO donor SNP, which also strongly increased T3 levels. Regression analysis of NOX and oxLDL indicated a highly significant association of oxLDL and NOX in the normal diet control group. Further, our results indicate that diet and thyroid dysfunction state influence the amount of the endogenous NOS inhibitor ADMA. Results of the present study also indicates towards a reduced bioavailability of NO in thyroid dysfunctions. Potential clinical implications and consequences need further investigations.

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7. APPENDIX

1. NORMAL RODENT DIET

Altromin Spezialfutter GmbH & Co. KG
#1320 P Rats/Mice - Maintenance

Crude Nutrients	[%]
Dry Matter	89.0
Crude Protein	19.5
Crude Fat	4.0
Crude Fibre	6.0
Crude Ash	6.5
NfE Nitrogen Free Extracts	53.0
Minerals	**[%]**
Calcium	0.9
Phosphorus	0.7
Magnesium	0.3
Sodium	0.2
Potassium	0.7
Trace Elements	**[ppm]**
Iron	155
Manganese	85
Zinc	70
Copper	14
Iodine	1.5
Selenium	0.6
Cobalt	0.3

Vitamins per kg	
Vitamin A	15.000 IU
Vitamin D3	600 IU
Vitamin B1	18 mg
Vitamin B2	12 mg
Vitamin B6	9 mg
Vitamin B12	24mg
Vitamin C	36 mg
Vitamin K3	3 mg
Vitamin E	75 mg
Folic Acid	2 mg
Biotine	60 mg
Nicotinic Acid	36 mg
Pantothenic Acid	21 mg
Choline Chloride	600 mg

Amino Acids	[%]		[%]
Alanine	1.3	Methionine + Cysteine	0.8
Arginine	0.9	Phenylalanine	0.9
Aspartic Acid	1.3	Phenylalanine + Tyrosine	1.6
Glutamic Acid	4.2	Proline	1.6
Glycine	0.8	Serine	0.9
Histidine	0.4	Threonine	0.7
Isoleucine	0.7	Tryptophan	0.2
Leucine	2.1	Valine	0.9
Lysine	0.5		

Metabolizable Energy (ME)	11.2 MJ/ kg (2676 kcal/g)
Calories from Protein	24 %
Calories from Fat	11 %
Calories from Carbohydrates	65 %
Annotation:	
Calculated values using Atwater factors.	

2. HIGH-FAT DIET (HFD)

Harlan Laboratories

TD.110093 20% Lard Diet (2018, Glucose, Yolk)

Formula	g/kg
2018, Tekland Global 18% Protein Rodent Diet	600.0
Lard	200.0
Dextrose, monohydrate	100.0
Egg Yolk Powder	100.0

Nutrient Information	
metabolizable energy (kcal/g)	4.7
% kcal from protein	12
% kcal from carbohydrate	32
% kcal from fat	56

Analytical constituents	[%]
Crude protein	14.00
Crude oils and fat	29.00
Crude fibres	3.00
Crude ash	3.00

Composition
Wheat
Maize
Lard
Wheat middlings
Dextrose
Egg yolk
Soybean, dehulled
extracted toasted
Maize gluten
Soybean oil
Calcium carbonate
Mineral dicalcium phosphate
Yeasts
Sodium chloride
Corn gluten free
Magnesium oxide

Additives (per kg)			
Nutritional additives			
E672	Vitamin A	9000	I.U.
E671	Vitamin D3	900	I.U.
E1	Fe (ferrous sulph.monohydr.)	30	mg
E5	Mn (mangan sulph.monohydr.)	26.4	mg
E6	Zn (zinc sulph.monohydr.)	18.6	mg
E4	Cu (cupric sulph. Pentahydr.)	4.2	mg
E3	Co (basic cob. Carb. Monohydr.)	0.3	mg
E2	I (calcium iodate anhydrous)	3.72	mg
Technological addidives			
E562	Sepiolite	585	mg

3. HORMONE AND METABOLIC RESEARCH - DECISION ON MANUSCRIPT

Ragginer Christine, MSc

Von: onbehalfof+hmr+thieme.de@manuscriptcentral.com im Auftrag von hmr@thieme.de
Gesendet: Donnerstag, 20. Juni 2013 11:37
An: Ragginer Christine, MSc
Betreff: Hormone and Metabolic Research - Decision on Manuscript ID HMR-2013-03-0072.R2

20-Jun-2013

Dear Ms. Ragginer,

Thank you for making the additional changes. It is a pleasure now to accept your manuscript entitled "Treatment with the Nitric Oxide Donor SNP Increases Triiodothyronine Levels in Hyper- and Hypothyroid Sprague Dawley Rats" in its re-revised form for publication in Hormone and Metabolic Research.

You find your manuscript as well as this decision letter in your Author Center under Manuscripts with Decisions.

Your manuscript will be forwarded to Georg Thieme Publishers. They will prepare your manuscript for printing. Thieme will contact you in the next weeks for further details.

Thank you for your contribution. Also on behalf of the Editors of Hormone and Metabolic Research, we look forward to your continued cooperation with the journal.

Yours sincerely,
Werner A. Scherbaum, MD, PhD
Editor-in-Chief
Hormone and Metabolic Research
E-mail: hmr@thieme.de

****

4. CURRICULUM VITAE

PERSONAL DATA:

Christine Ragginer MSc
Born at Zell am See, Austria , on the 3rd of December 1981

EDUCATION:

2010 - 2013	PhD studies “Medical Science”, Medical University Graz Doctoral School: “Translational Molecular and Cellular Biosciences”
2008 – 2010	Master of Science „Biochemistry and Molecular Biomedicine“ (Karl-Franzens University Graz)
2002 – 2008	Bachelor studies „Molecular Biology“ (Karl-Franzens University Graz)
2001 – 2002	Bachelor studies “Physioloy and Cell Biology”, Anglistics (Diploma), University Salzburg
1996 - 2001	Handelsakademie Zell am See
1992 - 1996	Bundesrealgymnasium Zell am See

MASTER THESIS / BACHELOR THESIS

05/2009 – 07/2010
Master thesis „Hexanoic Acid Activation in Hectochlorin Biosynthesis: Studies on the Structural Basis of Substrate Specificity in Acyl-Acyl Carrier Protein Synthetases” at the Institute of Biotechnology and Biochemical Engineering (University of Technology Graz);

07/2008
Bachelor thesis „Vorkommen, Bedeutung und Analyse von Wachsestern“ at the Institute of Molecular Biosciences (Karl-Franzens University Graz);

04/2008

Bachelor thesis „Biopatentierung nach der Richtlinie 98/44/EG“ at the Institute of Molecular Biosciences (Karl-Franzens University Graz);

RESEARCH PROJECTS/ INTERNSHIPS

11.2010 -10.2013

FWF Projekt NOTHYS; Clinical Institute of Medical and Chemical Laboratory Diagnostics (Medical University Graz);

01/2009 – 03/2009

Project work „Identification of Novel Enoate-Reductases Using a Functional Motif Approach“ at the Institute of Structural Biology (Karl-Franzens University Graz);

07/2007 – 09/2007

Internship at the Institute for Hygiene Microbiology and Environmental Medicine (Medical University Graz);

08/2005 – 09/2005

Internship at the Joanneum Research Institute for Energy Research, Graz;

PROFESSIONAL CAREER

since 11/2010 PhD student at the Clinical Institute of Medical and Chemical Laboratory Diagnostics (Medical University Graz);

2008 - 2010 Co-worker at the Biobank, Medical Universtiy Graz;

SCIENTIFIC PUBLICATIONS

ORIGINAL PAPER

Ragginer, C; Bernecker. C; Ainoedhofer, H; Pailer, S; Kieslinger, P; Truschnig-Wilders, M; Gruber, HJ Treatment with the Nitric Oxide Donor SNP Increases

Triiodothyronine Levels in Hyper- and Hypothyroid Sprague Dawley Rats. Hormone and Metabolic Research [ahead of print]

Ragginer C, Lechner A, Bernecker C, Horejsi R, Möller R, Wallner-Blazek M, Weiss S, Fazekas F, Schmidt R, Truschnig-Wilders M, Gruber HJ. Reduced urinary glutamate levels are associated with the frequency of migraine attacks in females. Eur J Neurol. 2012 Mar 21

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PRESENTATIONS / POSTER:

Ragginer, C; Bernecker, C; Kieslinger, P; Pailer, S; Rehberger, B; Labacher, M; Truschnig-Wilders, M; Gruber, HJ. Thyroid dysfunctions influence the patients' anthropometric parameters, lipid metabolism and oxidative stress - a clical trial pilot follow-up study. Supplement Journal of Diabetes 2013 - 5th International Congress on Prediabetes and the Metabolic Syndrome; APRIL 18-20, 2013, Vienna, AUSTRIA. [Poster]

Ragginer, C; Bernecker, C; Ainoedhofer, H; Kieslinger, P; Gruber, HJ; Truschnig-Wilders, M. In vivo model demonstrating an association between thyroid-diseases, NO pathway and metabolic dysfunctions. Supplement Wiener Klinische Wochenschrift. 2012; 01(12):11-12.-40. Jahrestagung der Österreichischen Diabetes Gesellschaft; NOV 15-17, 2012; Salzburg, AUSTRIA. [Oral Communication]

Ragginer, C; Bernecker, C; Horejsi, R; Möller, R; Lechner, A; Pailer, S; Truschnig-Wilders, M; Gruber, HJ. Increased oxidative capacity during migraine attacks contributes to migraine related metabolic dysfunctions. Clinical Chemistry and Laboratory Medicine. 2012; 50(10): A273--4.Jahrestagung der Österreichischen Gesellschaft für Laboratoriumsmedizin und Klinische Chemie; NOV 6-10,2012; Salzburg.[Poster]

Ragginer, C; Bernecker, C; Ainoedhofer, H; Pailer, S; Kieslinger, P; Truschnig-Wilders, M; Gruber, HJ. Thyroidal dysfunctions reduce the bioavailability of nitric oxide which contributes to atherogenesis in Sprague Dawley rats. 80th European Atherosclerosis Society Congress; MAY 25-28, 2012; Milan, ITALY. [Poster]

Bernecker, C; Ragginer, C; Fauler, G; Zelzer, S; Lechner, A; Truschnig-Wilders, M; Gruber, HJ. 4-Hydroxy-2-nonenal is associated with migraine-related metabolic and cardiovascular risk. 80th European Atherosclerosis Society Congress; MAY 25-28, 2012; Milan, ITALY. [Poster]

Ragginer, C; Bernecker, C; Ainoedhofer, H; Pailer, S; Kieslinger, P; Truschnig-Wilders, M; Gruber, HJ. Thyroid dysfunctions have an impact on oxLDL levels in Sprague Dawley rats. Doctoral Day 2012, Medical University Graz ; DEZ 7, 2012; Graz, AUSTRIA. 2012. [Oral Communication]

Ragginer, C; Bernecker, C; Ainoedhofer, H; Pailer, S; Kieslinger, P; Truschnig-Wilders, M; Gruber, HJ.Thyroid dysfunctions have an impact on oxLDL levels in Sprague Dawley rats. Doctoral Day 2012, Medical University Graz ; DEZ 7, 2012; Graz, AUSTRIA. 2012. [Poster]

Ragginer, C; Bernecker, C; Ainödhofer, H; Kieslinger, P; Pailer, S; Truschnig-Wilders, M; Gruber, HJ. Thyroidal dysfunctions reduce the bioavailability of nitric oxide in Sprague Dawley rats. Doctoral Day 2011, MUG; NOV 4, 2011 ; Graz, AUSTRIA. 2011. [Poster]

Mitwirkung am Workshop „Schimmel und holzzerstörende Pilze – Biologie und Medizin, Diagnostik und Bewertung, Prävention und Sanierung“, Veranstalter: Institut für Hygiene der MUG, ÖGMM –Österr. Gesellschaft für Medizinische Mykologie, FA 17a - Amt der Steiermärkischen Landesregierung; MAR 1, 2008, Ort: Graz, Raiffeisenhof;