

SUMMARY OF THESIS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY (PHD)

**Dimethlyarginines at the crossroad of
atherosclerosis and insulin resistance**

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1. Abbreviations

ADMA	asymmetric dimethylarginine
Apo-A1	apolipoprotein A1
Apo-B	apolipoprotein B
ARIC	Atherosclerosis Risk in Communities
CRP	C reactive protein
DDAH	dimethylarginine dimethylaminohydrolase
eNOS	endothelial nitric oxide synthase
HDL-C	high density lipoprotein
HISS	hepatic insulin sensitizing substance
HOMA	Homeostasis Model Assessment of β cell function and insulin resistance
iNOS	inducible nitric oxide synthase
IC ₅₀	concentration producing half-maximum inhibition
IMT	intima-media thickness
KI	confidence interval
Koeff	regression coefficient
LDL	low density lipoprotein
Lp(a)	lipoprotein a
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
SDMA	symmetric dimethylarginine
y ⁺ transporter	cationic amino acid transporter

2. Introduction

Atherosclerosis, insulin resistance and their sequelae ischaemic heart and cerebrovascular disease pose increasing demand on our ageing society. Detailed investigation of these two former disorders reveals that their risk factors are closely correlated and interdependent, the underlying processes greatly overlap. One significant common factor is nitric oxide (NO). In addition to its vasodilatory effect on resistance vessels; NO comprises several beneficial effects - indeed it is considered a quasi anti-atherosclerotic molecule. It inhibits low density lipoprotein (LDL) oxidation by its antioxidant action, reduces platelet aggregation and adhesion. It hinders monocyte migration and endothelial adhesion, decreases the expression of chemokines and endothelial adhesion molecules; furthermore NO suppresses myointimal hyperplasia by its antiproliferative action. Endogenous NO is related to insulin sensitivity as well. NO therefore is presumed to be a potential link between atherosclerosis and insulin resistance, thus substances that decrease nitric oxide's bioavailability may assume paramount significance in the evolution of these metabolic disorders.

Putative cardiovascular risk factors: dimethylarginines

Classical risk factors for cardiovascular diseases such as hypercholesterolemia, hypertension, smoking and diabetes explain only 20-40% of the risk associated with ischaemic cardiovascular disease, therefore the identification of novel independent vascular risk factors is a priority today.

Over a decade ago it was shown that the level of endogenous

asymmetric dimethylarginine (ADMA) is increased in the plasma of patients for whom cardiovascular disease is the major cause of death e.g. in end-stage renal disease.

Biosynthesis of dimethylarginines

Methylated arginine derivatives such as ADMA, are produced during the normal course of protein turnover. The guanidine-methylation of L-arginine may either be asymmetrical mono- and/or dimethylation carried out by protein-methyltransferase (PRMT) I or asymmetrical monomethylation and symmetrical dimethylation by PRMT II during the course of post-translational protein modification. Methylated proteins are cleaved during proteolysis, enter the cytoplasm and subsequently the plasma. Based on preexisting knowledge ADMA, symmetrical dimethylarginine (SDMA) and L-N-monomethylarginine (L-NMMA) are formed during the proteolysis of posttranslationally modified proteins. The asymmetrically methylated congeners are competitive inhibitors of NOS, whereas the symmetrically methylated SDMA lacks such a direct inhibitory effect. Starting from that the plasma concentration of ADMA is almost 10 times higher than that of L-NMMA, ADMA most likely assumes a more significant role in the regulation of nitric oxide synthesis.

Elimination of ADMA

ADMA is eliminated from the body as a combination of renal excretion (in the renal tubules ADMA competes with L-arginine on the γ^+ transporter for reabsorption), and intracellular enzymatic degradation

by dimethylarginine dimethylaminohydrolase (DDAH) to dimethylamine and citrulline. Catabolism seems to be superior as only 5% of parenterally administered ADMA was recovered in urine.

Pharmacodynamic effects of methylarginines

Asymmetrically methylated ADMA inhibits nitric oxide synthase (NOS) by competing with L-arginine for binding to the enzyme's catalytic domain. In vitro evidence was provided that in certain disease states ADMA level may increase sufficiently (to 3-15 $\mu\text{mol/L}$) to inhibit NOS. The IC_{50} for ADMA with respect to inhibition of endothelial NOS (eNOS) was 3.9 $\mu\text{mol/L}$ in endothelial cell culture, thus it was conveniently presumed that ADMA is an endogenous eNOS inhibitor. Conversely it was shown that the normal neuronal concentration of methylated arginines e.g. ADMA and L-NNMA results in over 50% inhibition of nitric oxide generation by neuronal nitric oxide synthase (nNOS) therefore endogenous methylarginines are sufficient modulators of the nNOS as well. There exists, however, yet another isoform of NOS, the inducible nitric oxide synthase (iNOS), that may exert cytotoxic and pro-atherogenic effects by contributing to the formation of highly reactive peroxynitrites. The iNOS is known to be induced in inflammation and more specifically in activated macrophages and smooth muscle vascular cells that reside in atherosclerotic lesions. With respect to the opposing effect of these three isoforms, asymmetric dimethylarginine, the major endogenous nitric oxide synthase inhibitor, may be either protective or deleterious in certain conditions.

Additional to its direct inhibitory effect, ADMA has further indirect

inhibitory effect on nitric oxide production by decreasing L-arginine's access to the cationic amino acid transporter (γ^+ transporter), a common pathway for entering the cell. Further observations imply that ADMA concentration dependently enhances superoxide production and subsequently activates redox-regulated transcription factors like nuclear factor kappa B (NF κ B), resulting in the concomitant up-regulation of endothelial adhesion factors and monocyte adhesion.

Evidences in support of asymmetrical dimethylarginine, a significant endogenous nitric oxide synthase (NOS) inhibitor, being a cardiovascular risk molecule are constantly accumulating. As the details of the mechanisms accompanying this effect are unraveling attention is starting to divert towards the symmetrical stereoisomer of ADMA- symmetrical dimethylarginine (SDMA). SDMA although lacks direct inhibitory effect on NOS, may hinder the synthesis of nitric oxide (NO) by competing with both ADMA and L-arginine for cell entry via the concentrative γ^+ transporter. While some studies failed to associate SDMA with all-cause mortality and fatal/non-fatal cardiovascular events, more sophisticated approaches focusing on SDMA have identified risks associated with its elevation. Accordingly, in the multicenter CARDIAC study, SDMA contributed to risk stratification in patients whose ADMA level was below the threshold level (1.75 μ mol/L) of increased risk for coronary heart disease.

Atherosclerosis and insulin resistance

In our hypothesis we propose that ADMA by inhibiting both the neuronal and the endothelial forms of NOS, results in insulin

resistance as well as in atherosclerosis, therefore ADMA is a molecule responsible for the coexistence of these two conditions. A likely rationale for the coexistence of insulin resistance and atherosclerosis is as follows. Elevation of ADMA is responsible for insulin resistance and atherosclerosis as it is able to inhibit both neuronal and endothelial nitric oxide synthase. Insulin resistance associated with hypercholesterolaemia, hyperlipidemia, hyperglycaemia, type 2 diabetes mellitus, uraemia, hypertension, obesity and the metabolic syndrome is attained by the elevation of ADMA via variable mechanisms (oxidative stress, impaired renal clearance etc) that inhibits nNOS and consequently impairs post-prandial insulin sensitization. On the other hand accelerated atherosclerosis associated with the above mentioned factors is a consequence of the inhibition of the endothelial isoforms. It is also worthy of notice that metabolic abnormalities induced by insulin resistance further elevate the systemic ADMA concentration, thus accelerate the progression of both atherosclerosis and insulin resistance.

Additional to these two isoforms of NOS there is the third isoform, the inducible NOS. While mainstream research focused on eNOS; the third isoform the inducible NOS remained unaddressed. As mentioned above iNOS has deleterious effects when upregulated in inflammatory states, e.g. in atherosclerosis accompanied by oxidative stress, due to the transiently unregulated liberation of NO and the subsequent formation of peroxynitrite. As induction of iNOS yields a highly cytotoxic and pro-atherogenic molecule, the enzyme per se may trigger or accelerate the atherosclerotic process. In accordance with the opposing effects of the distinct isoforms the kinetics of enzyme inhibition is different, since 3.9 $\mu\text{mol/L}$ ADMA is needed to achieve

half-maximal inhibition of eNOS, while 1 $\mu\text{mol/L}$ ADMA attains near-complete inhibition of iNOS.

Starting from this, it may be proposed that in the range of normal concentration (0.35-1.0 $\mu\text{mol/L}$) ADMA possibly confers protection in disease states where iNOS is induced, e.g. atherosclerosis, by selectively inhibiting the inducible isoform. Indeed previous preclinical investigations have shown that NOS inhibitors confer anti-inflammatory activity in an edema model of inflammation in mice.

3. Aims

Determination of ADMA's role in the evolution of atherosclerosis and insulin resistance

As previously discussed, based on clinical evidences and preclinical models utilizing NOS inhibitors for inducing insulin resistance our group has proposed that ADMA may contribute to the simultaneous evolution of atherosclerosis and insulin resistance by inhibiting the endothelial as well as the neuronal isoform of NOS present in the anterior hepatic plexus with the latter being associated with the regulation of insulin sensitivity.

Starting from this hypothesis we set out to investigate the significant predictors of insulin resistance in the context of atherosclerosis, with special focus on the role ADMA, SDMA and L-arginine play in a case-control study of young atherosclerotic patients and their age-matched controls. Patients suffering from early onset atherosclerosis comprise the cases with an appropriate age and sex-matched control group. The primary outcome measure was insulin resistance characterized by the HOMA index (Homeostasis Model Assessment of β cell function and insulin resistance).

Assessment of near-physiological ADMA level's potential role in the inflammatory state accompanying atherosclerosis

As discussed beforehand endothelium derived nitric oxide (NO) is presumed an ubiquiter, quasi antiatherosclerotic molecule produced by endothelial nitric oxide synthase (eNOS). Elevated level of asymmetric dimethylarginine (ADMA), by inhibiting the endothelial nitric oxide synthase (eNOS), assumes significant role in atherosclerosis. However, ADMA inhibits the inducible NOS (iNOS) as well, which triggers atherosclerosis via peroxynitrite formation.

Starting from this we set out to investigate whether ADMA is a risk or protective factor in early-onset atherosclerosis by assessing its relationship with intima-media thickness (IMT), a powerful predictor of coronary and cerebrovascular complications. Given the fact that age is a primary determinant of IMT as well as carotid atherosclerosis, we implemented a case-control study design comprising of subjects younger than 55 years, with cases having at least 30% stenosis of the internal carotid artery (ICA).

4. Materials and methods

The investigated patient population

Study design and protocol

The present study was approved by the Ethical Committee of the University of Debrecen, and written consent was obtained from each participant. The investigation conforms with the principles outlined in the Declaration of Helsinki. Young (upper age limit of 55 years) atherosclerotic patients (cases) and their age and gender matched controls were recruited at the Neurosonological Laboratory of the Department of Neurology, University of Debrecen (Hungary). The presence of atherosclerosis was established on basis of the status of the ICA and patients having at least 30% stenosis upon Duplex ultrasound examination were designated into the atherosclerotic group.

One of the main outcome measure of interest was IMT a marker now accepted by regulatory authorities, as a validated surrogate marker for atherosclerotic vascular disease. The other was insulin resistance defined by the HOMA (Homeostasis Model Assessment of β cell function and insulin resistance) model. Insulin resistant state was defined as a HOMA index > 4.4 .

Blood samples were drawn in the morning between 7:30 and 8 AM after an overnight fast, prior the administration of the morning

medications. Fibrinogen was determined from plasma. Serum samples were frozen within 60 minutes and stored at -70°C until analysis.

Determination of ADMA and other arginine derivatives

The solid phase extractions (SPE) were achieved based on the method of Nonaka and coworkers. Serum of 250 µL was mixed with 50 µL L-homoarginine hydrochloride (Sigma, HArg) as internal standard (1000 µmol/L) and 700 µL borate buffer (pH 9.00) then the solutions were passed through the SPE cartridges (OASIS[®] MCX 3cc) using a 12-column manifold (J. T. Baker). After the washing procedure the arginine derivatives were eluted with solution of cc ammonia-water-methanol (10/40/50, v/v/v) (using ammonia solution (Reanal) and methanol (Scharlau). The solvent was evaporated to dryness at 60 °C in vacuum, then it was dissolved in 200 µL deionized water and used for derivatization as described by Molnar-Perl and colleagues. The samples of 200 µL were mixed with 63 µL OPA/MPA (*ortho*-phthaldialdehyde/3-mercaptopropionic acid) reagent solution. Subsequently, the samples were incubated at 22 °C for 10 min then were cooled down to 5 °C. For chromatography, samples of 20 µL were injected into the chromatographic system consisting of a Waters 2695 Separations Module equipped with thermostable autosampler (5 °C) and column module (35 °C), a Waters 2745 Fluorescent detector with a Waters Symmetry C-18 (4.6 x 150 mm, 3.5 µmol/L) column, (each from Waters Milford, MA, USA). Gradient elution at a flow rate of 1 mL/min was applied using mobil phase **A** (20 mmol/l (NH₄)₂CO₃ in acetonitrile (Scharlau) :water 10:90, pH adjusted 7.50 ± 0.05) and mobil phase **B** (acetonitrile). The gradient condition was as follows: 0-13 min 100% **A**, 13-15 min linear change to 70 % **A** and 30 % **B** and

hold this setting for additional 5 min (i.e., 15-20 min). 20-20.1 min linear change to 100 % **A** and hold until 25 min. Analytes were detected at $\lambda_{\text{ex}}=337$ nm, $\lambda_{\text{em}}=520$ nm was used for arginine and homoarginine and $\lambda_{\text{em}}=454$ nm for asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA). Baseline separation was obtained.

Carotid Duplex Ultrasound Investigations

Ultrasound examinations were performed immediately after blood sampling with a color-coded HP SONOS 2000 (Hewlett Packard) carotid duplex equipped with a 7.5-MHz linear transducer. For screening, the ICA stenosis was classified in categories of 10%, taking into account the peak systolic velocity in the jet of the stenosis, broadening of the stenotic and poststenotic spectra, peak systolic velocity in the poststenotic ICA, and direction of ophthalmic flow. A peak systolic velocity of at least 120 cm/s was the threshold for a 50% stenosis.

Common carotid artery (CCA) IMT was analyzed offline using video images based on the Atherosclerosis Risk in Communities (ARIC) study protocol. IMT was measured in the far walls of the right and left CCAs on the 1 cm segment proximal to the dilatation of the carotid bulb. Eleven measurements were conducted at 1 mm increments in each of these 1 cm segments on both sides, and the mean IMT of the 22 values in each patient was calculated and used for statistical analysis. The reader of IMT was blinded to the designation of subjects.

Statistical analysis

Normality of continuous variables was checked by the Shapiro-Wilk test. In case of normal distribution, Student's t-test was used for comparison. Frequencies were compared by the Pearson χ^2 test. Two-way ANOVA was used to control for smoking in group comparisons.

The correlation of IMT and ADMA was established using Spearman's correlation. Alternatively simple linear regression was performed with possible predictors of the outcome variables (both with HOMA index and IMT). Following all significant variables were introduced into a multiple linear regression model to further quantify the relationship between serum ADMA concentration and the outcome measure (HOMA index or IMT). Finally Spearman's correlation and multiple regression was performed on data stratified according the status of the ICA (non-atherosclerotic vs. atherosclerotic). Values are given as means, regression coefficients and their 95% confidence intervals. Statistical analysis was performed by Stata 8.2 (Stata Corporation) software.

5. Results

Characterization of the method used for the quantification of dimethylarginines

We quantified serum ADMA concentration by adapting previously published methods to allow baseline separation and exact quantification of the two dimethylarginine stereoisomers. The method utilizes derivatization with methanolic phtaldialdehyde, followed by HPLC separation and fluorescent detection. The method yielded sharp HPLC peaks and baseline separation. The intra-day precision for Arg, ADMA and SDMA were 2.2%, 2.1% and 1.2% respectively. Recoveries were better than 98% for each component, and the limit of detection and quantification were 0.05 $\mu\text{mol/L}$ and 0.10 $\mu\text{mol/L}$, respectively. The serum level in control subjects averaged 0.42 ± 0.088 $\mu\text{mol/L}$ (range 0.29-0.62 $\mu\text{mol/L}$). These values were similar to some previously reported ones, while somewhat higher or lower than described by other groups. Our finding that serum ADMA concentration failed to significantly differ in the atherosclerotic and non-atherosclerotic group is in agreement with other previously reported studies recruiting coronary artery disease patients.

Change of dimethylarginines in insulin resistance and atherosclerosis

Demographic characteristics and risk profile of insulin sensitive and resistant patients

The mean age in the total group was 50.7 years with insulin resistant patients being slightly younger than their insulin sensitive counterparts (49.73 years vs. 51.09). Men and women were distributed equally and there were no differences in the prevalence of carotid atherosclerosis, diabetes, heart disease, hypertension, smoking, cardiovascular diseases and antiplatelet use. Cerebrovascular disease was more frequent in the case history of insulin resistant patients than controls.

The mean serum ADMA level was 0.40 ± 0.01 $\mu\text{mol/L}$ (CI: 0.39, 0.42), 0.41 ± 0.09 $\mu\text{mol/L}$ (CI: 0.39, 0.43) and 0.39 ± 0.09 $\mu\text{mol/L}$ (CI: 0.36, 0.43) in the complete data set, the insulin sensitive and insulin resistant stratum respectively. Similarly, L-arginine level didn't differ significantly with respect to insulin sensitivity, while serum SDMA level showed pronounced reduction among insulin resistant individuals (0.37 ± 0.12 $\mu\text{mol/L}$ (CI: 0.34, 0.39) and 0.31 ± 0.09 $\mu\text{mol/L}$ (CI: 0.27, 0.35) for the insulin sensitive and insulin resistant stratum, respectively). Conversely, the L-arginine to ADMA and the ADMA to SDMA ratio was unaltered or significantly decreased respectively. Regardless serum creatinine level was not statistically different between insulin sensitive and insulin resistant patients. Abnormalities of the lipid homeostasis were more prevalent in the insulin resistant group with triglyceride level being significantly higher while Apo-A1

and HDL-C were significantly lower among insulin resistant individuals.

Quantification of the relationship between HOMA-index and serum ADMA to SDMA ratio using simple and multiple linear regression

After simple linear regression we found significant negative correlation between SDMA in the presence of insulin resistance (β : -0.55, CI -1.06, -0.44; $p=0.033$) but not between ADMA. The strong positive association seen between the HOMA index and the ADMA/SDMA ratio remained statistically significant even after adjusting for all significant predictors and a priori identified confounders (β : 6.76, CI 2.13, 11.39; $p=0.005$). Interestingly this relationship was even more pronounced in the atherosclerotic stratum (β : 8.29, CI 1.43, 15.15; $p=0.019$), while lack of association was seen in subjects free of carotid atherosclerosis upon multiple linear regression (β : 1.39, CI -5.46, 8.26; $p=0.671$).

Assessment of the influence near-normal serum asymmetric dimethylarginine levels have on cardiovascular morbidity characterized by IMT

Demographic characteristics and risk profile of patients suffering from carotid atherosclerosis and their age-matched controls

The mean age in the total group was 50.7 years with no difference between controls and cases. Men and women were distributed equally and there were no differences in the prevalence of diabetes and heart disease. Hypertension, smoking, cardiovascular diseases and antiplatelet use were more frequent among atherosclerotic patients

than controls.

Correlation analysis of serum ADMA and IMT in the whole patient population and that stratified with respect to the presence or absence of carotid atherosclerosis

The mean serum ADMA level was 0.403 ± 0.009 $\mu\text{mol/L}$ (CI: 0.385, 0.421), 0.395 ± 0.085 $\mu\text{mol/L}$ (CI: 0.373, 0.417) and 0.419 ± 0.088 $\mu\text{mol/L}$ (CI: 0.386, 0.452) in the complete data set, the atherosclerotic and the non-atherosclerotic stratum respectively. IMT showed a strong negative correlation with ADMA upon the analysis of the pooled data (Spearman correlation coefficient -0.300, $p=0.0041$) and data obtained from the atherosclerotic stratum (Spearman correlation coefficient -0.323, $p=0.012$), whereas no statistically significant correlation was seen in the non-atherosclerotic stratum.

Characterization of the relationship between IMT and serum ADMA level with the use of simple and multiple linear regression

Similarly to the results of correlation analysis, we found a significant negative linear relationship between serum ADMA level and IMT using linear regression (β : -0.51, 95% CI: -0.88, -0.14; $p=0.007$). The negative association seen between IMT and ADMA remained statistically significant even after adjusting for all significant predictors and determinants identified in advance (β : -0.510, CI -0.894, -0.127; $p=0.010$). Interestingly this negative relationship was even more pronounced in the atherosclerotic stratum (β : -0.67, CI -1.16, -0.18; $p=0.008$), while lack of association was seen in subjects free of carotid atherosclerosis upon multiple linear regression (β : -0.367, CI -1.31,

0.576; $p=0.418$). In addition to ADMA we found that the presence of atherosclerosis significantly contributes to IMT (β : 0.094, CI 0.014, 0.17; $p=0.022$), while serum HDL-C level seemed to confer protection by decreasing IMT as well (β : -0.081, CI -0.156, -0.006; $p=0.035$; β : -0.11, CI -0.21, -0.021; $p=0.018$ in the full data set and the atherosclerotic stratum respectively).

6. Discussion

The major finding of the present study is that the ratio of ADMA to SDMA is positively correlated with HOMA index, a commonly used measure of insulin sensitivity and β -cell function in early-onset atherosclerosis. Additionally we found inverse relationship between these two parameters in patients free of carotid atherosclerosis. That this significant positive correlation is limited only to the atherosclerotic stratum supports the hypothesis that dimethylated arginine derivatives are indeed at the intercept of the processes contributing to insulin resistance and atherosclerosis.

Previously we proposed that ADMA may cause insulin resistance by the inhibition of the neuronal isoform of nitric oxide synthase (nNOS), while the simultaneously observed atherosclerosis is the consequence of endothelial nitric oxide synthase (eNOS) inhibition. This hypothesis stemmed from animal models utilizing intraportal administration of nonselective and selective neuronal nitric oxide synthase inhibitors for inducing insulin resistance by hindering a potent insulin sensitizing mechanism referred to as meal induced sensitization, anatomically linked to the nitrergic fibers of the anterior hepatic plexus. Additionally various preclinical and clinical studies have demonstrated a role for ADMA in atherogenesis as well.

Starting from that the two stereoisomers ADMA and SDMA compete with each other for entering the cell via the γ^+ transporter we propose that their ratio is a better measure of ADMA entering the cell than ADMA is alone. Consequently the increase in the ratio of ADMA to

SDMA could be viewed as a relative increase of ADMA. More specifically, the decrease of SDMA in the presence of unaltered ADMA level may contribute to the evolution of both insulin resistance and atherosclerosis, by failing to oppose to ADMA at the site of entry (the y+ transporters) thereby facilitating ADMA's cellular uptake and the subsequent eNOS/nNOS inhibition. Our finding that the ratio of ADMA to SDMA is the only significant determinant of insulin resistance in atherosclerosis further articulates the possibility that ADMA assumes a central role in the evolution of both of these disease entities.

Summarizing, we found that the ADMA/SDMA ratio was significantly correlated with the HOMA index in patients suffering from early-onset atherosclerosis. Accordingly we propose that the ratio of ADMA to SDMA by accounting for the competition at the y+ transporters may be an indicator of intracellular ADMA level, thus it may be a significant factor determining insulin sensitivity.

The major finding stemming from our second research hypothesis was that serum ADMA concentration in young atherosclerotic patients is inversely related to IMT, a surrogate for cardiovascular disease. Currently, ADMA is emerging as a non-traditional cardiovascular risk factor in renal failure and is a candidate for becoming a diagnostic marker for the atherosclerotic burden in general, as evidence accumulated to date favors its pro-atherogenic role exerted via eNOS inhibition.

In accordance with the opposing effects of the two distinct isoforms (eNOS and iNOS) the kinetics of enzyme inhibition is different, since 3.9 $\mu\text{mol/L}$ ADMA is needed to achieve half-maximal inhibition of

eNOS (Cardounel et al 2002), while 1 $\mu\text{mol/L}$ ADMA attains near-complete inhibition of iNOS.

Accordingly, it may be proposed that in the range of normal concentration (0.35-1.0 $\mu\text{mol/L}$) ADMA possibly confers protection in disease states where iNOS is induced, e.g. atherosclerosis, by selectively inhibiting the inducible isoform. Indeed previous preclinical investigations have shown that NOS inhibitors confer anti-inflammatory activity in a carrageenin-induced edema model of inflammation in mice.

Conversely we found that serum ADMA levels were negatively correlated with the unfavorable outcome (increase of IMT). Furthermore this inverse relationship was more pronounced when the analysis was restricted to the atherosclerotic stratum.

In summary, we propose that ADMA may be beneficial rather than detrimental in the context of inflammatory conditions known to induce iNOS, if the ADMA concentration is in the range where only the inducible isoform is comprised. Accordingly, attempts to lower ADMA should target this range. This finding will possibly assume significance as new long-awaited strategies or pharmaceutical interventions will be established to remove this putative “uremic” toxin from the circulation of end stage renal disease and other patients.

7. Summary

The link between the evolution of atherosclerosis and endothelium derived NO synthesized by endothelial NOS (eNOS) is long acknowledged since endothelial dysfunction (that is the deterioration of endothelium dependent NO release) is viewed as the forerunner of atherosclerosis. Additional to this a novel insulin sensitizing mechanism was described that is also linked to NO dependent mechanisms e.g. the activation of nNOS and is able to enhance the insulin sensitivity of striated muscle. There exists yet a third isoform of NOS, the inducible isoform (iNOS) that yields the highly reactive peroxynitrite. The different effect of these three distinct isoforms of NOS came into the focus of our attention due to the identification of a novel endogenous nitric oxide synthase inhibitor the asymmetrical dimethylarginine (ADMA). ADMA inhibits the three isoforms with different kinetics.

Within the frame of our research on one hand we investigated if the simultaneous inhibition of eNOS and nNOS is able to contribute to the evolution of atherosclerosis and insulin resistance. On the other hand we assessed if the inhibition of iNOS is able to confer beneficial effect in a condition when the enzyme is probably induced e.g. in early-onset atherosclerosis

We found that SDMA or rather the proportion of ADMA/SDMA (a more appropriate indication of the intracellular ADMA level) showed positive correlation with the HOMA index used for the characterization of insulin resistance, an effect that remained significant even after correction for confounders if only atherosclerotic patients were included in the analysis.

On the other hand we found that ADMA level negatively correlated with intima-media thickness a surrogate for cerebro- and cardiovascular diseases, and this negative correlation remained significant even after correction for confounders.

Summarizing our results we propose that ADMA assumes a central role in the simultaneous evolution of atherosclerosis and insulin resistance, furthermore we found that near-normal ADMA level is beneficial in atherosclerotic patients, probably by inhibiting peroxynitrite formation.

8. Author's own publications

***In extenso* publications in support of the thesis**

1. **Zsuga J**, Török J, Magyar MT, Valikovics A, Gesztelyi R, Keki S, Csiba L, Zsuga M, Bereczki D. Serum asymmetric dimethylarginine negatively correlates to intima-media thickness in early-onset atherosclerosis. *Cerebrovasc Dis* 2007; 23: 388-394 (**IF: 2.319**)

2. **Zsuga J**, Török J, Magyar MT, Valikovics A, Gesztelyi R, Lenkey Á, Csiba L, Kéki S, Zsuga M, Bereczki D. Dimethylarginines at the crossroad of insulin resistance and atherosclerosis. *Metabolism* 2007; 56: 394-399 (**IF: 2.294**)

3. **Zsuga J**, Gesztelyi R, Török J, Kéki S, Bereczki D. Asymmetric dimethylarginine: a molecule responsible for the coexistence of insulin resistance and atherosclerosis via nitric oxide inhibition. *Med Hypotheses* 2005; 65: 1091-1098 (**IF: 0.92**)

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***In extenso* publications associated with the thesis**

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1. Karsai D, Gesztelyi R, **Zsuga J**, Jakab A, Szendrei L, Juhász B, Bak I, Szabó G, Lekli I, Vecsernyés M, Varga E., Szentmiklósi AJ, Tósaki Á. Influence of hyperthyroidism on the effect of adenosine transport blockade assessed by a novel method in guinea pig atria. *Cell Biochem Biophys* 2007; 47: 45-52 (**IF: 2.138**)

2. Karsai D, **Zsuga J**, Juhász B, Dér P, Szentmiklósi AJ, Tósaki Á, Gesztelyi R. Effect of Nucleoside Transport Blockade on the Interstitial Adenosine Level Characterized by a Novel Method in Guinea Pig Atria. *J Cardiovasc Pharmacol* 2006; 47: 103-109 (**IF: 1.313**)

3. Gesztelyi R, **Zsuga J**, Juhász B, Dér P, Vecsernyés M, Szentmiklósi AJ. Concentration estimation via curve fitting: quantification of negative inotropic agents by using a simple mathematical method in guinea pig atria. *Bull Math Biol* 2004; 66(5): 1439-1453 (IF: 1.485)

4. Gesztelyi R, **Zsuga J**, Hajdu P, Szabo JZs, Cseppento A, Szentmiklosi AJ. Positive inotropic effect of the inhibition of cyclic GMP-stimulated 3',5'-cyclic nucleotide phosphodiesterase (PDE2) on guinea pig left atria in eu- and hyperthyroidism. *Gen Physiol Biophys* 2003; 22: 501-513 (IF: 0.794)

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