ADMA 2014

Endogenous Modulators of Nitric Oxide: From Mechanisms to Medicine

7th International Symposium on Asymmetric Dimethylarginine

June 30 through July 2, 2014
Saint Petersburg, Russia

Park Inn Radisson Pulkovskaya Conference Centre

Welcome from the Co-Chairs

Dear Colleagues,

On behalf of the Organizing Committee, it is our great pleasure to welcome you to St-Petersburg and to the 7th International Symposium on Asymmetric Dimethylarginine - Endogenous Modulators of Nitric Oxide: From Mechanisms to Medicines.

The Symposium has been designed to provide an innovative and comprehensive overview of the latest research developments in endogenous modulators of nitric oxide, primarily in the areas of molecular biology, drug discovery, and the pathogenetic role of methylarginines in cardiovascular disease.

Many distinguished cardiologists and scientists have joined the faculty and will take part in this Symposium. Papers will be presented in the form of a keynote lecture, scientific sessions with invited lectures and oral presentations, and posters and will include superb scientific material that was carefully selected from over 40 abstracts submitted for presentation at the meeting.

We would like to express our thanks to the pharmaceutical industry for their generous support, to our dedicated staff, colleagues, friends and families for their untiring help, support and advice in planning and arranging this meeting.

An array of activities will be organized to provide everyone an opportunity to mingle and network with colleagues from all over the world in a more relaxed setting. We also hope and trust that you will enjoy your visit to the very beautiful and exciting city of St-Petersburg, in June-July 2014.

Yours sincerely,

Evgeny Shlyakhto
Almazov Centre
Co-Chair

Steven Lentz
University of Iowa
Co-Chair

Scientific Organizing Committee

Evgeny Shlyakhto	Almazov Centre, Russia
Steven Lentz	University of Iowa, USA
Rainer Böger	Hamburg, Germany
John Cooke	Methodist Hospital, USA
Yingjie Chen	University of Minnesota, USA
Michael Galagudza	Almazov Centre, Russia
James Leiper	MRC, United Kingdom
Roman Rodionov	Dresden, Germany

Thank You to our Sponsors

Monday, June 30, 2014

Satellite Symposium Frontiers in Cardiovascular Therapy: Nitric Oxide and Endothelial Function	
3:30 - 5:00 pm	Current approaches and new horizons in the treatment of acute heart failure decompensation **(Supported by Novartis)**

ADMA 2014 Opening Session		
5:30 - 5:40 pm	**Evgeny Shlyakhto** Almazov Centre Russian Federation	Welcome and Opening Remarks
5:40 - 5:50 pm	**Steven Lentz** University of Iowa USA	Introduction of Keynote Speaker
5:50 - 6:50 pm	**Mark Bedford** MD Anderson Cancer Center University of Texas, USA	**Keynote Lecture:** Arginine Methylation – Enzymes, Binders & Inhibitors

7:30 pm **Buffet Dinner and Evening Reception**
Park Inn Pulkovskaya

Tuesday, July 1, 2014

Session 1: Methylarginines: Makers, Markers, or Misleading?		
Chairpersons	**Michael Galagudza** Almazov Centre Russian Federation	**Renke Maas** Friedrich-Alexander-University Erlangen-Nürnberg, Germany
8:00 – 8:30 am	**Christopher Wilcox** Georgetown University, USA	ADMA and DDAH-1 in the kidney
8:30 – 9:00 am	**Dao Wen Wang** Tongji Medical College, Wuhan, China	A novel loss-of-function DDAH1 promoter polymorphism is associated with increased susceptibility to thrombosis, stroke and coronary heart disease
9:00 – 9:15 am	**Olga Bolshakova** Almazov Centre Russian Federation	Endothelial function and MTHFR gene polymorphisms in postmenopausal patients with hypertension
9:15 – 9:30 am	**Zinaida Malakhova** Pavlov Medical University Russian Federation	Time course and relative contribution of acetyl-choline-induced NO-, PGI2-, and EDHF-mediated vasodilation in humans
9:30 – 9:35 am	**Renke Maas**	**Wrap-up and Clinical Perspectives**

9:35 – 10:00 am **Coffee Break, Exhibits**

Session 2: Metabolic Control in Gain and Loss of NO Synthase Function		
Chairpersons	**Steven Lentz** University of Iowa,USA	**Renke Maas** Friedrich-Alexander-University Erlangen-Nürnberg, Germany
10:00 – 10:30 am	**Alexander Zhloba** Pavlov State Medical University, Russian Federation	ADMA and DDAH-1 in the kidney
10:30– 11:00 am	**Tatiana Subbotina** Almazov Centre, Russian Federation	A novel loss-of-function DDAH1 promoter polymorphism is associated with increased susceptibility to thrombosis, stroke and coronary heart disease
11:00 – 11:30 am	**Elizaveta Alekseevskaya** Pavlov State Medical University Russian Federation	Endothelial function and MTHFR gene polymorphisms in postmenopausal patients with hypertension
11:30– 11:45 am	**Filippo Martino** Hannover Medical School Germany	Time course and relative contribution of acetyl-choline-induced NO-, PGI2-, and EDHF-mediated vasodilation in humans
11:45 – 11:50 am	**Steven Lentz** University of Iowa, USA	**Wrap-up and Clinical Perspectives**

12:00 – 1:30 pm **Lunch, Exhibits and Poster Viewing**

Session 3: Metabolism and Transport of Methylarginines		
Chairpersons	**Ellen Closs** University Medical Center of the Johannes Gutenberg-University, Mainz, Germany	**Mark Bedford** MD Anderson Cancer Center, University of Texas, USA
1:30 – 2:00 pm	**Roman Rodionov** University Hospital Carl Gustav Carus, Dresden, Germany	Metabolism of methylarginines
2:00 – 2:15 pm	**Michael Galagudza** Almazov Centre, Russian Federation	The role of ADMA in the pathogenesis of myocardial ischemia-reperfusion injury in diabetes mellitus: focus on microRNA
2:15 – 2:30 pm	**Alexey Kolobov** Saint-Petersburg State University, Russian Federation	HNF4a is the major regulator of AGXT2 expression in liver
2:30 – 2:45 pm	**Igor Bondarenko** North West Research Centre, Russian Federation	Is there a pathway for ADMA degradation alternative to DDAH?
2:45 – 3:00 pm	**Ben Caplin** University College London, UK	Disruption of renal ADMA metabolism leads to salt-sensitive hypertension
3:00 – 3.05 pm	**Ellen Closs**	Wrap-up and Clinical Perspectives

3:05 pm **Social Activities in St. Petersburg and Peterhof**
Evening and dinner individually

Wednesday, July 2, 2014

Session 4: Pharmacological Modulation of Methylarginines		
Chairpersons:	**Edzard Schwedhelm** University Hospital Hamburg – Eppendorf, Germany	**Roman Rodionov** University Hospital Carl Gustav Carus, Dresden, Germany
8:00 – 8:30 am	**Yohannes Ghebremariam** Methodist Hospital, USA	Regulation of NOS/DDAH Pathway by Proton Pump Inhibitors: Safety Concerns and Therapeutic Opportunities
8:30 – 8:45 am	**Blerina Ahmetaj-Shala** Imperial College, UK	Cyclo-oxygenase-2 anti-inflammatory drugs or gene deletion is associated with increased circulating methylarginines: identification of novel biomarkers of cardiovascular toxicity)
8:45 – 9:00 am	**Roman Sukhovershin** Methodist Hospital, USA	Proton pump inhibitors increase asymmetric dimethylarginine and induce inflammation in vascular endothelial cells
9:00 – 9:15 am	**James Tomlinson** Imperial College, UK	Reduced renal DDAH1 activity protects against progressive kidney fibrosis and eGFR decline)
9:15– 9.20 am	**Edzard Schwedhelm**	Wrap-up and Clinical Perspectives

9:15– 9.20 am **Coffee Break, Exhibits**

Session 5: Modulation of Methylarginines by Genes and Pathophysiological Conditions		
Chairpersons:	**Chris Wilcox** Georgetown University, USA	**Yingjie Chen** University of Minnesota, USA
9:50 – 10:20 am	**Ben Caplin** University College London, UK	ADMA: Unyielding opponent or double-agent in the battle that is cardio-renal disease?
10:20 – 10:35 am	**Aygun Kazimli** Almazov Centre, Russian Federation	Assessment of ADMA in patients with pulmonary hypertension
10:35 – 10:50 am	**Michael Gilinsky** Institute of Physiology and Fundamental Medicine, Russian Federation	The effect of rapamycin on ADMA in two strains of rats
10:50 – 11:05 am	**Roman Rodionov** University Hospital, Dresden, Germany	Increased amounts of asymmetric Nα-acetyl-ADMA after ADMA infusion in mice
11:05 – 11:20	**Laura Howe** Hammersmith Hospital, UK	Elucidation of the Role of the DDAH/ADMA/NO Axis in Pregnancy-related Haemodynamic Dysfunction
11:20 – 11:25	**Yingjie Chen**	Wrap-up and Clinical Perspectives

11:30 – 1:00 pm **Lunch, Exhibits and Poster Viewing**

Session 6: Clinical Implications of SDMA and Other Methylarginines		
Chairpersons	**Alexandra Konradi** Almazov Centre, Russian Federation	**Norbert Weiss** University Hospital Carl Gustav Carus, Dresden, Germany
1:00 – 1:30 pm	**Renke Maas** Friedrich-Alexander-University Erlangen-Nürnberg, Germany	ADMA as a risk marker - are we any wiser than in 2001?
1:30 – 2:00 pm	**Dorothee Atzler** University of Oxford, UK	Homoarginine – a cardiovascular risk marker and a new therapeutic target
2:00 – 2:15 pm	**Yingjie Chen** University of Minnesota, USA	DDAH1 plays an important role in protecting the heart from systolic overload-induced congestive heart failure
2:15 – 2:30 pm	**Sophie Piper** MRC Clinical Sciences Centre, Imperial College London, UK	DDAH1 and insulin resistance
2:30 – 2:45 pm	**Laura Dowsett** MRC Clinical Sciences Centre, Imperial College London, UK	ADMA is a novel regulator of mTOR expression in adipocytes
2:45 – 2:50 pm	**Norbert Weiss**	Wrap-up and Clinical Perspectives

2:50 – 3:20 pm **Coffee Break, Exhibits**

Session 7: Drug Discovery and Experimental Therapeutics		
Chairpersons	**John P. Cooke** Methodist Hospital, USA	**Rainer Böger** University Hospital Hamburg–Eppendorf, Germany
3:20 – 3:50 pm	**James Leiper** MRC Clinical Sciences Centre, Imperial College London, UK	Drugging the DDAH/ADMA/NO pathway
3:50 – 4:05 pm	**Isabel Bernges** University of Hamburg, Germany	High-throughput screening identified an analogue of the thyreostatic drug thiamazole as potential regulator of DDAH1
4:05 – 4:20 pm	**Christopher Wilcox** Georgetown University, USA	Activation of Nrf2 Reduces ADMA and Increases Nitric Oxide in Human Renal Glomerular Endothelial Cells (HRGECs) by Transcriptional Activation of DDAH, eNOS and PPAR-γ
4:20 – 4:25 pm	**John Cooke**	Wrap-up and Clinical Perspectives

Session 8: Debate: The Arginine Paradox – Is there a Therapeutic Role for Arginine?		
Moderators:	**James Leiper** MRC Clinical Sciences Centre, Imperial College London, UK	**Evgeny Shlyakhto** Almazov Centre, Russian Federation
4:30 – 5:00 pm	**John P. Cooke** Methodist Hospital, USA	Arginine supplementation is not evidence-based medicine
5:00 – 5:30 pm	**Rainer H. Böger** University Hospital Hamburg–Eppendorf, Germany	There is a role for arginine if we select our patients wisely

Concluding Remarks		
5:30 pm	**Steven Lentz**	**Evgeny Shlyakhto**

7:00 pm **Departure for Congress Banquet and Awards Ceremony**

Location:
The House of Scientists at the Palace of the Grand Duke Vladimir

Poster Presentations

Poster Number	Authors	Institutions	Title
P1	R.A. Sukhovershin, N.I. Dubrovina, **Michael Gilinsky***	Institute of Physiology and Fundamental Medicine, Siberian Branch of Russian Medical Academy, Novosibirsk, Russian Federation	Methylarginines and animal behaviour in the poloxamer-407 model of atherosclerosis
P2	**Alexey Maslyanskiy***, Ekaterina Kolesova, Ilia Penin, Elena Vasylieva, Alexandra Konradi	Federal Almazov Medical Research Centre, St-Petersburg, Russian Federation	ADMA levels and arterial wall stiffness in rheumatology patients
P3	**Sara Tommasi***, Chiara Zanato, Benjamin C. Lewis, Andrew Rowland, Pramod C. Nair, Matteo Zanda and Arduino A. Mangoni	University of Aberdeen, Institute of Medical Sciences, School of Medical Sciences Aberdeen, Scotland, UK; Flinders University and Flinders Medical Centre, Department of Clinical Pharmacology, School of Medicine, Adelaide, Australia	Arginine analogues incorporating carboxylate bioisosteres as novel DDAH inhibitors
P4	Zhijing Zhao, Ming Zhang, Wenting Shen, Lianyan Song, Monan Zhang, **Guang Hu***	Bioengineering Center, Shaoxing Institute of Technology, College of Engineering, Peking University, China	A luminescence assay for the determination of asymmetric dimethylarginine
P5	**Natalya Khromova***, O. Berkovich, E. Bazhenova, N. Vakhrameeva, A. Zhloba	Federal Almazov Medical Research Centre, St-Petersburg, Russian Federation	Homocysteine levels and endothelial dysfunction
P6	**Karl Florian Wintgens***, Natalia Kuzmina, Robert Klüsener, Jana Ruppert, Karl-Heinz Kellner, Franz Paul Armbruster, Christoph Melzer, Thomas Dschietzig	Immundiagnostik AG, Germany University of Giessen and Marburg Lung Center, Department of Internal Medicine, Member of the German Lung Center, Justus Liebig University of Giessen, Giessen, Germany	ADMA, a biomarker of vascular dysfunction, in an out-patient cohort at very high cardiovascular risk
P7	**Ralph Theo Schermuly*** Dmitry Sonin*, Michael Galagudza	Federal Almazov Medical Research Centre, St-Petersburg, Russian Federation; I. P. Pavlov State Medical University, St-Petersburg, Russian Federation	Increased alveolar epithelial expression of dimethylarginine dimethylaminohydrolase 2 in idiopathic pulmonary fibrosis
P8	**Dmitry Sonin***, Michael Galagudza	Federal Almazov Medical Research Centre, St-Petersburg, Russian Federation; I. P. Pavlov State Medical University, St-Petersburg, Russian Federation	Endogenous nitric oxide is not involved in the mechanisms of flow-dependent vasodilation in hypertensive rats
P9	**N.D. Gavriliuk***, O. Irtyuga, T. Druzhkova, V. Uspenskiy, E. Alekseevskaya, A. Zhloba, O. Moiseeva	Federal Almazov Medical Research Centre, St-Petersburg, Russian Federation; I. P. Pavlov State Medical University, St-Petersburg, Russian Federation	Lack of association between asymmetric dimethylarginine and ascending aortic aneurysm

Poster Presentations

Poster Number	Authors	Institutions	Title
P10	**Eliza A Kalk***, James Leiper, Mark Ungless	MRC Clinical Sciences Centre; Faculty of Medicine Imperial College London. UK	nNOS and DDAH1 expression in the ventral tegmental area
P11	**Ludmila A. Aleksandrova***, Tatiana F. Subbotina, Aleksandr A. Zhloba, Elizaveta S. Alekseevskaya, Ekaterina V. Zhiduleva,Olga M. Moiseeva	Federal Almazov Medical Research Centre, St-Petersburg, Russian Federation; I. P. Pavlov State Medical University, St-Petersburg, Russian Federation	Disorders of thiol metabolism in endothelial dysfunction
P12	**John Garlick***, Amy Wilson-O'Brien, Andrew Wilson, Nicholas Williamson	University of Melbourne, St Vincent's Hospital, University of Melbourne, Bio21, Australia	The separation and quantitation of methylated arginine analogues using ion mobility – mass spectrometry
P13	Sophia Georgi, Normund Jabs, **Natalia Jarzebska***, Silke Brilloff, Renke Maas, Roman N. Rodionov, Christian Zietz, Norbert Weiss	University Center for Vascular Medicine, Department of Internal Medicine III, and Department of Pathology, University Hospital Carl Gustav Carus, Technische Universität Dresden, Germany; Institute of Experimental and Clinical Pharmacology and Toxicology, Friedrich-Alexander University Erlangen-Nürnberg, Germany	Liver and kidney are the major sources of AGXT2 in humans
P14	**Roman N. Rodionov***, Annett Rexin, Silke Brilloff, Jens Martens-Lobenhoffer, Stefanie M. Bode-Böger, Vladimir Todorov, Christian Hugo, Norbert Weiss, Bernd Hohenstein	University Center for Vascular Medicine and Division of Nephrology, Department of Internal Medicine III, University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany; Institute of Clinical Pharmacology, Otto-von-Guericke University, Magdeburg, Germany	ADMA reduction does not protect mice with streptozotocin-induced diabetes mellitus from development of diabetic nephropathy
P15	Tracy D Bell; Jones AP Tomlinson, James Leiper, William E Welch and **Christopher S Wilcox***	Georgetown University Hypertension, Kidney and Vascular Research Center, Washington, DC, USA and MRC Clinical Science Center, Imperial College, London, UK	DDAH-1 and ADMA regulate proximal tubular fluid of reabsorption: comparison of pharmacological blockade of DDAH-1 with gene knockdown

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P1
Methylarginines and Animal Behavior in the Poloxamer-407 Model of Atherosclerosis

R.A. Sukhovershin, N.I. Dubrovina, M.A. Gilinsky*

Institute of physiology and fundamental medicine, Siberian Branch of Russian Medical Academy, Novosibirsk, Russia.

At the end of the last century systematic evidence appeared that the disturbance of nitric oxide (NO) metabolism triggers the chain of events: reduced bioavailability NO - endothelial dysfunction - atherosclerosis (Boger et al., 1996; Cooke, 1996). In accordance with this point of view an important role of methylated forms of L-arginine (L-Arg), the substrate of NO-synthase, is suggested for the development of atherosclerosis. To investigate the involvement of ADMA in the formation of atherosclerosis, we applied a relatively simple, stable model of the development of this pathology. The model is based on the use of nonionic surfactant poloxamer 407 (Johnston, 2004). Perennial (since 1983) study of Johnston et al. [1983 , 2004, 2010] showed that "many of the physiological and biochemical processes characteristic for natural atherosclerosis occur as well in the poloxamer 407 - induced model of dyslipidemia and atherosclerosis in mice". Authors described numerous facts indicating the adequacy of the model studied (Johnston, 2010).

By means of HPLC with fluorimetric detection (all Shimadzu) we measured the concentration of L-Arg and methylarginines, after 2 or 14 weeks of poloxamer 407 administration to the mice CBA (0.5 g/kg each 3-rd day). As a control, saline injection has been used. After two weeks of administration of poloxamer the only concentration of symmetric dimethylarginine (SDMA) was significantly higher than that in control group. After 14 weeks of administration of poloxamer 407 levels of endogenous NO synthase inhibitors monomethylarginine (MMA) and asymmetric dimethylarginine (ADMA) were significantly higher than control levels at this time. Levels of L-Arg and SDMA did not differ significantly from controls during this period. Arginine level increased after 14 weeks of poloxamer administration, but the difference with 2 weeks level did not become significant. The development of atherosclerosis in used mice was histologically confirmed (Korolenko et al., 2012). Correlations between MMA levels and total cholesterol and triglycerides were significant (respectively $r = 0.63$, $p<0.002$ and $r = 0.46$; $p<0.03$). For ADMA correlation values were similar ($r = 0.48$; $p<0.25$ and $r = 0.50$; $p <0.02$). After 14 weeks of chronic poloxamer 407 administration the locomotor activity of mice decreased and anxiety increased. It was found the significant deficit in the processes of passive avoidance forgetting.

We discuss here: 1) the possibility to use MMA as an internal standard in the assessment of NO synthase inhibition in mice; 2) the mechanisms of dimethylarginine levels association with the indicators of endothelial dysfunction, and 3) the probable relation of behavior changes to increasing concentrations of MMA or/and ADMA.

This work was supported by the Russian Foundation for Basic Research, grant No. № 13-04-01079.

P2
ADMA levels and arterial wall stiffness in rheumatology patients

Maslyanskiy Alexey*, Kolesova Ekaterina, Penin Ilia, Vasylieva Elena, Konradi Alexandra

Federal Almazov Medical Research Centre

The aim of the present study was to compare the ADMA levels in patients with rheumatoid arthritis (RA), system lupus erythematous (SLE), systemic sclerosis (SSc), ankylosing spondylitis (AS) with healthy control and subjects with major cardiovascular risk factors, and to determine the main correlations, linked to ADMA elevation in each condition. We studied ADMA level in 67 patients with SSc, 31 patients with diffuse cutaneous SS and 27 patients with limited cutaneous disease and also 9 patients with overlap syndrome (median age was 53 (42-60), 42 patients with RA (median age was 55 (48-59), 41 patients with AS (median age was 37 (32-48), and 50 patients with SLE (median age was 36 (27-45), 50 subjects with major cardiovascular risk factors (median age was 51 (44-56). All rheumatology groups, were comparable according 10-years Framingham cardiovascular risk score. Ten healthy blood donors were used as a control group (median age was 25 (21-30). All patients were characterized with basic clinical, instrumental and laboratory tests. Vascular involvement was accessed by measurement of pulse wave velocity (PWV) and augmentation index (AI) with applanation

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tonometry employing the SphygmoCor system (AtCor Medical Pty Ltd., Sydney, Australia). ADMA level was assessed in serum samples of patients and controls by an ELISA (Immunodiagnostik, Germany), according manufacturer instructions. The highest levels of ADMA was detected in patients with SLE 0,77 (0,64-0,97) mcmol/L, SSc 0,66 (0,54-0,76) mcmol/L, RA 0,63 (0,56-0,74) mcmol/L. It was significantly lower in patients with AS 0,54 (0,46-0,57), subjects with major cardiovascular risk factors 0,54 (0,48-0,60) mcmol/L and in healthy controls 0,42 (0,13-0,47) mcmol/L (p<0,001). In SSc patients, ADMA correlated with main activity markers and with augmentation index (AI) (r=0,48 p=0,0007). In patients with RA a correlation of ADMA with pWV was documented (r=0,52 p=0,0007). In AS patients, ADMA level correlated significantly with traditional risk factors (age, glucose) and also with pWV Sphygmocor (r=0,44 p=0,001). Besides, in group of subjects with cardiovascular risk factors, ADMA level correlated with traditional risk factors (age and smoking), but not with arterial stiffness. At the same time PWV was comparable between all groups. In rheumatology diseases increased levels of ADMA as a marker of endothelial damage are observed, especially in diseases associated with autoantibodies production. At the same time ADMA appears to be associated with increases vascular stiffness in rheumatic diseases but not in patients with classic atherosclerosis.

P3

Arginine analogues incorporating carboxylate bioisosteres as novel DDAH inhibitors

Sara Tommasi,* Chiara Zanato, Benjamin C. Lewis, Andrew Rowland, Pramod C. Nair, Matteo Zanda and Arduino A. Mangoni

University of Aberdeen, Institute of Medical Sciences, School of Medical Sciences Aberdeen, Scotland, UK Flinders University and Flinders Medical Centre, Department of Clinical Pharmacology, School of Medicine, Adelaide, Australia

Nitric oxide, a free radical endogenously generated by mammals, is involved in several key physiological processes. The excessive synthesis of this molecule is associated with a variety of pathological conditions including septic shock, pulmonary fibrosis, migraine, tumoral angiogenesis, as well as some neurodegenerative disorders. The inhibition of human dimethylarginine dimethylaminohydrolase (DDAH), an enzyme responsible for indirect modulation of nitric oxide synthesis, represents a promising molecular target to limit nitric oxide overproduction. In this study, the synthesis and kinetic characterization of twenty-four new chemical entities (NCEs) is reported, along with their DDAH inhibitory profiles. In particular the corresponding acylsulfonamide of the current prototypic DDAH inhibitor L-257, demonstrated a significantly lower inhibition constant (Ki = 1μM), relative to L-257 (Ki = 26 μM), when measuring DDAH-1 mediated citrulline formation by mass spectrometry. Molecular modelling studies suggests that the enhanced activity of acylsulfonamide derivative over L-257 may occur from an improved positioning of acyl sulphonamide atoms in the active site of DDAH-1 with superior polar and hydrophobic contacts.

P4

A luminescence assay for the determination of asymmetric dimethylarginine

Zhijing Zhao, Ming Zhang, Wenting Shen, Lianyan Song, Monan Zhang, Guang Hu*

Bioengineering Center, Shaoxing institute of Technology, College of Engineering, Peking University

Asymmetric dimethylarginine (ADMA), an L-arginine analogue which inhibits nitric oxide formation and thus causes endothelial dysfunction, is associated with various cardiovascular risk factors, metabolic diseases, and systemic or local inflammation. Currently, the ADMA level can be determined by HPLC, LC/MS, GC/MS and ELISA. Here we reported the development of a fast and highly sensitive assay for ADMA based on enzymatic conversion of ADMA into ornithine through L-citrulline by dimethylarginine dimethylaminohydrolase and ornithine carbamoyltransferase. The released carbamoyl phosphate was converted into ATP by carbamate kinase, which can be measured by luminance in the presence of luciferin and luciferase. The assay exhibited a good linearity over three log10 of ADMA concentration with a R2 greater than 0.995. The limit of detection, report range, recovery, and coefficients of variation of intra-assay and inter-assay, was 0.02 micromol/L, 0.1-2.5

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micromol/L, >95%, <3% and <8%, respectively. In comparison with a commercially available ELISA assay, excellent correlation was found in measuring ADMA in urine samples (n = 40, r = 0.95). The cross-reactivity in our assay for symmetric dimethylarginine and L-arginine was less than 0.02% and <0.2%, respectively. Overall, one 96 well-plate can be completed in less than one hour using 10 microliter of urine samples. We are currently optimizing the assay for measuring ADMA in plasma samples with promising preliminary results. Moreover, this assay can be easily adopted to determine the concentrations of L-Arginine and L-citrulline. If validated in larger clinical studies, this assay will allow ADMA level to be routinely tested in clinical lab settings.

P5
Homocysteine levels and endothelial dysfunction

Khromova N.,* Berkovich O., Bazhenova E., Vakhrameeva N., Zhloba A.

Federal Almazov Medical Recearch Center

Objective: Hyperhomocysteinemia is known as an independent risk factor of atherosclerosis, but the relationship between hyperhomocysteinemia and endothelial dysfunction among patients with premature coronary artery disease is not well studied. So, the aim of our study was to estimate association between hyperhomocysteinemia and endothelial dysfunction among patients with myocardial infarction (MI) under 45 years.

Materials and Methods: We examined 254 males with MI under 45 years (group 1) and 203 healthy males as a control group (group 2). Diagnosis of MI was verified by medical history and clinical, electrographical and biochemical dates. Physical examination consist of anthropometry (weight, height), blood pressure and heart rate registration. Homocysteine level, number of circulating endothelial cells (CEC), carotid intima media thickness and brachial artery endothelium dependent vasodilation were measured for all patients. Homocysteine level was determined by high performance liquid chromatography; quantitative measurement of CECs was performed by the method developed by J.Hladovec (1978); carotid intima media thickness was estimated by ultrasonic method; endothelium dependent vasodilation was evaluated by the method proposed by D.Celermajer (1992), modification by O.Pogorelova (1997) and O.Ivanova (1998).

Results: We observe no difference of homocysteine levels in both groups. In all groups homocysteine mean levels was normal. Among group 1 patients males with vasocontriction has homocysteine level higher that males with vasodilatation (15,6±1,5μmol/l and 11,7±0,8μmol/l, respectively; p=0,04). There are correlation between homocystene level and carotid intima media thickness (r=0,4; p=0,012) and correlation between homocystene level and number CECs (r=0,4; p<0,0001) in group 2.

Conclusions: This dates shows negative impact of homocycteine level on endothelium functional condition among patients with MI under 45 years.

P6
ADMA, a biomarker of vascular dysfunction, in an out-patient cohort at very high cardiovascular risk

Karl Florian Wintgens,* Natalia Kuzmina, Robert Klüsener, Jana Ruppert, Karl-Heinz Kellner, Franz Paul Armbruster, Christoph Melzer, Thomas Dschietzig

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ADMA, asymmetric dimethylarginine, is generated when methyl residues are transferred by protein arginine methyltransferases-1 and -2 to arginine originating from nutrition, protein catabolism, or other metabolites such as citrulline. ADMA is the most important endogenous inhibitor of all 3 different nitric oxide synthases. It has been shown to constitute a major link between oxidative burden and vascular endothelial dysfunction because the ADMA-degrading enzyme, dimethylarginine dimethylaminohydrolase, is inactivated by oxidative stress. Moreover, ADMA is an independent predictor of all-cause and cardio-vascular mortality.

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This cross-sectional study aimed at investigating plasma ADMA and its dependence of various clinical and biochemical factors in 225 patients carrying automatic implantable cardioverters/defibrillators (AICD) for primary or secondary prevention of sudden cardiac death, with 75% of them suffering from systolic heart failure, 69% from coronary artery disease (CAD), and 27% from type-2 diabetes (T2D).

Univariate analysis showed that ADMA levels were associated with plasma NT-proBNP, carbonyl protein (a marker of oxidative protein modification), and zonulin (an inhibitory regulator of epithelial/endothelial tight junctions), as well as with NYHA functional class, but not with high-sensitivity C-reactive protein (hsCRP), nitrotyrosine, left ventricular ejection fraction, pack years, severity of CAD, renal function (creatinine, eGFR), body mass index, weight, height, sex, or age. After multivariate analysis in a general linear model, the positive associations with NT-proBNP ($p = 0.001$) and carbonyl protein ($p = 0,044$) remained significant. In the subgroup of 51 individuals with T2D, multivariate analysis revealed a significant positive affection of ADMA by GFR class (KDIGO stadium) ($p = 0.025$) as well as by age ($p = 0.002$).

The finding of ADMA levels being associated with circulating carbonyl protein may support reports on ADMA's up-regulation by elevated oxidative stress; its elevation with NT-proBNP reflects vascular endothelial dysfunction in heart failure. Of note, there was no significant relation to hsCRP which illustrates the distinct regulation of these 2 biomarkers in cardio-vascular pathophysiology. In the (much smaller) subgroup of T2D patients, ADMA was affected by the severity of chronic kidney disease and age which is likely to indicate incremental vascular pathology.

P7

Increased alveolar epithelial expression of dimethylarginine dimethylaminohydrolase 2 in idiopathic pulmonary fibrosis

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Dimethylarginine dimethylaminohydrolase (DDAH), which metabolizes the endogenous Nitric Oxide Synthetase (NOS) inhibitor asymmetric dimethylarginine (ADMA) to citrulline and dimethylamine, is a crucial enzyme regulating NO production. Since inducible NOS (iNOS) activity is increased in lung fibrosis and the NO system has an impact on the severity of lung fibrosis in the bleomycin model, we analysed the expression of ADMA-DDAH in lung tissue from patients with Idiopathic Pulmonary Fibrosis (IPF) lungs lung transplant donors (Control) and from mice challenged with bleomycin. As compared to control, mRNA and protein expression analysis showed a 3-fold increase in DDAH2 and iNOS expression in IPF lungs, in absence of any significant change in DDAH1 expression. Moreover, by immunohistochemistry and double-immunofluorescence, a co-localization of both DDAH2 and iNOS in the type II cells of IPF could be demonstrated and increased peroxynitrite formation were observed in face of unchanged ADMA levels. Similar changes were observed in bleomycin challenged murine lungs, in which DDAH2 and iNOS expression and activity began to increase at day 7, increased up to day 21, and remained unchanged thereafter (up to d28). Immunohistochemical analysis revealed DDAH2 induction in type II epithelial cells and co-localization with iNOS. These data demonstrate that DDAH2 is upregulated in both, the murine model of pulmonary fibrosis and patients suffering from IPF and may play a pivotal role by guarding iNOS activity in this disease.

P8

Endogenous nitric oxide is not involved in the mechanisms of flow-dependent vasodilation in hypertensive rats

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Hypertension is characterized by endothelial dysfunction, associated with a progressive decrease in nitric monoxide (NO) bioavailability. It remains unknown which of vasodilator factors play a major role in stabilization of blood pressure in SHR via mediation of the flow-dependent vasodilation.

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Objective: Evaluation of the role of NO in the mechanisms of flow-dependent vasodilation in normotensive and SHR.

Methods: The experiments were performed on male Wistar rats (n=9), Wistar-Kyoto (WKY) rats (n=10), and SHR (n=15) anesthetized with pentobarbital (60 mg/kg). The abdominal aorta was cannulated and sequentially perfused with gradually increasing blood flow rates in the range from 2 to 6 ml/min. Each bout of perfusion lasted for 1 min. Subsequently, the pressure-flow charts were plotted, and arterial distensibility (AD), intravascular pressure stability (IPS), and hydraulic resistance (HR) of the hindquarters vascular bed were determined mathematically.

Results: The inhibition of nitric oxide (NO) synthase (NOS) with NG-nitro-L-arginine (L-NNA) at a dose of 10 mg/kg resulted in the increase in basal HR of the hindquarters vascular bed in all groups. It was associated with the decrease in the IPS. The obtained results provide further evidence for an important role of NO in the maintenance of the arterial pressure stability both in normo- and hypertensive rats. However, the major difference between SHR and normotensive rats in the setting of NOS inhibition was the paradoxical increase in AD in SHR in response to blood flow enhancement.

Conclusion: The involvement of NO in flow-dependent vasodilation in SHR is unlikely.

P9

Lack of association between asymmetric dimethylarginine and ascending aortic aneurysm

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Objectives: Thoracic aortic aneurysm (TAA) is a very heterogeneous group of disorders with strong genetic component. Traditional risk factors of cardiovascular diseases (CVD) are also discussed for TAA. Asymmetric dimethylarginine (ADMA), a nitric oxide synthase inhibitor and marker of endothelial dysfunction could play role in TAA development.

Methods: This study included 105 patients with ascending aortic aneurysm: 63 patients with tricuspid aortic valve (TAV) (m:f ratio 2,5:1, mean age 59±7 years, body mass index(BMI)= 28,8±4,3 kg/m2) ,24 patients with bicuspid aortic valve (BAV) (m:f ratio 2,4:1, mean age 53±10 years, BMI=28,3±5,6 kg/m2). Also 18 patients, who had tricuspid aortic valve, normal aorta and traditional risk factors of CVD(m:f ratio 1,6:1 ,mean age 56±7,BMI=30,4±6,3 kg/m2) and 17 healthy donors without risk factors (m:f ratio 2:1 ,mean age 22±1) were studied . Plasma levels of ADMA, symmetric dimethylarginine (SDMA) and homocystein (Hcy) were measured.

Results: There were no significant differences in thoracic aorta diameter (47.3 ± 3.6 mm in TAV vs. 46.5 ± 3.1 mm in BAV), ADMA and SDMA levels between TAA groups (fig.1). There also were no significant differences in ADMA and SDMA levels between patients in TAV group and patients with risk factors without aortic dilatation. But ADMA and SDMA were higher in all groups comparing with young control (p=0,000003 and p=0,00, respectively) with significant biomarker/age correlation (r=0,2, p=0,03 for ADMA and r=0,4, p=0,00 for SDMA).

Fig.1

Groups	ADMA, µmol/l M ± σ	SDMA, µmol/l M ± σ
TAV	0,45± 0,1	0,49± 0,14
BAV	0,49 ± 0,11	0,49 ± 0,12
Patients with risk factors	0,47± 0,13	0,48 ± 0,21
Young controls	0,27± 0,13	0,16 ± 0,1

Smoking was also associated with higher ADMA and SDMA levels (p=0,015,p=0,001)(fig.2)

Fig.2

Groups	ADMA, µmol/l M ± σ	SDMA, µmol/l M ± σ
Smokers	0,49± 0,11	0,54± 0,17
Non-smokers	0,43± 0,11	0,45± 0,11

ADMA and SDMA concentrations were associated with higher Hcy level (r=0,3, p=0,0007, r=0,5, p=0,00). Tendency to increase descending aortic diameter associated with ADMA and SDMA revealed in TAV group and in group with CVD risks without TAA (r=0,2, p=0,06, r=0,2, p=0,09).

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Conclusions: According to obtained data, ADMA and SDMA don't play important role in ascending aortic aneurysm development. This evidence confirms conception of non-atherosclerotic nature of such localization aneurysms. The main factors influence ADMA and SDMA levels were age and smoking. Tendency of increased ADMA and SDMA concentration was associated only with descending aortic aneurysm with due to other origin and therefore other pathogenesis.

P10
nNOS and DDAH1 expression in the ventral tegmental area

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Nitric oxide (NO) is an important signalling molecule. One way that NO's production is regulated is through the asymmetric dimethylarginine (ADMA)-dimethylarginine-dimethylaminohydrolase (DDAH) pathway, although this has not been thoroughly investigated in the brain. One role of NO in the brain is as a negative feedback signal in ventral tegmental area (VTA) dopamine neurons. In response to depolarisation, NO diffuses retrogradely and potentiates GABA release, which in turn inhibits dopamine neuron activity. We are using this system as a model to investigate the physiological role of DDAH1 as a regulator of NO production in the brain.

Although both DDAH1 and nitric oxide synthase (nNOS; which is the source of neuronal NO) are expressed in the VTA, it is not clear in which cell types. We, therefore, used immunohistochemistry to investigate nNOS and DDAH1 expression in the VTA of mice. In addition, we recorded mini inhibitory presynaptic currents (mIPSCs) onto VTA dopamine neurons in the presence of a nitric oxide donor (SNAP), as an alternative method for investigating NO's locus of action in potentiating inhibitory currents in dopamine neurons (previous studies have used evoked paired-pulse ratios.)

Firstly, differences in brightness and detail between DDAH1 staining in the VTA of wild-type and DDAH1 global knockout mice suggests that DDAH1 is expressed in this area, in the cell bodies of both dopaminergic and non-dopaminergic neurons. However, due to high background immunostaining this particular DDAH1 antibody does not lend itself to a more detailed analysis of DDAH1 positive cells in the VTA. nNOS appears to be more commonly expressed in non-dopaminergic neurons, but in close proximity to dopaminergic neurons. Secondly, the frequency, but not the amplitude, of mIPSCs increased in the presence of SNAP, consistent with the suggestion that NO induces presynaptic changes in GABA release.

To investigate DDAH1 and nNOS expression in the VTA more fully, we are in the process of experimenting with different DDAH1 antibodies, and looking at DDAH1 and nNOS gene expression in relation to VTA dopamine neurons using fluorescence in situ hybridization.

P11
Disorders of thiol metabolism in endothelial dysfunction

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Introduction: Endothelial dysfunction and accumulation of asymmetric dimethylarginine (ADMA) have been identified as independent predictors of future cardiovascular events in patients with coronary artery disease and endothelial dysfunction.
The aim of the study was to assess relationship between endothelial dysfunction, oxidative stress (OS) markers and parameters of thiol metabolism in patients with aortic dilatation (AD) and aortic valve stenosis (AS).

Material and Methods: We studied the specimens of blood plasma of 78 patients with AD and AS with elevated plasma level of ADMA and 34 healthy controls. OS was defined as an imbalance between prooxidants [the plasma concentration of thiobarbituric acid reactive substances (TBARS)] and antioxidants [the plasma activity of superoxide dismutase (SOD), SH-plasma level]. In addition we studied activity of glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Red) and glutathi

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one-SH concentration (GSH) in erythrocytes. Results. We estimated negative correlation (r= -0.270, p =0.035) between TBARS and SOD; TBARS and SH (r= -0.900, p=0.019). It was found that TBARS was higher [8.2(QIR 6.8-9.1)] µmol/L, vs. 6.34(QIR 5.6-7.2) µmol/L, SH- level was lower [159.9(QIR 75.0-219.0) µmol/L vs. 217.4(187.8-240.2) µmol/L]; SOD activity in plasma was lower [31.5(QIR 26.7-42.3) u/mL vs. 56,2(QIR 36,9-73,9) u/mL] in patients when compared with the healthy controls, but there were no considerable differences among patients with AD and AS. We estimated significant declines in activity of GSH-Px- [10.5(QIR 8.4-11.4) mmol/g Hb/min vs. 14.25(QIR 10.9-17.97) mmol/g Hb/min] and GSH-Red [1.16(QIR 0.95-1.61) mkmol/g Hb/min vs. 1.4(QIR 1.2-1.9) mkmol/g Hb/min] accompanied by falling GSH concentration 1.33(QIR 1.1-1.6) µmol/ml of packed cells vs. 1.82 (QIR 1.34-2.34) µmol/ml of packed cells] in erythrocytes of patients AD. We had defined significance positive bilateral correlation (r= 0.724, $p < 0.001$) between GSH concentration and activity GSH-Red in erythrocytes.

Conclusion. Our findings are in agreement with the point of view that ADMA initiates an oxidative damage by indirect suppression SOD and GSH-Px activity. Glutathione is the effective antioxidant and in RSNO state the donator of free NO and so is closely related to the metabolism of NO. Its level depends on the activity GSH-Px and GSH-Red in erythrocytes under OS. We expect that our data may be used as endothelial dysfunction characterization.

P12

The Separation and Quantitation of Methylated Arginine Analogues Using Ion Mobility – Mass Spectrometry

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Plasma ADMA has a narrow distribution within the nanomolar range. Small changes in plasma and intracellular ADMA concentrations have long been associated with large pathological changes. As such, there is a need for robust and accurate assays to measure small perturbances in the DDAH/ADMA pathway. Using guidelines set out by Teerlink any intra assay CV should be below 5% to measure small but significant changes and avoid potential Type II errors. Of the current measurements for ADMA only few claim to have sufficiently low CVs such as Capillary electrophoresis and High Performance Liquid Chromatography (HPLC) which require long separation times meaning potenitally long batch times. However HPLC-Mass Spectrometry is currently the gold standard in ADMA measurement.

Ion Mobility Spectrometry (IMS) is a rapidly expanding analytical technique for the separation of volatile and semi-volatile molecules. Small molecules separate in IMS in gas phase under the presence of a weak electric field according to their mass, charge, size and shape. Coupled with traditional existing Mass Spectrometry and ionisation techniques such as HPLC, IMS allows for the measurement of compounds of identical mass to charge ratio such as SDMA and ADMA that would otherwise need additional potentially lengthy separation.

We describe a technique which is robust and can be applied to any existing HPLC without the need for specialty expensive columns for separation and can therefore be quantitated in gradients of less than a minute.

P13

Liver and kidney are the major sources of AGXT2 in humans

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It has been shown in various epidemiological studies that increased levels of an endogenous inhibitor of nitric oxide synthases: asymmetric dimethylarginine (ADMA) and its structural analogue symmetric dimethylarginine (SDMA) are associated with cardiovascular diseases. ADMA can be hydrolysed by dimethylarginine dimethylaminohydrolase (DDAH). It can also be metabolized through an alternative pathway by alanine:glyoxylate aminotransferase 2 (AGXT2), which converts ADMA to α-keto-δ-(N,N-dimethylguanidino)valeric acid (DMGV). Previous Northern Blot and in-situ RNA-hybridisation studies identified the kidney as the major organ of Agxt2 expression in rats, while RT-PCR and Western Blot suggested that Agxt2 is expressed in the mouse kidney and liver at comparable levels. In order to better understand the physiological role of AGXT2 we analysed the expression of the enzyme in human tissues. We performed immunohistochemical staining in both frozen and paraformaldehyde-fixed samples from a normal tissue bank using a custom made and characterized rabbit polyclonal anti-AGXT2 antibody. We observed the strongest expression of AGXT2 in the proximal convoluted tubule of the kidney and in the periportal zone of the liver with a homogenous pattern throughout all the samples. Additionally we see weak staining in the skeletal and heart muscle. Small intestine, lungs and aorta are negative for AGXT2 expression. We are currently confirming our immunohistochemistry data by RT-PCR and measuring the tissue levels of AGXT2 activity as well as the tissue concentrations of the common AGXT2 substrates and products. Our current data suggest that both hepatocytes and kidney tubular epithelial cells are the major sources of AGXT2 in humans.

P14

ADMA reduction does not protect mice with streptozotocin-induced diabetes mellitus from development of diabetic nephropathy

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Background: Diabetic nephropathy is the major cause of end stage renal disease in the Western countries. Association studies suggest that asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthases (NOS), may play an important role in the pathogenesis of diabetic nephropathy. Solid direct evidence is, however, still lacking. The goal of this study was to test the hypothesis that lowering systemic ADMA levels leads to amelioration of diabetes-induced functional and structural abnormalities of diabetic nephropathy in a murine model of type I diabetes mellitus.

Methods: Diabetes was induced with a single injection of streptozotocin (STZ; 180mg/kg bw) in C57BL/6 wild type and DDAH1 transgenic (DDAH1-Tg) mice. Healthy mice served as age matched controls. Mice were sacrificed after 20 weeks of diabetes. Functional data, ADMA levels and histology were assessed.

Results: Transgenic overexpression of DDAH1 led to a significant decrease in plasma ADMA levels in healthy (0.57 ± 0.11 vs 0.37 ± 0.04; $P<0.05$) and diabetic mice (0.43 ± 0.12 vs 0.25 ± 0.8;$P<0.001$). However, diabetes did not increase plasma ADMA levels. Diabetic mice developed and albuminuria (27 and 25 vs. 9 and 6 µg albumin/mg creatinine). By histology, diabetic kidneys showed a slight increase in matrix expansion as assessed on silver stainings and a clearly enhanced glomerular proliferation rate (0.47 and 0.54 vs. 0.13 and 0.07 cells/glomerular cross-section; $P<0.05$). Lower ADMA levels in DDAH1-Tg mice did not prevent these changes. Compared to wt mice, DDAH1-Tg mice showed a reduced number of Mac2 positive cells in the kidney.

Conclusions: In summary, STZ-induced diabetes led to the development of early features of diabetic nephropathy. Overexpression of DDAH1 and subsequent lowering of systemic ADMA levels did not prevent these changes, indicating that ADMA is not the major mediator of diabetic nephropathy in this experiment model.

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P15

DDAH-1 and ADMA regulate proximal tubular fluid of reabsorption: comparison of pharmacological blockade of DDAH-1 with gene knockdown

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Background: proximal tubule (PT) reabsorption of Na+ and fluid is enhanced by NO but diminished by knockout of NOS-I or III. ADMA is an endogenous inhibitor of NOS, but its effects on tubular function are unknown. ADMA is degradated by DDAH whose type 1 isoform (DDAH-1) is predominantly and richly expressed in the PT. SNIPs of DDAH-1 predicted the rate of decline of glomerular filtration rate (GFR) in patients with chronic kidney disease (CKD) suggesting that PT DDAH-1 may regulate the progression of renal disease.

Hypothesis: We tested the hypothesis that DDAH-1 metabolizes ADMA in the PT thereby reducing its plasma concentration and enhancing NO synthase (NOS) activity and PT reabsorption of fluid (Jv).

Methods: Jv was measured in anesthetized rats and mice in S2 segments of the PT by direct in vivo micropuncture and microperfusion of artificial tubular fluid (ATF).

Results: Addition of, L-257 (10^{-4} M; selective DDAH-1 inhibitor) versus vehicle to ATF perfusing the PT of rats for 5-10 mins did not affect Jv (3.4 ± 0.20 vs 3.1±0.39 nl/min/mm). However, a bolus i.v. injection (60mg/kg) of L-257 2hrs before and 5-10 mins of PT perfusion of L-257 significantly ($P< 0.005$) reduced Jv by 33% to 2.3±0.17 nl/min/mm and increased plasma ADMA (systemic Veh: 0.46 ± 0.030 vs. systemic L-257: 0.67 ± 0.029 µmol/l; $P < 0.0001$) without changing plasma SDMA. Microperfusion of ADMA (10-4 M) or L-NAME (10^{-4} M) into the PT of rats both reduced Jv significantly by >40% to 2.0±0.24 and 1.8±0.22 nl/min/mm, respectively ($P<0.05$) three days later. A rapid intravenous injection of 1 ml of saline containing Transit and siRNA targeted to DDAH-1 (hydrodynamic method for in vivo gene silencing in mice) reduced Jv significantly by 36% compared to scrambled siRNA (2.2 ± 0.16 vs 1.4 ± 0.26 nl/min/mm; $P<0.05$). Surprisingly, PT cell-specific DDAH-1 knockdown in mice did not affect Jv significantly compared to wild type (PTC-D1WT: 1.7 ± 0.21 vs PTC-D1KO: 2.0 ± 0.15).

Conclusions: PT fluid reabsorption is regulated by ADMA and its tubular metabolism by DDAH-1. The two-hour delay in the effects L-257 to diminish Jv likely can be ascribed to the time required after inhibition of DDAH-1 for accumulation of sufficient tubular ADMA to impair PT reabsorption. Whereas systemic DDAH-1 gene knockdown was as effective as systemic DDAH-1 blockade with L-257 in reducing Jv in the PT, lifelong deletion of PT DDAH-1 was ineffective, suggesting robust adaptive mechanisms that eventually restore PT reabsorption. Thus, L-257 is a novel regulator of PT function. SNIPs that alter DDAH-1 in the PT may change renal function and contribute to CKD progression.

Supported by grants from the NIH and the MRC.

Oral Abstracts

O1
Arginine Methylation – Enzymes, Binders & Inhibitors

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We have developed a protein domain microarray approach to screen for “readers” of histone tail marks and non-histone proteins. Recently, using this approach, we identified Tudor domain containing protein 3 (TDRD3) as a major methyl-arginine effector molecule that recognizes methyl-histone marks and facilitates gene transcription. However, TDRD3 has no enzymatic activity of its own and the underlying mechanism by which it functions as a transcriptional coactivator is unknown. To address this issue, we identified topoisomerase IIIB (TOP3B) as a component of the TDRD3 complex. TDRD3 serves as a molecular bridge between TOP3B and arginine-methylated histones. The TDRD3-TOP3B complex is recruited to the c-MYC gene promoter primarily by the H4R3me2a mark, and the complex promotes c-MYC gene expression. TOP3B relaxes negative supercoiled DNA and reduces transcription-generated R-loops in vitro. TDRD3 knockdown in cells increases R-loop formation at the c-MYC locus, and Tdrd3-null mice exhibit elevated R-loop formation at this locus in B cells. Tdrd3-null mice show significantly increased c-Myc/Igh translocation, a process driven by R-loop structures. By reducing negative supercoiling and resolving R-loop, TOP3B promotes transcription, protects against DNA damage, and reduces the frequency of chromosomal translocations.

In a separate project, we are using our domain microarrays to identify and characterize small molecules that can inhibit methyl-dependent protein-protein interactions and function as drug-like epigenetic modulators. We have determined that the UNC1215 compound, developed by Stephen Frye’s group, binds a small subset of MBT, Tudor and chromo domains. Using biotinylated forms of a UNC1215 analog library, we are now identifying compounds with greater specificity for distinct methyl-reading domains.

O3
A novel loss-of-function DDAH1 promoter polymorphism is associated with increased susceptibility to thrombosis, stroke and coronary heart disease

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Asymmetrical dimethylarginine (ADMA), an endogenous arginine analogue, inhibits nitric oxide synthases and plays an important role in endothelial dysfunction. In the present study, we tested whether a novel genetic variant in dimethylarginine dimethylaminohydrolase 1 (DDAH1), an important ADMA hydrolyzing gene, was associated with stroke and coronary heart disease (CHD) susceptibility in the Chinese Han population. By resequencing, we identified a novel 4-nucleotide deletion/insertion variant in the DDAH1 promoter. The insertion allele disrupted binding of metal-regulatory transcription factor 1, which resulted in significant reduction of in vitro DDAH1 transcriptional activity and in vivo DDAH1 mRNA level, and in turn, increased plasma ADMA level and the ratio of ADMA to L-arginine. We initially genotyped the polymorphism in 1388 stroke patients and 1027 controls as well as 576 CHD patients and 557 controls and then replicated our study in additional independent case-control cohorts comprising 961 stroke patients and 822 controls and 482 CHD patients and 1072 controls. We identified that the -396 4N ins allele was significantly associated with increased risk of thrombosis stroke and CHD after adjusting for environmental factors in both samples for both diseases (thrombosis stroke discovery set: odds ratio [OR]=1.35, P=0.032; replication set: OR=1.51, P=0.006; CHD discovery set: OR=1.45, P=0.035; replication set: OR=1.47, P=0.003). Our results suggest that the DDAH1 loss-of-function polymorphism is associated with both increased risk of thrombosis stroke and CHD.

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O4

Endothelial function and MTHFR gene polymorphisms in postmenopausal patients with hypertension

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Menopause is known to be associated with development and progression of cardiovascular risk factors and diseases in females. Pathogenesis of arterial hypertension (AH) in postmenopausal women is characterized by high peripheral vascular resistance and early remodeling of cardiovascular system. Endothelial dysfunction is probably the "first step" of cardiovascular continuum. Homocystein (HC) is one of the markers of functional state of endothelium, it also affects platelets aggregation. Study objective: to evaluate MTHFR genes polymorphisms in association with HC levels, parameters of endothelial function and blood pressure (BP) levels in hypertensive postmenopausal women. Material and methods. C677T, G1793A and A1298C MTHFR genes polymorphisms were studied in 150 postmenopausal women with AH and in 100 healthy women (50 pre- and 50 postmenopausal). Results and conclusions. HC levels were higher in hypertensive females than in healthy women (12,4±0,9 vs 7,4±0,4 μmol/L, p=0.002). Hypertensive postmenopausal women had higher HC levels than hypertensive premenopausal females (13,7±1,1 vs 9,7±0,4 μmol/L, p=0,03). Direct correlations were found between HC, BP levels and some parameters of endothelial function. C677T MTHFT gene polymorphisms: C677-allele frequency in hypertensive postmenopausal women was 0,71, 677T-allele frequency was 0,29 (in general population it is 0,76 and 0,24 accordingly). Carriers of 677T-allele had higher levels of BP, bigger size of left ventricle and increased platelet aggregation parameters than patients with CC genotype. G1793A MTHFT gene polymorphisms: G1793-allele frequency was 0,90, 1793A-allele frequency was 0,10. Presence of 1793A-allele was associated with higher level of diastolic BP (105,8 3,0 vs 95,2 1,3 mm Hg, p=0,009) than in patients with GG genotype. A1298C MTHFT gene polymorphisms: A1298-allele frequency was 0,61, 1298 C-allele frequency was 0,39 in hypertensive postmenopausal females. No association was revealed between A1298C MTHFT gene polymorphisms and BP levels or parameters of endothelial function. Postmenopausal women with AH are characterized by specific features of hemodynamics and pathogenesis: high level of total peripheral vascular resistance associated with endothelial dysfunction (impaired endothelial-derived vasodilatation, high level of HC and circulating markers of endothelial dysfunction). Endothelial function differs in post- and premenopausal women. Endothelial-derived vasodilatation was lower and levels of HC and and circulating markers of endothelial dysfunction were higher in postmenopause both in healthy women and patients with AH. Besides, hypertensive postmenopausal females had higher levels of platelets aggregation. An association was found between presence of different alleles of MTHFR genes and BP levels, endothelial function and platelet aggregation parameters.

O5

Time course and relative contribution of acetylcholine-induced NO-, PGI2-, and EDHF-mediated cutaneous vasodilation in humans.

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Endothelium-derived hyperpolarizing factor (EDHF) currently remains the most poorly studied endothelial vasodilator. Evaluation of activity of EDHF can be done through other vazodilators «dual block» of the synthesis of nitric oxide and prostacyclin nitro-L-arginine and inhibitors of COX or, in contrast with, the block of the synthesis EDHF introduction tetraethylammonii chloride (TEA). Traditionally vasoactive substances blockers are injected intraarterially or intravenously producing systemic effect. We conducted a study in 30 healthy volunteers using ionophoretic administration of nitro-L-arginine inhibitor of COX (diclophenac) and TEA combined

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with ionophoresis of acetylcholine. In the test with acetylcholine in all patients there was a significant ($p < 0.05$) increase in blood flow from the 1st minute after the procedure, which reached its maximum value (146% of baseline) at 2 minutes. In the test of nitro-L-arginine, diclophenac sodium and acetylcholine increase of tissue perfusion lagged and only began with the 2nd minute registration. Tissue blood flow reaches the maximum value for 3 minutes, making 132% of the background, and returned to baseline by 5 minutes. In the test of TEA and acetylcholine the flow rate started to increase after 1 minute registration, reached a maximum at 2 minutes (142% of the background), and then begins to decline significantly faster than in the test with acetylcholine. At the 4th minute the difference from baseline became already significant.The data obtained allow to conclude that both methods (block EDHF and block of NO-synthase activity plus COX) allow to evaluate the contribution of EDHF in endothelium-dependent vasodilation. Results obtained by applying these methods complement each other. Conclusion. Ionophoresis of blockers of vasoactive substances with the following measurement of dynamics of blood flow in the skin can be used to assess the contribution of EDHF in endothelium-dependent vasodilation.

O6
The plasma profile of metabolites influencing nitric oxide production

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Introduction: Plasma metabolome depends on cellular metabolism and creates conditions for endothelial generation of nitric oxide by influencing directly on endothelial NO-synthase (eNOS) and/or through mitochondria on eNOS subunits aggregation to active state.

The aim of this study was to evaluate plasma metabolite shifts directly modulating NOS activity on the one hand and associated with mitochondrial metabolism on the other hand in cardiovascular disease (CVD) patients with endothelial dysfunction.

Material and Methods: Plasma samples from 122 patients with CVD and 40 donors have been used to study the profile of the 20 amino acids, 3 methylated derivatives of basic amino acids, 2 aminothiols, nitric oxides and nitrosothiols levels. Amino acids profiles have also been studied in patients (n = 34) with a prothrombotic state. 68 plasma species of CVD patients and 22 donors were used for evaluation of homocysteine binding to plasma proteins with Mr> 300 kDa. All samples used in this retrospective study were characterized by other routine data. SAS Statistical Software version 9.3 was applied to obtain descriptive statistic data, compare values (Mann-Whitney's test) and assess correlations.

Results: In CVD patients, including 57 with aortic dilatation and 48 with aortic valve stenosis increased level of asymmetric dimethylarginine (ADMA) compared with donors ($p < 0.001$) was found. In 17 hypertensive patients without end-organ damage this difference was not significant ($p = 0.15$). Increasing symmetric dimethylarginine (SDMA) and decreasing trimethyllysine (TML) levels comparing to donors' samples was detected in all these three subgroups ($p < 0.0001$, for each). Correlation between ADMA and SDMA values in all three subgroups ($R=0.38$, $p=0.004$; $R=0.39$, $p=0.007$; $R=0.46$, $p=0.053$), but not for TML with each ones were established. Observed changes were accompanied by Ala, Gly and branched chain amino acids positive shifts and as to Arg, Citrulline pair, upward trend of the last one was detected. In CVD patients with moderate hyperhomocysteinemia, an increase of homocysteine binding by proteins with molecular weight above 300 kDa was observed. The significant increasing of the level of NOS activity products was revealed in samples from CVD patients, but no correlations with the levels of methylated arginine derivatives or the amino acids rates were observed. It should also be noted elevated C-reactive protein in these patients.

Conclusion: Plasma metabolic shifts in studied CVD patients were characterized by: 1) an imbalance of sulfur amino acids and increased homocysteine transport with high molecular mass proteins, 2) a positive shifts of amino acids metabolized in the mitochondria, 3) a significant changes in metabolism of methylated derivatives of basic amino acids, including an increase in ADMA and SDMA and reduced formation of TML.

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O7

Fibrinolysis-induced shifts in plasma amino acid profile: possible contribution to vascular metabolism

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Background: Components of the coagulation and fibrinolytic systems are proteases with different substrate specificity that may activate some exopeptidases, which can produce some amounts of free amino acids derived from fibrin, its degradation products or other proteins.

Aims were the estimation of coagulation/fibrinolysis-induced modification of broad amino acid profile in plasma samples of healthy persons and patients with cardiovascular pathology.

Materials and methods: Fresh platelet-poor citrated individual plasma samples from 30 patients with cardiovascular diseases (CVD), 18 donors and 14 pools from patients with normal total homocysteine level have been investigated. Coagulation and subsequent fibrinolysis were initiated by addition of thrombin (or tissue factor) and tissue plasminogen activator, respectively. Amino acid profile before and after completion of coagulation/fibrinolysis was evaluated by RP-HPLC using C18 column after OPA-derivatization. The parameters of fibrinolysis were evaluated by the clot turbidity assay at 405 nm.

Results: Coagulation/fibrinolysis in the human plasma is associated with a significant increase in the concentrations of some amino acids, including serine, alanine, arginine, valine, methionine, leucine, and lysine ($p<0.001$ for all); but only three of them, namely Arg, Lys, and Met are elevated so much that their concentrations exceed the reference boundaries. The duration of fibrinolysis significantly correlated with the degree of increase of basic amino acids arginine and lysine: $rS=-0.447$ ($p=0.012$) and -0.495 ($p=0.008$), respectively, but not with methionine concentrations. The degree of increasing the concentration of Lys and Met in CVD patients was significantly reduced compared with donors ($p<0.0001$ and $p=0.006$, resp.).

Conclusions: At metabolomics studies with amino acid profiling it should be considered the possibility of a substantial modification of amino acid profile due to the activation of proteolytic processes such as coagulation and fibrinolysis during sample preparation.

The clinical significance of elevated concentrations of Arg, Lys, and Met is explained, primarily, by the fact that arginine is a substrate of endothelial nitric oxide synthase (eNOS) which provides adequate vasodilation. Lys and Met can metabolically participate in vascular wall remodeling, being the precursor of polylysines and suppliers of C1-fragments.

O8

The factors of mitochondrial dysfunction and their association with ADMA

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Introduction: Elevated levels of homocysteine and methylated arginine derivatives ADMA and SDMA are cardiovascular risk factors. Endothelial dysfunction and impairment of blood circulation may cause secondary mitochondrial dysfunction.

The aim of the present study was to evaluate parameters of mitochondrial dysfunction (MD) and methylated amino acid metabolism in patients with thoracic aortic aneurysms (TAA) and aortic stenosis (AS) complicated by arterial hypertension.
Material and Methods. The results of testing blood plasma samples from patients (n=94, from 30 to 77 years, mean age in group 59.2±8.6 years, 62 males) were compared with those of healthy donors (n=34, from 18 to 25 years, 7 males). Patients with TAA (n=69, 52 males) and AS (n=25, 10 males) were comparable by age. The concentrations of lactic (LA) and pyruvic acids (PA), cytochrome C (CytC, ELISA), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1a, ELISA), ADMA (HPLC), SDMA (HPLC) and total homocysteine (tHcy, HPLC) were determined. Statistical analysis was performed using SAS

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Statistical Software version 9.3 and included comparison (Mann-Whitney's test) analysis. A p value <0.05 was considered significant.

Results: The patients were characterized of elevated levels of LA (p<0.0001), PGC1a (p<0.0001), ADMA (p<0.0001), SDMA (p<0.0001), tHcy (p<0.0001) and higher values of LA/PA (p=0.0002) and SDMA/ADMA (p<0.0001) ratios in comparisons with donors. Evaluation of the CytC level in patient species for excluding influence of physiological apoptosis during the menstrual cycle as comparison group were used healthy donors from 56 to 61 years (n=20, 10 male) data. Concentration of CytC also was higher in patients group (p=0.0026). The patients with TAA and AS differed only in values SDMA/ADMA ratios (p=0.05) and this parameter was higher in group patients with TAA. All patients were apportioned into two groups: with level of ADMA above and below than 95 percentile of ADMA concentration in donors. The patients with high levels of ADMA (n=30) had higher levels of PGC1a (p=0,028) and LA/PA values (p=0,033) than patients with lower ADMA (n=64).

Conclusion: In patients with aortic dilatation and aortic valve stenosis complicated by arterial hypertension found higher levels of ADMA, SDMA, tHcy and metabolic shifts typical for MD. The strongest differences in MD parameters with donors detected in concentrations proteomic markers especially CytC. It should be noted that Cyt C and PGC1a characterize MD from different angles as indicators of mitochondrial permeability and biogenesis efficiency respectively. The most informative metabolomic marker of MD in present study was LA. Thus, in patients with high risk of cardiovascular events is advisable to monitor not only endothelial dysfunction markers but also markers of MD.

O9

Role of microRNA-126 in Cell-Cell Signalling in Asymmetric Dimethylarginine induced endothelial dysfunction

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Introduction: Asymmetric Dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS), is elevated in patients with chronic kidney disease and is known to contribute to endothelial dysfunction and cardiovascular disease in this patient population. It remains unclear if microRNAs, small regulatory noncoding RNA molecules, have an impact on the detrimental effects of ADMA concerning vascular injury. We investigated the role of microRNA-126, which is essential for endothelial development and biology in ADMA induced endothelial dysfunction.
Methods: We measured ADMA-Plasma levels in 39 patients with coronary artery disease (CAD) by ELISA assay and correlated them with circulating miR-126 levels assessed by qRT-PCR. We infused ADMA into healthy rats (250µMol ADMA/kg/day) and healthy human volunteers (0.1mg ADMA/kg/min, 40 minutes) and quantified plasma levels of different microRNAs. We investigated the underlying molecular mechanisms in vitro by using HUVECs. Stimulating them with ADMA, we quantified extra-and intracellular levels of miR- 126. Furthermore we validated our findings by using another eNOS-Inhibitor L-NAME (50 µMol). To get insight into biogenesis level of microRNA-126 we quantified pri-miR-126 after ADMA treatment. Transcriptional activation was assessed by electrophoretic mobility shift assay (EMSA). Immunefluorescence staining and confocal microscopy was performed to detect multivesicular bodies and exosomes after ADMA and L-NAME treatment.

Results: Plasma Levels of ADMA in patients with coronary artery disease correlated inversely with levels of circulating levels of microRNA-126 ($r = -0.52$; $p < 0.001$). ADMA infusion reduced circulating levels of miR-126 in rats. Levels of circulating miR-126 in healthy human volunteers were significantly decreased at 40 minutes after ADMA infusion ($p < 0.05$), whereas other microRNAs were not significantly altered. We could detect a time dependent alteration of extra- and intracellular amounts of miRNA-126 (extracellular: decrease 24h compared to CTL $p < 0.05$; intracellular: increase 2h compared to CTL $p < 0.05$) in vitro. Stimulation with L-NAME showed a significant reduction of extracellular levels of miR-126 although intracellular levels stay unchanged. Changes of

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intracellular levels of miR-126 were related to transcriptional activation after 24 h but not after 20 min and 4 h by ETS-1, which is known to regulate miR-126 expression. Consistent to these findings intracellular pri-miR-126 levels were significantly increased only after 24 h stimulation with ADMA. Confocal microscopy of CD 63 positive multivesicular bodies (MVB) and exosomes revealed an obvious change in MVB structure due to fusion and/or swelling of the vesicles. This was detectable after ADMA and L-NAME stimulation.

Conclusion: We were able to show that, miR-126, which is known to convey alarm signals to injured endothelium and thereby contribute to repair mechanisms and cellular survival, is regulated by ADMA. Therefore, detrimental effects of ADMA on endothelial function might be partly mediated by miR-126. Observed effects seem to be a consequence of altered miR- 126 trafficking through exosomes/microvesicles impaired by the inhibition of the endothelial NO-Synthases.

O10
Metabolism of methylarginines

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The endogenous guanidine-methylated analogues of L-arginine, NG–monomethyl–L–arginine (NMMA), asymmetric NG,NG–dimethyl–L–arginine (ADMA) and symmetric NG,N'G–dimethyl–L–arginine (SDMA), have been recognized as markers and potentially mediators of cardiovascular diseases. NMMA, ADMA and SDMA are derived from the proteolysis of the proteins that are methylated on arginine residues by protein arginine N-methyl transferases (PRMTs) Two distinct PRMT activities have been identified in mammalian tissues. PRMTs type 1 monomethylate and asymmetrically dimethylate arginine residues within proteins, whereas PRMTs type 2 monomethylate and symmetrically dimethylate arginine residues within proteins. PRMTs type 1, therefore, are the major sources of ADMA, while PRMTs type 2 generate SDMA. Endogenous methylarginines could be excreted by kidney in the unchanged form or further enzymatically catabolized. The major pathway of NMMA and ADMA catabolism is there hydrolysis to L-citrulline and methylamines by dimethylarginine dimethylaminohydrolases (DDAHs). There are two isoforms of DDAH (DDAH1 and DDAH2) in mammals, each encoded by a separate gene. DDAH1 is thought to play the major role in ADMA hydrolysis to citrulline, while the relative contribution of DDAH2 to ADMA metabolism remains controversial. None of the DDAH isoforms is able to metabolize SDMA. An alternative pathway of metabolism of methylarginines is their conversion to the corresponding N-methylated derivatives of α-keto-δ-guanidinovaleric acid. AGXT2 can utilize all the three endogenous methylarginines as substrates. Downregulation of AGXT2 leads to elevation of systemic levels of ADMA and SDMA, which suggests that endogenous AGXT2 is required for regulation of their homeostasis. The third, presumably minor, pathway of metabolism of endogenous methylarginines, is their Nα-acetylation. The enzyme responsible for this reaction still needs to be identified. Further investigation of metabolism of endogenous methylarginines should lead to better understanding of their roles in normal and pathological conditions and potentially to development of novel therapeutic approaches to modulation of their levels.

O11
The role of ADMA in the pathogenesis of myocardial ischemia-reperfusion injury in diabetes mellitus: focus on microRNA

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Recent epidemiological studies have demonstrated that elevated levels of asymmetric dimethylarginine (ADMA) are associated with increased risk of ischemic heart disease. To date, it remains to be determined whether the increased ADMA level plays a causative or merely biomarker role in myocardial ischemia-reperfusion injury.

In this study, we tested the hypothesis that genetically determined decrease in ADMA level may modulate myocardial ischemia-reperfusion injury (IRI) both in the intact and type 1 diabetic mice. Myocardial expression of ADMA-dependent

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microRNA-21 in the settings of myocardial IRI with and without type 1 diabetes mellitus (T1DM) was also analyzed in the animals with normal or decreased ADMA level.

Experimental groups were: 1) wild type mice (WT, n = 9), 2) WT with streptozotocin-induced T1DM (WT T1DM, n = 5), 3) dimethylarginine dimethylaminohydrolase (DDAH) transgenic mice (DDAH Tg, n = 7), 4) DDAH Tg with T1DM (DDAH Tg T1DM, n = 6). Under pentobarbital anaesthesia and mechanical ventilation, the left coronary artery was occluded for 45 min followed by 90-min reperfusion. Infarct size was determined histochemically using triphenyltetrazolium chloride staining and quantified blindly. Myocardial expression of microRNA-21 was studied using in situ hybridization technique.

There were no inter-group differences in anatomical area at risk. Infarct size averaged 37 ± 13.4 % in WT group, being significantly smaller in WT T1DM group (17 ± 7.2%, p=0.012 vs. WT). Infarct size was not different from WT in DDAH Tg and DDAH Tg T1DM groups and averaged 26 ± 8.5% and 25 ± 9.2%, respectively. Myocardial expression of microRNA-21 was unaffected by either treatment.

In conclusion, decreased ADMA level in mice overexpressing ADMA-metabolizing enzyme DDAH failed to protect the myocardium from IRI in intact and diabetic mice. Diabetes itself may be cardioprotective in the experimental settings, suggestive of the phenomenon of metabolic preconditioning. MicroRNA-21 expression in the heart was not changed in response to ischemia-reperfusion and/or decreased ADMA level.

O12
HNF4a is the major regulator of AGXT2 expression in liver

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Background: Recent studies demonstrated that alanine:glyoxylate aminotransferase 2 (AGXT2) is involved in metabolism of both endogenous methylarginines and lipids, suggesting potentially important role of this enzyme in cardiovascular and renal pathology. However, the regulation of AGXT2 expression and enzymatic activity in healthy and pathological conditions remains unknown. Our pilot experiments suggested that the region of the murine *Agxt2* promoter, harboring putative hepatic nuclear factor 4 alpha (HNF4a) binding site, is required for *Agxt2* transcription. The goal of the current study was to test the hypothesis that HNF4a is the major regulator of AGXT2 expression.

Methods and results: We generated a series of *Agxt2* promoter / luciferase reporter constructs, expressed them in the murine hepatic cell line Hepa 1-6 and assessed the reporter activity in the cell lysates. Constructs with mutations in putative HNF4a binding site showed an average of 80% decrease in the reporter activity in comparison with the constructs containing the wild type promoter sequence ($p<0.001$). Transfection of Hepa 1-6 with Hnf4a-specific siRNAs led to 40% ($p<0.001$) reduction in *Hnf4a* mRNA level and 50% ($p<0.001$) reduction in *Agxt2* mRNA level as assessed by qPCR. Finally, we used in vivo mouse model to demonstrate that inducible liver-specific *Hnf4a*-knockout results in 90% ($p<0.001$) decrease in liver *Agxt2* expression and 85% ($p<0.01$) decrease in liver Agxt2 activity.

Conclusions: Our study identified HNF4a as the major regulator of *Agxt2* gene expression in the mouse liver. This finding suggests potential novel mechanisms of how HNF4a may play a role in cardiovascular disease, e.g. by regulation of AGXT2-mediated metabolism of methylarginines, nitric oxide and lipids.

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O13
Is There a Pathway for ADMA degradation Alternative to DDAH?

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Background: Asymmetric dimethylarginine (ADMA) is a naturally occurring amino acid detected in the bloodstream. ADMA is an endogenous inhibitor of nitric oxide synthesis, and its plasma level (P-ADMA) is elevated in patients with stable angina (SA), being a molecular marker for and a mediator of endothelial dysfunction in those patients.

ADMA is thought to be broken down mostly by dimethylarginine dimethylaminohydrolases. We suggested that an alternative, namely, demethylation pathway may exist for ADMA disruption, in which cyanocobalamin (Ccbl) acts as an acceptor of methyl groups from ADMA.

Aim: To check if Ccbl intake may lead to P-ADMA decrease and associated elevation of plasma methylcobalamin (P-MetCbl) in SA patients different from controls.

Methods: 36 SA patients (P-ADMA:) and 34 age- and gender-matched controls were given Ccbl orally at 500 µg 2x a day for 2 weeks. P-ADMA and P-MetCbl were analysed on the supplementation days 0 and 15 by HPLC.

Results: In the control subjects, the intake of Ccbl has lowered P-ADMA from 0,55±0,07 µM/L to 0,42±0,05 µM/L (by 23,6%, p<0,05) and increased P-MetCbl from 297±69 ng/L to 592±97 ng/L (by 99,3%; p<0,05).
In SA patients, P-ADMA decreased from 0,74±0,09 µM/L to 0,45±0,07 µM/L (by 39,2%; p<0,05) and P-MetCbl rose from 197±82 ng/L to 772±110 ng/L (by 291,9%; p<0,05).

Discussion and conclusion: Supplementation with Ccbl results in a decline of P-ADMA in both control and SA groups (more markedly in SA). This was accompanied by a substantial increase of P-MetCbl (much higher in SA), while the elevation of plasma level of 5'-deoxyadenosylcobalamine was not significant. The findings suggest the existence of the ADMA demethylation pathway that may merit a further therapeutic exploring, for the pathway is more active in SA patients than in controls, and Ccbl substantially reduces the total number of angina attacks in the study group within the 3 month follow-up period from 43 (registered during the pre-supplementation 3 month period) to 14.

O14
Disruption of renal ADMA metabolism leads to salt-sensitive hypertension

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Background: Asymmetric dimethylarginine (ADMA) is found at increased concentrations in patients with renal failure. High ADMA levels have been implicated as a factor underlying the increased cardiovascular (CV) risk in chronic kidney disease (CKD). Although increases in ADMA have been proposed to reduce vascular NO bioavailability, experimental studies have failed to demonstrate endothelial dysfunction at the ADMA concentrations observed in CKD. As NO also regulates tubular sodium handling, an alternative explanation for ADMA effects is through a salt-dependent mechanism. We have recently demonstrated that the predominantly renal tubular enzyme alanine-glyoxylate aminotransferase-2 (AGXT2) plays an important role in regulating circulating ADMA concentrations. Therefore we examined the detailed CV phenotype in AGXT2 knockout (KO) mice.

Methods: Telemetry probes were implanted in AGXT2 KO and WT animals under general anaesthetic at 12-14 weeks of age. After recovery, continuous BP recordings were performed for 24-48 hours at baseline on a standard (0.3% sodium) diet and then again following 2 weeks of a low-sodium (0.03%) diet. Ex-vivo vascular responses to endothelial dependent agonists were also explored.

Results: There was no evidence of impaired endothelial-dependent dilatation in arteries from AGXT2 KO mice. Mean systolic BP was 8.8mmHg (0.6-17.0; P<0.05) higher in AGXT2 KO vs WT mice at baseline. Overall systolic BP fell by a mean of 2.1mmHg (1.8 - 2.5; P<0.005) on a low-sodium diet but there was an additional 7.3mmHg (6.7-7.9; P<0.005) fall in the AGXT2 KO mice (Figure).

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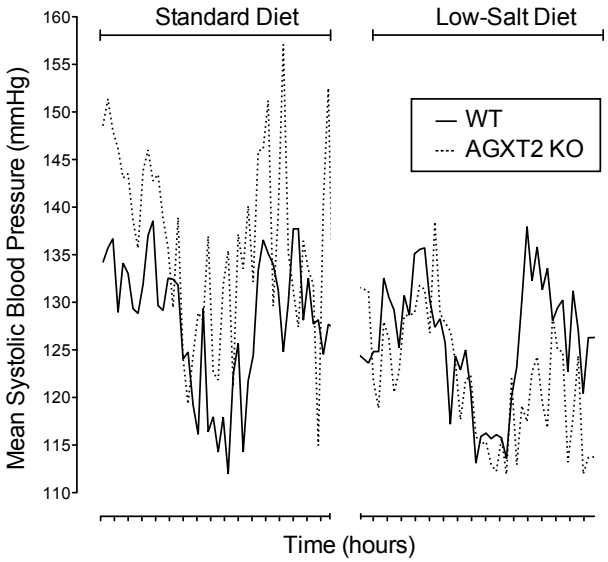

Figure: Mean Systolic BP in WT and AGXT2 KO mice from 24-hour telemetry recordings at baseline (left) and afer 2-weeks on a low-salt diet (right). P<0.05 for a difference between genotypes at baseline, NS on low-salt diet.

Conclusions: Vessels from AGXT2 KO mice are functionally normal ex-vivo confirming this enzyme does not mediate an effect by altering ADMA metabolism in the endothelium. The hypertension due to disruption of AGXT2 is entirely abrogated by a low-sodium diet. These findings suggest that increases salt-dependent mechanisms underlie vascular dysfunction in AGXT2 KO mice. Increasing renal tubular AGXT2 activity might represent a novel therapeutic strategy in CKD associated vascular disease.

O15

Regulation of NOS/DDAH Pathway by Proton Pump Inhibitors: Safety Concerns and Therapeutic Opportunities

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Background: Proton pump inhibitors (PPIs) are gastric acid suppressing drugs widely used for the treatment of gastro-esophageal reflux disease (GERD). Recently, several studies in patients with acute coronary syndrome (ACS) have raised the concern that use of PPIs in these patients may increase their risk of major adverse cardiovascular events (MACE). The mechanism of this possible adverse effect is not known. Whether the general population might also be at risk has not been addressed.

Methods and Results: Plasma ADMA is an endogenous inhibitor of nitric oxide synthase (NOS). Elevated plasma ADMA is associated with increased risk for cardiovascular disease, likely due to its attenuation of the vasoprotective effects of endothelial NOS. We find that PPIs elevate plasma asymmetric dimethylarginine (ADMA) level and reduce nitric oxide (NO) levels and endothelium-dependent vasodilation in a murine model and ex vivo human tissues. PPIs increase ADMA because they bind to, and inhibit dimethylarginine dimethylaminohydrolase (DDAH), the enzyme that degrades ADMA.

Conclusions: We discovered a plausible biological mechanism to explain the possible association of PPI use with increased MACE in patients with unstable coronary syndromes. Of concern, this adverse mechanism is also likely to extend to the general population using PPIs. This finding compels additional clinical investigations and pharmacovigilance directed toward understanding the cardiovascular risk associated with use of the PPIs in the general population.

Therapeutic opportunity: The inhibition of DDAH by the PPIs compels the need to assess potential therapeutic use of the PPIs in diseases characterized by excessive DDAH activity. Idiopathic pulmonary fibrosis (IPF); a deadly lung disease characterized by overly active DDAH will be discussed as a model to repurpose the PPIs for therapeutic application.

O16

Cyclo-oxygenase-2 anti-inflammatory drugs or gene deletion is associated with increased circulating methylarginines: identification of novel biomarkers of cardiovascular toxicity

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Non-steroidal anti-inflammatory drugs and newer cyclo-oxygenase (COX)-2 selective drugs are used to treat inflammation and pain and are associated with heart attacks and strokes. Since COX-2 is largely absent in blood vessels (where COX-1 regulates prostacyclin release), the mechanisms associated with COX-2 inhibitors and cardiovascular events are unknown but may involve the kidney where COX-2 is highly expressed. The kidney is critical for the clearance of the nitric oxide synthase (NOS) inhibitors asymmetric dimethylarginine (ADMA) and L-N^Gmono-methylarginine (L-NMMA) via the enzymes dimethylarginine dimethylaminohydrolase (DDAH) and alanine:glyoxylate aminotransferase 2 (AGXT2), and renal excretion. Increased circulating levels of ADMA are associated with cardiovascular disease where they are presumed to act predominantly via inhibition of protective endothelial nitric oxide synthase (eNOS).

Transcriptomic and pathway analysis of genes altered in kidneys from COX-2 knockout (COX-$2^{-/-}$) mice implicated an association between COX-2 and NO pathways. Confirmation of these NO associated genes using RT-qPCR demonstrated a reduction in AGXT2 and DDAH1, and an increase in protein methyl-transferase (PRMT)-1 which synthesises ADMA. This data was validated in COX-$2^{-/-}$ mice which exhibited impaired renal function, reduced DDAH1 protein and increased plasma levels of ADMA and L-NMMA. Endothelial dependent, eNOS mediated, relaxation of the aorta was reduced in COX-$2^{-/-}$ mice and this was rescued by exogenous L-arginine. In line with data from knockout mice, the COX-2 selective inhibitor, parecoxib increased blood pressure and plasma levels of ADMA and L-NMMA in wild-type mice. Finally in healthy human volunteers 7 days treatment with the COX-2 inhibitor celecoxib (Celebrex™; 200mg b.i.d) increased plasma ADMA.

These findings are the first to identify a viable biomarker, associated with cardiovascular disease that is increased by COX-2 inhibition. They also suggest that the cardiovascular side-effects associated with NSAIDs could be mediated by inhibitors of eNOS and that NSAID- induced cardiovascular toxicity might be prevented by L-arginine supplementation to at risk individuals.

O17

Proton pump inhibitors increase asymmetric dimethylarginine and induce inflammation in vascular endothelial cells

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Proton pump inhibitors (PPIs) are over-the-counter drugs widely used to treat gastroesophageal diseases (Sachs G. et al., 2006). There are a number of studies showing that PPI use is associated with increased cardiovascular risk (Charlot M. et al., 2010; Kwok C. et al., 2013).. Recently, our group discovered that PPIs can directly inhibit dimethylarginine dimethylaminohydrolase (DDAH), the enzyme that metabolizes asymmetrical dimethylarginine (ADMA), a proinflammatory molecule and an endogenous inhibitor of nitric oxide (NO) synthases (NOS). Consequently, inhibition of DDAH by PPIs results in ADMA accumulation, reduced NO production and impaired vascular function (Ghebremariam Y. et al., 2013). These findings reveal a possible mechanism by which PPIs may increase cardiovascular risk. The aim of the present study is to examine whether a prolonged use of PPI at a clinically relevant concentration reduces ADMA elimination and induces inflammatory response in vascular endothelial cells.

Primary human microvascular endothelial cells were incubated with the PPI esomeprazole (3 μM; a clinically relevant concentration) for 8 days. Gene expression profile of pro-inflammatory cytokines (interleukins: IL1β, IL6 and IL8) was assayed by quantitative real time PCR (qRT-PCR), intracellular ADMA level and concentration of IL8 in cell culture medium were measured by ELISA.

Prolonged treatment of vascular endothelial cells with PPI resulted in a significant increase of ADMA levels (1.071±0.005 μmol/mg protein vs. 0.697±0.031 μmol/mg protein in control, $p<0.05$).

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In addition, gene expression profile of inflammatory cytokines was upregulated: IL1β - 2.73±0.09 fold increase; IL6 - 1.96±0.19 fold increase; IL8 - 2.20±0.27 fold increase ($p<0.05$ for all values). Furthermore, increased IL8 mRNA transcript observed after incubation with esomeprazole was associated with accumulation of IL8 protein in cell culture medium (6357.7±877.43 pmol/mL vs. 2738.4±487.4 pmol/mL in control, $p<0.05$).

In conclusion, our data suggests that long term use of PPIs at clinically relevant concentrations could lead to ADMA accumulation in endothelial cells and induces vascular inflammation which is known to increase cardiovascular risk.

O18

Reduced renal DDAH1 activity protects against progressive kidney fibrosis and eGFR decline

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Introduction: Asymmetric dimethylarginine (ADMA) competitively inhibits nitric oxide (NO) synthesis whilst dimethylarginine dimethylaminohydrolase 1 (DDAH1) metabolises ADMA; thus representing an alternative regulatory pathway for NO production. Although an association between elevated circulating ADMA and poor cardiovascular and renal outcomes has been widely reported, a causal link is unresolved. We recently published evidence of a DDAH1 gene variant that leads to lower plasma ADMA but counter-intuitively, associates with a steeper rate of eGFR decline. Furthermore, we reported an association between renal allograft methylarginine metabolising enzyme gene expression and eGFR decline following transplantation.

Hypothesis and Methods: The principal site of renal DDAH1 expression is within the renal proximal tubule (PT). We tested the hypothesis that reduced kidney DDAH1 activity slows the progression of kidney function decline by; (A) generating a novel PT-specific DDAH1 gene knock-out (PTD1KO) mouse; (B) subjecting it to a folate model of CKD and (C) confirming associations between renal gene expression and functional decline in two independent human renal allograft cohorts.

Results: (A) KO mice had elevated PT cell ADMA (60%, $p<0.05$), with a reduction in NO synthesis (60%, $p<0.05$) whilst no effect was observed in extra-renal tissues, urine, plasma or systemic BP. Urinary proteomic analysis revealed a baseline 8-fold reduction in uromodulin (UMOD; $p<0.001$) in PTD1KOs. (B) At 12 weeks following folate injury, PTD1KO mice were protected from kidney function decline (serum creatinine; 12.9 ± 0.7 µmol/L versus 24.4 ± 3.3 µmol/L in controls; $p<0.001$); renal pro-fibrotic gene upregulation (Col12α and TGFβ; $p<0.05$); and kidney collagen deposition (4.5% vs 7.2% in controls, $p<0.01$). (C) A significant correlation between DDAH1 gene expression and eGFR decline was confirmed in human renal allograft protocol biopsies ($p<0.05$). Furthermore, a positive association of renal tissue DDAH1 and UMOD gene expression was confirmed in the live-donor human allograft sub-group.

Conclusions: Renal DDAH1 activity correlates with the progression of kidney function decline following injury in both an experimental in vivo model and human kidney allograft cohorts. Our work highlights the significance of NO-ADMA imbalances at a tissue level and suggests that circulating ADMA is an imprecise marker of renal disease. An association between UMOD and DDAH1 gene expression suggests a plausible mechanistic role of uromodulin in chronic kidney disease progression.

O19

ADMA: Unyielding opponent or double-agent in the battle that is cardio-renal disease?

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Raised levels of circulating ADMA predict vascular events as strongly as well characterised risk factors such as hypertension or dyslipidameia. However the exact causal relationship between methylarginines and vascular disease remains poorly defined. Furthermore no specific ADMA lowering therapies have yet been developed meaning the benefits (or harms) from reducing ADMA are unknown.

Those with kidney impairment have the some of the highest rates of cardiovascular disease of any patient group. The kidney is critical in methylarginine metabolism and ADMA levels are highest in patients with renal disease. This talk will review the population based, clinical and experimental studies of methylarginines with a focus on renal disease. It will ask what these studies can tell us about methylarginine metabolism in general, the contribution of ADMA to vascular disease and the importance of the kidney in ADMA regulation. Finally, the insight these studies give us into potential mechanisms through which asymmetric methylarginines might exacerbate, as well as possibly ameliorate, renal and vascular disease pathology will be discussed.

O20

Assessment of asymmetrical dimethylarginine in patients with pulmonary hypertension

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Background: Asymmetrical dimethylarginine (ADMA) is an endogenous nitric oxide (NO) inhibitor. Increased level ADMA may contribute to endothelial dysfunction in patients with pulmonary hypertension. The aim of the study was to assess the possible between ADMA and basic determinants of PH severity.

Methods: We examined 56 pts with PH (mean age 42,8±14,5 yrs, 19 M): 32 with idiopathic pulmonary arterial hypertension, 7 with corrected congenital heart disease, 7 with scleroderma, 11 with inoperable chronic thromboembolic pulmonary hypertension (iCTEPH) and 12 healthy controls (41.8 ± 10.3 yrs, 3M). Serum ADMA by ELISA, serum NT pro-BNP (Elecsys), uric acid levels were determined. ECHO, 6MWT, right heart catheterization and cardiopulmonary exercise testing (CPET) were performed.

Results: 23 % pts had symptoms of II FC WHO, 59 % pts III FC and 13 % pts IV FC, decreased 6MWT (362± 97 m) and increased NT-proBNP level 1224 (288-2392 pg/ ml). Serum ADMA level in patients with PH were elevated in comparison with healthy control subjects (0.68 ± 0.25 μmol/l vs 0.35 ± 0.12 μmol/l; p< 0.05). There were correlations between ADMA levels and such prognostic markers as NT-proBNP (r=0.44; p=0.001), mean right atrium pressure (r=0.31; p<0.05), stroke volume LV (r=-0,34; p=0,01). Serum ADMA were correlated with 6-min walking distance (r=-0.37; p=0.004), work load by CPET (r=-0.42; p=0.02).

Conclusions: Serum ADMA level was elevated in patients with pulmonary hypertension and correlated with traditional prognostic markers. Also ADMA level predicts exercise intolerance. We assume that ADMA as a marker of endothelial could be used for assessment of PH severity.

O21

The Effect of Rapamycin on ADMA in Two Strains of Rats

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Background: Rapamycin, an inhibitor for mTOR complex, is an immunosuppressant approved for organ transplantation. Rapamycin inhibits a protein kinase complex mTORC1, and thus appears to provide most of the lifespan extending effects. However, rapamycin also influences a complex known as mTORC2. The disruption of mTORC2 action can produce diabetic-like symptoms such as decreased glucose tolerance and augmented insulin resistance. Increased plasma concentration of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthase, is now recognized as a risk marker for cardiovascular disease. It is therefore important to assess how the use of rapamycin would influence on the ADMA level. 3 groups of Wistar male rats and 3 groups of senescence-accelerated OXYS rats were used in this study. One of each 3 groups was control and 2 others were treated daily with 100 or 500 μg/kg of

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rapamycin with food from the age of 1,5 to 3,5 months. Plasma L-arginine and methylarginines were measured by means of HPLC with fluorimetric detector (Shimadzu) after solid phase extraction.

Results and disussion: Body weight depended in this experiment on genotype and was higher in Wistar than in OXYS rats (356 ± 32 g and 296 ±27 g, respectively). No differences were observed in body weight of rapamycin treated rats as compared to control groups. It was surprising that basic plasma ADMA concentration was significantly lower in intact OXYS rats than in Wistar (0,268±0,031 vs 0,354±0,063 μmol/l; p=0,0006; each n=11). Daily use of 100 μg/kg rapamycin resulted in increase of plasma ADMA concentrations in both strains of rats OXYS and Wistar (each n=11; correspondingly 0,307±0,049 vs 0,268±0,031μmol/l; p=0,045 and 0,413±0,048 vs 0,354±0,063 μmol/l; p=0,033). When daily dose of 500 μg/kg of rapamycin, was used the significant increase in ADMA level was registered only in Wistar rats (0,431±0,076 vs 0,354±0,063 μmol/l; p=0,018). It is unclear why senescence-accelerated rats OXYS demonstrated lower level of ADMA than control Wistar. Reduced metabolism (lower weight) could partially explain this. The only two clear manifestation of decreasing ADMA due to application of rapamycin has been described before during heart (Potena et al., 2008) or kidney (Esposito et al., 2009) transplantation. This effect was accompanied with the inhibition of the development of cardiac allograft vasculopathy. Several investigators (Pavlakis, Goldfarb-Rumyantzev, 2008; Lamming et al., 2012) observed the developments of negative (diabetic) symptoms after rapamycine use. It should be taken into account that: 1) diabetes like symptoms and effect of life extension can alternate in time during the same rapamycin treatment because of its biphasic influence mTORC1 and mTORC2; and 2) in spite of appearing diabetic symptoms the lifespan could be extended because of rapamycin. Special experiments could clarify the cause-effect relations between ADMA and insulin resistance like those performed earlier (Sydow, Mondon, Cooke, 2005). Conclusion. Increased level of plasma ADMA under the effect of rapamycin in our experiments could reflects rather temporary changes of NOS activity (may be related to the increased insulin resistance) than to the reduction of lifespan.

This work was supported by the Russian Foundation for Basic Research, grant No. № 13-04-01079

O22

Increased amounts of asymmetric N α-acetyldimethylarginine (Ac-ADMA) after asymmetric dimethylarginine (ADMA) infusion in mice

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Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide production. Elevated levels of ADMA are associated with a range of cardiovascular diseases. Thus, knowledge of the biological regulation of ADMA levels is of major clinical and scientific interest. One of the elimination pathways of ADMA only recently proven to be present in humans is N-acetylation of ADMA to form asymmetric Nα-acetyldimethylarginine (Ac-ADMA) [1] and the subsequent renal elimination of Ac-ADMA.

In order to characterize this pathway under different biological settings, we infused ADMA versus saline in mice with- and without total nephrectomy. Animal protocols were carried out according to the requirements of the National Act on the Use of Experimental Animals (Germany). ADMA (250 μmol × kg^{-1} × d^{-1}) was infused in C57/BL6 mice using osmotic minipumps (Alzet) intraperitoneally over 3 days. Some mice underwent bilateral nephrectomy 24 hours before completion of the infusion. ADMA and Ac-ADMA were measured by HPLC coupled to tandem mass spectrometry.

The resulting ADMA plasma concentrations after ADMA infusion were 5.32 ± 3.39 μmol/L in the nephrectomy group and 5.21 ± 3.25 μmol/L in the sham group. The ADMA levels in the control mice (saline infusion) showed 0.63 ± 0.09 μmol/L in the nephrectomy group and 0.53 ± 0.07 μmol/L in the sham group. The corresponding Ac-ADMA levels were 229.65 ± 91.30 nmol/L (nephrectomy, ADMA infusion), 4.77 ± 0.88 nmol/L (sham, ADMA infusion), 45.08 ± 9.25 nmol/L (nephrectomy, saline infusion) and 1.35 ± 0.45 nmol/L (sham, saline infusion), respectively.

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These results demonstrated that Ac-ADMA is formed in vivo in response to increased ADMA concentrations and may therefore be a relevant elimination pathway for ADMA. Furthermore, Ac-ADMA is predominantly eliminated via the kidneys, which is illustrated by the steep increase of the Ac-ADMA plasma concentrations after nephrectomy in mice.

[1] J. Martens-Lobenhoffer, R.N. Rodionov, S.M. Bode-Böger, Determination of asymmetric N α-acetyldimethylarginine in humans: A phase II metabolite of asymmetric dimethylarginine, Anal. Biochem. 452 (2014) 25-30.

O23

Elucidation of the Role of the DDAH/ADMA/NO Axis in Pregnancy-related Haemodynamic Dysfunction

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Introduction: This present study was performed to determine the involvement of the NO signalling pathway in complications in pregnancy including preterm labour (PTL) and preeclampsia (PE). There is already some evidence to suggest that altered circulating factors such as VEGF family proteins and their receptors, as well as the NO signalling pathway members may contribute to PE, as well as an association between PE and reduced DDAH activity. We aimed to further explore the roles of ADMA and NO in pregnancy, both in an endotoxaemic model of PTL and in DDAH1 knockout (KO) mice.

Methods: Female CD1, DDAH1 wild type (WT) and DDAH1 KO mice were implanted with a PA-C10 radiotelemetry probe (DSI, Netherlands) into the left common carotid artery. Non-pregnant (nP) baseline recordings were taken for 48h, 7-10 days post-surgery. Mice were then timed-mated and recordings were resumed upon detection of a copulatory plug until 2 days post-partum. On E16, mice were injected with LPS (0111:B4, 10μg) or vehicle control (PBS). Further cohorts of mice were used to harvest maternal tissues at several time points for analysis of NO and ADMA as well as several inflammatory markers.

Results: In female CD1 mice it was seen that mean arterial pressure fell from 109.4±1.86 mmHg (nP baseline) to post-implantation nadir at E8 (100.8±1.3 mmHg, $P<0.01$), which then gradually increased until just before labour. Intraperitoneal injection of LPS was found to induce PTL at 21.43±1.5 hours post-injection (PBS controls laboured at 51.95 ± 3.5 hours, $P<0.05$), causing 98±1.8% pup death but no maternal mortality. After LPS, MAP dropped significantly in pregnant mice compared to nP controls. NO levels in serum were significantly increased after LPS treatment at 6 and 12h ($p<0.05$); however this effect was significantly attenuated in pregnant mice compared to nP controls ($p<0.05$). Furthermore, serum ADMA levels were also increased in pregnancy but no further increase was observed after LPS, unlike in nP controls. VEGF was also shown to be significantly increased in nP mice after LPS, but not in pregnant mice. Baseline MAP was significantly increased in female DDAH1 KO mice compared to WT controls (116±2.8 mmHg and 108.6±0.9 mmHg respectively, $P<0.05$). SAP, pulse pressure and HR were also increased and these differences were accentuated in pregnancy. Levels of serum NO and ADMA were then determined to construct a profile of the effects of DDAH1 deletion in pregnancy.

Conclusions: Changes in MAP after LPS compared with vehicle control in pregnant mice show that there is a significantly altered cardiovascular response to systemic inflammation in pregnancy. As NO levels were also attenuated in pregnant animals in endotoxaemia, taken with a hypotensive response, these data may suggest an increase in sensitisation to the vasodilator action of NO. Deletion of DDAH1 increases MAP in female mice.

O24

ADMA as a risk marker – are we any wiser than in 2001?

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In 2001 first data indicated that an elevated plasma asymmetrical dimethylarginine (ADMA) concentration predicts mortality and cardiovascular events

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independently of traditional risk factors. Based on its ability to interfere with L-arginine-dependent pathways and signaling it was proposed that ADMA may not only be a risk marker but also a risk factor. Since then, more than 75 clinical studies prospectively assessed ADMA as a risk marker for diverse clinical outcomes in a wide range settings ranging from healthy volunteers to patients with sepsis and terminal organ failure. The majority of these studies confirmed an association of ADMA and progression of disease or mortality. Complementing animal models of altered ADMA metabolism and in vitro studies added to our understanding of the underlying pathophysiology but also provided new challenges to key pathophysiological assumptions. Moreover, more and more structurally and biochemically related compounds such as symmetrical dimethylarginine (SDMA) were found to predict mortality as well. Based on the data available so far, ADMA is likely to remain the predominant L-arginine-related risk marker, but new factors may have to be considered to explain its relation to mortality.

O25

Homoarginine - A Cardiovascular Risk Marker and a new Therapeutic Target

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Homoarginine is a naturally occurring non-proteinogenic amino acid. L-Lysine can be guanylated to homoarginine under the catalysis of the enzyme L-arginine:glycine amidinotransferase (AGAT). Once formed, homoarginine can serve as a weak substrate for nitric oxide (NO) synthase (NOS). Within the last few years, clinical evidence increased that low concentrations of endogenous homoarginine are a risk marker for cardiovascular (CV) and cerebrovascular events. In contrast to other CV biomarkers that are positively associated with CV outcomes (i.e., ADMA or NTproBNP), plasma concentrations of homoarginine are inversely associated with CV risk and mortality in patients (coronary artery disease, heart failure, and stroke) as well as in population-based cohorts (Study of Health in Pomerania and Dallas Heart Study). In line with the clinical data, experimental studies in mice have recently supported a mechanistic link between low homoarginine and cerebrovascular disease. At this point, further studies are needed to evaluate whether the regulation of plasma homoarginine or the modulation of the homoarginine metabolism could emerge as potential novel therapeutic targets in CVD.

O26

DDAH1 plays an important role in protecting the heart from systolic overload-induced congestive heart failure

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ADMA is the strongest independent predictor of mortality and major nonfatal cardiovascular events in patients after myocardial infarction. While studies have demonstrated that congestive heart failure is associated with chronic accumulation of plasma ADMA and reduced myocardial DDAH activity, it is not clear whether chronic DDAH1 dysfunction or ADMA accumulation can cause the development of congestive heart failure. Here we demonstrated that chronic ADMA accumulation by global DDAH1 gene knockout (DDAH1 KO) caused aging-dependent hypertension and moderate left ventricular hypertrophy but did not cause left ventricular dysfunction or failure, indicating that DDAH1 dysfunction or chronic ADMA accumulation is insufficient to cause congestive heart failure under unstressed conditions. Using an inducible cardiac specific DDAH1 gene deficient strain, we demonstrated that selective DDAH1 gene deletion in cardiac myocytes (cardio-DDAH1 KO) had no effect on plasma ADMA, myocardial ADMA (55±3.0 nmol/g protein in cardio-DDAH1 KO mice vs 52±5.5nmol/g protein in wild type mice), systolic blood pressure, or left ventricular hypertrophy under control conditions, suggesting that left ventricular hypertrophy observed in global DDAH1 KO mice is mainly due to moderate systolic hypertension. In response to more severe chronic systolic pressure overload produced by aortic banding, cardio-DDAH1 KO mice showed small but significant increase of myocardial ADMA (65±3.6 nmol/g protein in cardio-DDAH1 KO mice vs 57±4.7

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nmol/g protein in WT mice, p<0.05), but significantly greater left ventricular hypertrophy, pulmonary congestion (312±35mg in cardio-DDAH1 KO mice vs 191±16mg in WT mice, p<0.05), and heart failure (51±3.3% in cardio-DDAH1 KO mice vs 63±3.3% in WT mice, p<0.05) than WT mice, indicating that DDAH1 distributed in cardiac myocytes is important for protecting heart under stress conditions. Collectively, our data demonstrate that DDAH1 protects the heart against ventricular hypertrophy and dysfunction though both blood pressure-dependent and blood pressure-independent molecular mechanisms.

O27
DDAH1 and Insulin Resistance

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Type 2 diabetes is a prevalent metabolic condition and is the result of an impaired response to insulin. Insulin resistance and type 2 diabetes are clearly associated with obesity and the secondary cardiovascular complications of this condition are serious and life threatening.

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthases and increased levels are seen in multiple pathologies. Increased plasma levels of ADMA have been associated with patients with type 2 diabetes, insulin resistance and obesity, although a causal link between ADMA and diabetes has not been established. Dimethylarginine dimethylaminohydrolase (DDAH) is the enzyme that catalyses the metabolism of ADMA. There are two isoforms of the enzyme which are both involved in the control of ADMA and NO.

The interplay of insulin with NO release is well established but the initial causes for the onset of insulin resistance are not well defined. Elevated levels of ADMA are linked to insulin resistance and transgenic mice that over-express ddah1 show increased insulin sensitivity. Of note is that metformin, an insulin sensitising drug that is widely used in the treatment of insulin resistance, reduces plasma glucose and ADMA concentrations.

In order to elucidate the physiological role of DDAH1 in glucose homeostasis we investigated the glucose handling in a ddah1 global knockout model.

Intra-peritoneal glucose tolerance tests in ddah1 global knockout mice demonstrate insulin resistance. Baseline plasma glucose levels were 25% higher in ddah1 knockouts and peak levels were 53% higher in ddah1 knockouts. The kinetics of plasma glucose accumulation and clearance in ddah1 knockout mice suggests dysfunction in both the liver and skeletal muscle. On a normal chow diet, hepatocyte specific ddah1 knockout mice and skeletal muscle specific ddah1 knockout mice show no insulin resistance. On a high fat diet however the hepatocyte specific ddah1 knockout mice show significant insulin resistance and lower metabolic rate than their fat fed wild-type counterparts.

These studies demonstrate for the first time a causal link between ADMA accumulation and insulin resistance. Furthermore these data establish DDAH1 activity is a significant regulator of insulin resistance.

O28
Asymmetric dimethylarginine is a novel regulator of mTOR expression in adipocytes

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The metabolic syndrome, in which cardiovascular disease and obesity play major roles, has become an important healthcare issue. Systemic concentrations of asymmetric dimethylarginine (ADMA) correlate with obesity and insulin resistance and it is considered to be an independent marker of cardiovascular disease. Currently, ADMA is understood to be an endogenous inhibitor of nitric oxide synthase; however, the direct effect of ADMA on adipocytes and in obesity has not been investigated. Here, we establish that ADMA in adipocytes regulates the expression and activity of the mTOR lipid synthesis pathway which results in adipocyte hypertrophy both in vitro and in vivo.

Treatment of 3T3-L1 adipocytes with ADMA (10μM) resulted in adipocyte hypertrophy (control 27μm2 ±1.4, 10μM ADMA 33μm2± 1.6). This could not be mimicked through the blockade of NO by L-NAME (28μm2 ± 1.5) and PB-ITU (25μm2± 1.2). Hypertro

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phy was driven by an increase in mTOR expression (x2.4 ± 0.4) which upregulates ACC (x4.6 ± 0.8) and FASN (x1.4 ± 0.1) suggesting ADMA increases lipid production in an NO-independent manner.

Investigations in vivo were explored through the genetic manipulation of DDAH1, an enzyme responsible for ADMA breakdown. Specific deletion of DDAH1 within adipocytes increased intracellular ADMA concentrations. This in turn caused an upregulation of mTOR, ACC and FASN expression and resulted in adipocyte hypertrophy. Conversely, genetic overexpression of DDAH1 reduced ADMA production within adipocytes and caused a down-regulation in mTOR expression.

These data suggests that ADMA is a novel regulator of mTOR expression and activity at physiological concentrations. Pathological concentrations of ADMA are associated with adipocyte hypertrophy a known risk factor for insulin resistance and inflammation.

O29
Drugging the DDAH/ADMA/NO pathway

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Since the identification of endogenous inhibitors (principally ADMA) of nitric oxide synthesis as mediators of cardiovascular dysfunction by Vallance and Moncada in 1992 our understanding of the biology and pathology of ADMA has increased enormously. Elegant experiments utilizing molecular, biochemical and in vivo techniques in experimental animals and humans have combined to elucidate the complex roles of ADMA in health and disease. It is now clear that accumulation of ADMA inhibits nitric oxide signalling in several cardiovascular disease states and that, in experimental animals, ADMA lowering has protective effects. Conversely, in situations of NO overproduction pharmacological or genetic elevation of ADMA might be therapeutically useful. Prompted by these observations we and others have embarked on screening programmes to identify novel chemical entities that might modulate ADMA levels in therapeutically useful directions. The fruit of these endeavors has been the identification of several structurally distinct chemical series of DDAH inhibitors that have shown promise in a number of in vitro and in vivo models of disease. In contrast to our success in identifying DDAH inhibitors activators of DDAH have not yet been identified. A number of reports suggest that alternative strategies to enhance DDAH activity, including transcription, miRNA's and post-translational protein modifications may represent realistic therapeutic approaches. Finally, the work of Ghebremariam and others has identified DDAH as a direct target of drugs currently in clinical use and these observations may explain some of the reported cardiovascular side-effects associated with their use. In this presentation I will discuss progress towards the development of drugs that target the DDAH/ADMA/NO pathway.

O30
High – throughput screening identified an analogue of the thyreostatic drug thiamazole as potential regulator of DDAH1

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Introduction: Asymmetrical dimethylarginine (ADMA) is a competitive inhibitor of nitric oxide synthase (NOS) and is mainly metabolized by the enzyme dimethylarginine–dimethylaminohydrolase (DDAH). Evidence has accumulated that decreased ADMA concentration due to an overexpression of DDAH plays an important role in diseases such as idiopathic pulmonary fibrosis, Alzheimer´s disease and cancer. Thus, selective inhibitors would be of great interest as potential therapeutic interventions to influence those diseases. Although a few potent DDAH inhibitors have been identified (L – 257, ebselen, omeprazole), their effects have not been demonstrated in these diseases so far or they lack in selectivity.

Goal: We sought to carry out a High–Throughput Screening (HTS) to identify further structural diverse

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inhibitors which show the potential as lead molecule.

Method: A HTS with app. 50.000 compounds was performed. A fluorescence–based activity assay with human recombinant DDAH1 and S–methylthiocitrulline (SMTC) as substrate was used and modified to endpoint measurement.

Results: HTS and subsequent Hit Profiling revealed up to 26 potential inhibitors of DDAH1. Next to new diverse structures and an analogue of the already described DDAH inhibitor SCH – 202676, several ebselen mimetics were found containing a sulphur instead of a selenium group. Interestingly a structure related to the drug thiamazole, which is indicated for hyperthyroidism, was identified.

Conclusion: In this study selected hits strengthen the assumption that the inhibitory effect on DDAH mainly results from the interaction with its cysteine residue. The hits can be used to find a more selective or less toxic inhibitor given that the use of selenium–containing drugs is still controversial. The influence of thiamazole analogues on DDAH1 activity suggests that the enzyme might be involved in off – target effects of thyreostatic drugs.

O31

Activation of Nrf2 Reduces ADMA and Increases Nitric Oxide in Human Renal Glomerular Endothelial Cells (HRGECs) by Transcriptional Activation of DDAH, eNOS and PPAR-γ

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Background: Plasma levels of ADMA are a strong predictor of CVD and CKD but therapeutic strategies for its reduction are lacking. Nrf2 is a transcriptional factor that binds to the antioxidant response element (ARE) on many antioxidant genes and is thereby a key component of the cell's response to oxidative stress. DDAH degrades ADMA which inhibits nitric oxide synthase (NOS), whereas PPAR-γ increases eNOS expression and phosphorylation. Hypothesis: Since we detected 2-3 putative AREs on promoters of human DDAH-1 and -2, PPAR-γ and eNOS genes, we hypothesized that Nrf2/ARE binding regulates the DDAH/P-PAR-γ/eNOS/ADMA/NO pathways. Methods: Incubation of HRGECs for 24 hours with tert-butylhydroquinone (tBHQ, 5-20 μM) activated Nrf2 by translocation to the nucleus and enhanced cell proliferation. Results: tBHQ (20 μM; n=6) increased medium nitrite by 69 ± 3.7% ($P<0.01$), intracellular NO (from DAF-FM fluorescence) by 62 ± 17.1% ($P<0.05$), and NOS and DDAH activities(from conversion of [3H]-Arginine or [14C]-ADMA to [3H]- or [14C]-Citrulline) by 58 ± 3.6% and 47 ± 8.5% ($P<0.05$) and decreased medium ADMA (by capillary zone electrophoresis), from 2.3 ± 0.16 to 1.2 ± 0.03 ($P<0.001$, n=6). tBHQ increased the mRNAs and proteins for eNOS by 79 ± 9% and 43 ± 17%, for DDAH-1 by 129 ± 10% and 62 ± 21%, and for DDAH-2 by 119 ± 25% and 48 ± 18% (all $P<0.05$) tBHQ also increased PPAR-γ expression and eNOS phosphorylation. tBHQ did not change expression in the cytoplasm while increasing its nuclear translocation markedly, implying activation of Nrf-2. Knockdown of the cell Nrf2 by transfection with specific siRNA abolished all these effects and attenuated cell proliferation. Chromatin immunoprecipitation (CHIP)-based PCR assays demonstrated that tBHQ enhanced the binding of Nrf2 to one ARE region on the promoters for DDAH-1 and -2 but there was no binding to the eNOS promoter despite upregulation and phosphorylation of eNOS by tBHQ. Knockdown of PPAR-γ prevented upregulation of eNOS and eNOS phospharylation by tBHQ, implying that the effects of tBHQ on eNOS and eNOS phosphorylation were indirect and secondary to increased PPAR-γ expression.

Conclusion: tBHQ activates Nrf-2. Activation of a specific ARE on the promoters for DDAH-1 and -2 genes by tBHQ led to their transcriptional activation which increased DDAH activity and reduced ADMA concentrations. However, Nrf2 increased eNOS expression and phosphorylation, NOS activity and NO generation without binding to the eNOS ARE. These effects on eNOS were ascribed to binding to an ARE the on PPAR-γ promotoer. Thus, Nrf2 improves endothelial cell function by coordinating a diverse set of pathways that enhance NO generation. Supported by grants from the NIH.