On behalf of the Purine and Pyrimidine Society and the Local Organizing Committee, we are pleased to announce the 16th International Symposium on Purine and Pyrimidine Metabolism in Man will be held June 6-9, 2015 in New York City, U.S.A.

International symposia devoted to the study of human purine metabolism were initiated in 1973 in Tel Aviv, Israel and, with expansion of the focus to include pyrimidine metabolism (1985), were convened every three years. Although sponsorship of the international symposia has traditionally been local and without a sustained organizing body, a European Society for the Study of Purine and Pyrimidine Metabolism in Man (ESSPPMM) was formed in 1987, and has met every two years since, in some years, in conjunction with the international symposium. In the 2003 joint international and European symposium in Egmond aan Zee, The Netherlands, an agreement was reached to merge the activities of the international and European groups in a new organization called the Purine and Pyrimidine Society (PPS). This decision was approved in 2005 by the ESSPPMM membership at their final meeting in Prague. Because rapid advances in our fields of interest dictate, PPS decided to sponsor biennial symposia, the first of which (called PP07) was held in Chicago, in 2007. Subsequent meetings were held in Stockholm, Sweden (2009); Tokyo, Japan (2011); and Madrid, Spain (2013).

PP15 will provide a special opportunity for basic and clinical researchers to present and discuss recent progress in the understanding of clinical and molecular mechanisms of diseases related to purine and pyrimidine metabolism. These include a myriad of pathologies such as: disorders related to uric acid metabolism; gout; renal lithiasis; enzymopathies, immunodeficiencies, and inflammation related to purine and pyrimidine metabolism; purine and pyrimidine transporters and receptors; as well as purines and pyrimidines in cancer, mitochondrial diseases, and anti-infectious agents. The organizing Committee strives to attract participation by investigators spanning the spectrum from gene and protein structural science and molecular genetics to clinical investigation and therapeutics.

The meeting venue is the Faculty House at Columbia University in New York City. Founded in 1754 as King’s College, Columbia University is one of the oldest institutions of higher education in the United States and is located on Morningside Heights at 116th Street and Broadway. The picturesque Columbia campus features the Low Memorial Library, which overlooks a large plaza that is a popular site for students to gather as well as a popular setting for Hollywood movies. The campus is readily accessible via public transportation or taxis to innumerable other tourist attractions in New York City.

We hope that you will have the chance to attend New York PP15. You will have the opportunity to present your research to your colleagues, and discuss advances and to make new friends. In addition, you are encouraged to find the time to incorporate a pre- or post-conference tour of New York City.

Sincerely yours,

Michio Hirano, MD

President
Local Organizing Committee for the 15th Symposium on Purine and Pyrimidine Metabolism in Man, PP15, New York, City, U.S.A.
Michio Hirano  New York City
Michael Becker  Chicago

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TRAVEL GRANTS AWARD

The PP15 Organizing Committee acknowledges and thanks the Purine Metabolic Patient’s Association (PUMPA) and the Purine and Pyrimidine Society their support of travel grants to the following recipients. The awards will be given after Closing Conference “Anne Simmonds Memorial Lecture”

PUMPA/PPS TRAVEL AWARDS
• Marco De Giorgi
• Christelle Machon
• Andrea López Moreno
• Dzjemma Sarkisjan
• Magdalena Zabielska

PPS POSTER AWARD
• Maliha Zafar
• Marie Zikanova
• Makoto Hosoyamada
ACKNOWLEDGEMENTS

The following companies and organizations have contributed to the Congress:

TAKEDA
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GENERAL INFORMATION

PPS Location:

Columbia University, Faculty House
64 Morningside Drive
New York, NY 10027
+1.212.854.1200 • fachouse@columbia.edu

Free Wi-fi – no password necessary

REGISTRATION DESK
The registration Desk will have the following timetables:
Saturday, June 6, 2015- 8:00-18:00 H
Sunday, June 7, 2015 – 8:00-18:00 H
Monday, June 8, 2015 – 8:00-18:00 H
Tuesday, June 9, 2015 – 8:00-18:00 H

CONGRESS LANGUAGE
The official language of the Congress is English. There will be no simultaneous translation

BADGE
Delegates will receive badges and vouchers for the booked events. Participants are kindly requested to wear their badge throughout the congress, even at social events. Lost badges will not be replaced. A new registration will be mandatory.

WORKING LUNCH
Lunch will be at the Congress venue, being essential presentation of meal tickets

MOBILE PHONES
During scientific session mobile phones must be switched off

CERTIFICATE OF ATTENDANCE
Certificate of attendance will be handed out at the registration desk together with the rest of your documentation.

CURRENCY
The official currency is US Dollar. Usual credit card (Mastercard, American Express, VISA, Discover) are accepted in hotel, department stores and restaurants.

INSURANCE
The organizers will not be responsible for any injury to participants or damage, theft and loss of personal belongings. Participants should therefore make their own insurance arrangements.
TIME ZONE
Eastern Time Zone (UTC-05:00)
New York City, Time zone

WEATHER
70’s-75 degrees

EMERGENCY SERVICES
Dial: 911

REGISTRATION
You may pre-register to the 16th International Symposium on Purine and Pyrimidine Metabolism in Man online, by email with mh29@columbia.edu or sc3745@columbia.edu

Please note: on-site registration can be made only in cash

Through website:
Congress Website: www.ppsociety.org
Purine and Pyrimidine Society website: http://www.ppsociety.org
To start the online registration process, please follow the instructions on the website. The online payment process for the 16th International Symposium on Purine and Pyrimidine Metabolism in Man is fully encrypted in order to protect credit card transaction.

By email (sc3745@columbia.edu) sending the registration form as attachment, with proof of bank transfer

We recommend registration through the website, as it facilitates management of information. An email confirming your registration will be sent to you within the next (5) working days after having received both, the registration form and your payment.

If you do not receive this email in due time, please email or contact Sherry P Cabrasawan at sc3745@columbia.edu and telephone #+1.212.305.2010
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TEACHER’S COLLEGE GUEST HOUSE
Official House for Speakers
517 W 121st Street

INTERNATIONAL HOUSE
Office House for Speakers
155 Claremont Avenue (entrance after 7:00PM – back of the bldg.)
SATURDAY, JUNE 6, 2015 - (Seminar Room 1,2,3,4 & Reception)

15:00-16:00: Executive Committee Meeting @ the Garden Room 2

16:00-18:00: Pre-Symposium: “Purine and Pyrimidine Translational Medicine” Kimiyoshi Ichida and Andre van Kuilenberg
16:00-16:25: I-1: Rosa Torres, PhD “Hypoxanthine deregulates genes involved in early neuronal development. Implications in Lesch-Nyhan disease pathogenesis”
16:50-17:15: I-3: Kimiyoshi Ichida, MD, PhD “Mechanism of hyperuricemia and gout as caused by ABCG2 Dysfunction”
17:15-17:40: I-4: Andre Van Kuilenburg, PhD – “The biochemistry and pathophysiology of purine and pyrimidine metabolism”

17:40-18:00: Discussion.

19:30-21:00: Welcome reception (Faculty House) Dinner at Seminar Room

SUNDAY, JUNE 7, 2015 - (Presidential Room 1, 2, 3 R)

08:30-10:30 am. Session 1: “Advances in gout and hyperuricemia”. Becker MA (USA) and Tappei Takada (Japan)
08:30-09:00: I-5: Hirotaka Matsuo. “Genetics of urate transporter genes and their roles in gout and hyperuricemia”
09:00-09:15: O-1: Tappei Takada “Comparison for the effects on hyperuricemia progression between genetic and environmental factors”
09:15-09:30: O-2: Shunya Uchida. “Serum uric acid has a significant impact on renal outcomes using propensity score method”
09:30-09:45: O-3: Hirotaka Matsuo. “Genome-wide association study (GWAS) of gout identifies multiple risk loci: a clue for future companion diagnostics of gout”
09:45-10:30: I-6: Juan Puig. “Ultrasound-based classification and treatment of asymptomatic hyperuricemia and gout”

10:30-11:00 am. Coffee Break.

11:00-12:30: Session 2: “Inborn Errors of Purine and Pyrimidine Metabolism”. Puig JG (Spain) and Monika Loeffler (Germany)
11:00-11:30: I-7: Monika Loeffler “Orotic acid (1905-2015) - in the cell, in milk: pro-growth and anti-gout”
11:30-11:45: O-5: Ivan Sebesta “Hereditary xanthinuria is not so rare disorder”
11:45-12:00: O-6: Yoko Nakajima Clinical, biochemical, and molecular analysis of 30 children with β-ureidopropionase deficiency demonstrates high prevalence of the c.977G>A (p.R326Q) mutation”
12:00-12:15: O-7: Juan Puig “Self-injurious behavior following dental extraction in Lesch-Nyhan disease”
12:15-12:30: O-8: Kiyoko Kaneko “Urinary excretion of uric acid, allantoin, and 8-OH-deoxyguanosine in uricase-knockout mice”

12:30-13:30: Poster I

13:30-14:30. Lunch.

14:30-16:00: Session 3: “Purines, Pyrimidines, and Cancer”. Peters GJ (Netherlands) Lars-Petter Jordheim (France)
14:30-15:00: I-8: G.J. Peters “Fluoropyrimidines: Beyond inhibition of thymidylate synthase”
15:00-15:15: O-9: Lars Petter Jordheim “Downregulation of S'-nucleotidase cN-II in cancer cells favors tumorigenicity and suggests impact on metabolic adaptability”
15:30-16:00: Purine and Pyrimidine Society General Assembly

16:30-17:00: Coffee Break

17:00-18:00: Poster II

MONDAY, JUNE 8, 2015 - (Presidential Room 1, 2, 3 R)

08:30-10:30 am Session 4: “Nucleoside transporters and receptors”. Marçal Pastor Anglada (Spain) and Imogen Coe (USA/Asia).

08:30-09:00: I-9: Imogen Coe “The SLC29 family of concentrative purine and pyrimidine nucleoside transporters: New modalities of regulation and therapeutics”

09:00-09:30: I-10: Marçal Pastor “The SLC28 family of concentrative purine and pyrmidine nucleoside transporters: New modalities of regulation and therapeutics”

09:30-10:00: I-11: Arancha Mediero “Blockade of adenosine transporters regulates osteoblast and osteoclast function and promotes bone formation in vivo by increasing adenosine levels”

10:00-10:15: O-13: Alex Bicket “ENT1, a novel link between purinergic and calcium signalling”

10:15-10:30: O-14: Masayuki Sakiyama “NPT1 is a renal urate efflux transporter in humans and its common gain-of-function variant (I126T) decreases the risk of renal underexcretion gout”

10:30-11:00: Coffee Break

11:00-12:00: Session 5: “Purine and pyrimidine enzyme regulation”. Steffan Eriksson and Stefan Lutz

11:00-11:30: I-12: Steffan Eriksson “An update of the role of deoxynucleoside kinases in purine and pyrimidine DNA precursor metabolism”

11:30-12:00: I-13: Stefan Lutz “Exploring and exploiting kinases for nucleoside analog activation”

12:00-12:15: O-15: Lars Petter Jordheim “The 5’-nucleotidase cN-II is inhibited by fludarabine”

12:15-12:30: PPS General Assembly

12:30-13:30: Poster III

13:30-14:30: Lunch

14:30-16:00: Session 6: “Purines and pyrimidines in inflammation and autoimmune disease”. Bruce Cronstein and Wajahat Mehal (USA)

14:30-15:00: I-14: Bruce Cronstein, MD

15:10-15:30: I-15: Wajahat Mehal, MD, PhD “The intersection of inflammasome activation and purinergic receptors”


15:45-16:00: O-18: Carmen Coriulo “Mice lacking adenosine A2A receptors develop spontaneous osteoarthritis”

16:30-17:00: Coffee Break

17:00-18:00: Poster IV

18:00-18:15: YOUNG INVESTIGATOR AWARD

18:15-19:00 - Anne Simmonds Memorial Lecture, MICHAEL BECKER

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TUESDAY, JUNE 9, 2015 - (Presidential Room 1,2,3 R)

08:30-10:30; Session 7: “Purine and Pyrimidine metabolism and mitochondrial disorders”. Michio Hirano and Liya Wang

08:30-09:00: I-16: Liya Wang “Mitochondrial purine and pyrimidine metabolism and beyond”
09:00-09:30: I-17: Michio Hirano “Mitochondrial purine and pyrimidine diseases: A tale of two genomes”
09:30-09:45: O-19: Caterina Garone “Thymidine kinase 2 deficiency: from diagnosis to treatment”
09:45-10:00: O-20: Chiara Rampazzo “Functional interaction of nuclear SAMHD1 and mitochondrial deoxyguanosine kinase in the regulation of mt dNTP pool balance”
10:00-10:15: O-21: Yolanda Camara “Deoxynucleoside supply rescues mtDNA depletion in human POLG-deficient fibroblasts”
10:15-10:30: O-22: Claus Desler “Balanced levels of dNTPs links mitochondrial dysfunction to cellular stress”

10:30-11:00; Coffee Break

11:00-12:45; Session 8: “Purine and Pyrimidine analogs”. Arnon Lavie and Varsha Gandhi

11:00-11:30: I-18: Varsha Gandhi “Vulnerability of LKB1 deficient NSCLC to adenosine analogs”
11:30-12:00: I-19: Arnon Lavie “Exploring and exploiting kinases for nucleoside analog activation”
12:00-12:15: O-23: A.E. Vidal “dUTP nucleotidohydrolase is a major factor in the cellular response to decitabine”
12:15-12:30: O-24: Dzjemma Sarkisjan “Concentration and cell line dependent effects of fluorocyclopentenylcytosine (RX-3117) in non-small cell lung cancer cells”
12:30-12:45: O25: Godefridus J. Peters, Elisa Giovannetti “Inhibitors of lactate dehydrogenase overcome the resistance towards gemcitabine in hypoxic mesothelioma cells, and modulate the expression of the human equilibrative transporter-1 (hENT1)”

13:30-14:30; Lunch

14:30-16:00; Session 9: “Non-mammalian purine and pyrimidine metabolism”. This session is dedicated to the memory of Professor Jure Piskur. Wolfgang Knecht and Birgitte Munch-Petersen

14:30-14:40: Introduction to the session: “Prof. Jure Piskur and purine and pyrimidine metabolism – some personal reflections” Wolfgang Knecht & Birgitte Munch-Petersen
15:15-15:35: O-26: María Valente “Balance of dUTP/dTTP ratio in bloodstream forms of Trypanosoma brucel”
Poster Session 1

Miscellaneous Purine and Pyrimidine Metabolism

1. Christelle Machon, Claire Bordes, Emeline Cros-Perrial, Charles Dumontet, Pierre Lanteri, Jérôme Guitton & Lars Petter Jordheim “Demonstration of the importance of sample preparation for the study of endogenous nucleotides in cell cultures”

2. Iwona Pelikant-Maecka, Alicja Sielicka, Ewa Kaniewska, Ryszard T. Smoleski, Ewa M. Somiska “Metabolic pathway and effects of 4-pyridone-3-carboxamide-1β-D-ribonucleoside in human endothelial cells”


4. Zdzisław Kochan, Joanna Karbowska, Patrycja Gogga, Barbara Kutryb-Zajac, Ewa M. Slominska, Ryszard T. Smolenski “Polymorphisms of NT5E gene that alter enzymatic activity and may predispose to calcification of the aortic valve”

5. T. Fukuuchi, M. Kobayashi, N. Yamaoka, K. Kaneko “Simultaneous determination of extra- and intra-cellular purines in Caco-2 and HepG2 cells by high-performance liquid chromatography”

Non-mammalian Purine and Pyrimidine Metabolism


Purine and Pyrimidine Analogs

9. Richard J Honeywell, Dzjemma Sarkisjan, letje Kathmann, Michael H Kristensen, Godefridus J Peters. “Sensitive liquid chromatography mass spectrometry (LC-MS) assay reveals novel insight of antimetabolite incorporation into DNA”


11. Vidal AE, Requena CE, Pérez-Moreno G, Ruiz-Pérez LM and D. González-Pacanowska. “dUTP nucleotidohydrolase is a major factor in the cellular response to decitabine”

12. Elisa Giovannetti, Leticia G. Leon, Paolo A. Zucali, Filippo Minutolo, Godefridus J Peters “Inhibitors of lactate dehydrogenase overcome the resistance towards gemcitabine in hypoxic mesothelioma cells, and modulate the expression of the human equilibrative transporter-1 (hENT1)”

13. N. Yamada, C. Iwamoto, T. Fukuuchi, N. Yamaoka, K. Kaneko, H. Kano” Evaluation of purine utilization by Lactobacillus gasseri strains with potential to decrease the absorption of food derived purines in the human intestine”.


Poster Session 2

Purine and Pyrimidine metabolism and mitochondrial disorders


17. Elisa Franzolin, Vera Bianchi and Chiara Rampazzo “Functional interaction of nuclear SAMHD1 and mitochondrial deoxyguanosine kinase in the regulation of mt dNTP pool balance”

18. Claus Desler, Jon Durhuus, Sisse Bøggild, Maria Angleys, Lene Juel Rasmussen “Balanced levels of dNTPs links mitochondrial dysfunction to cellular stress”.


Purines and pyrimidines in inflammation and autoimmune disease

21. Federico Cividini, Maria Grazia Tozzi1, Rosanna Pesi, Alvaro Galli, Emeline Cross-Perrial, Charles Dumontet, Simone Allegrini & Lars Petter Jordheim "Interaction between 5'-nucleotidase cN-II and the inflammasome protein NLRC4/Ipaf suggests new roles of cN-II in cell biology"
23. Emmanuel Martin, Noé Palmic, Christelle Lenoir, Fabian Hauck, Cédric Mongellaz, Mauro Degli Esposti, Naomi Taylor, Robert Wynn, Capucine Picard, Alain Fischer, Peter Arkwright and Sylvain Latour “CTP synthase 1 deficiency in humans reveals its central role in T lymphocyte proliferation”

Purine and pyrimidine enzyme regulation

24. Federico Cividini, Rosanna Pesi, Laurent Chaloin, Simone Allegrini, Emeline Cross-Perrial, Charles Dumontet, Maria Grazia Tozzi & Lars Petter Jordheim “The 5'-nucleotidase cN-II is inhibited by fludarabine”
25. N. Yamaoka, S. Akiba, M. Kikuchi, T. Fukushima, K. Kaneko “Inhibitory effect of plant extracts used in herbal medicine on xanthine oxidase activity”

Poster Session 3

Nucleoside transporters and receptors

27. Eiji Kubo, Hirofumi Yano, Yoshifuru Tamura, Takeshi Shiraishi, Makoto Hosoyama, Shunya Uchida "Expression of Intestinal and Renal Uric Acid Transporters in the 5/6 Nephrectomy Rat Model of Chronic Kidney Disease”
29. Alex Bicket, Zlatina Naydenova, and Imogen R. Coe “ENT1, a novel link between purinergic & calcium signalling”
30. Maliha Zafar, Zlatina Tarmakova, and Imogen Coe “Substrate translocation triggers endocytic regulation of the equilibrative nucleoside transporter 1 (ENT1)”
31. Aranzazu Mediero, Tuere Wilder, Carl Whatling, Bruce Cronstein. “Ticagrelor regulates osteoblast and osteoclast function and promotes bone formation in vivo by increasing adenosine levels”

Purines, Pyrimidines, and Cancer

32. Gabriel Bricard, Emeline Cross-Perrial, Charles Dumontet & Lars Petter Jordheim “Downregulation of 5’-nucleotidase cN-II in cancer cells favors tumorigenicity and suggests impact on metabolic adaptability”
33. Vidal AE, Requena CE, Pérez-Moreno G, Ruiz-Pérez LM and D. González-Pacanowska “dUTP nucleotidohydrolase is a major factor in the cellular response to decitabine”
34. André B.P. van Kullenburg and Rutger Meinsma “Differential expression of uridine-cytidine kinases in neuroblastoma. Implications for development of a targeted therapeutic approach”
35. Kees Smid, Erik Meijer, Thang V. Pham, Inge de Reus, Sander R. Piersma, Godefridus J. Peters, Connie R. Jimenez “Proteomics analysis of the effect of fluorouracil (5FU) and 5FU/leucovorin (LV) on colorectal cancer (CRC) in patients”
Inborn Errors of Purine and Pyrimidine Metabolism

37. Torres RJ and Puig JG “Hypoxanthine deregulates genes involved in early neuronal development. Implications in Lesch-Nyhan disease pathogenesis”
38. Torres RJ, Beltran LM, Sanchez A, Puig JG “Megaloblastic anemia in hypoxanthine-guanine phosphoribosyltransferase deficiency”
41. Blanka Stiburkova, Dana Gabrikova, Jitka Sokolova, Pavol Kristian, Clara Martinez-Mir, David Comas, Elizabeth Cordoba-Lanus, Felix Claverie-Martin “Extremely high frequency of SLC22A12 variants causing renal hypouricemia 1 in the Czech, Slovak and Spanish Roma population”
42. Marie Zikanova, Jakub Krijt, Vaclava Skopova, Matyas Krijt, Veronika Baresova and Stanislav Kmoch “Diagnosis of adenylsoucinate lyase deficiency by measuring of succinylpurines in neonatal dried blood spots”
44. I. Sebesta, B. Stiburkova, J.Krijt “Hereditary xanthinuria is not so rare disorder”
45. Makoto Hosoyamada, Yu Tsurumi, Naoko H. Tomioka, Yuko Sekine, Takayuki Morisaki, Shunya Uchida “Urat1-Uox double knockout mice are experimental animal model of renal hypouricemia and exercise-induced acute kidney injury”
46. Yoko Nakajima, Judith Meije, Doreen Dobritzsch, Chunhua Zhang, Tetsuya Ito, Yoriko Watanabe, Tomiko Kuhara, and André B.P. van Kuilenburg “Clinical, biochemical and molecular analysis of 30 children with β-ureidopropionase deficiency demonstrates high prevalence of the c.977G>A (p.R326Q) mutation”

Advances in gout and hyperuricemia

48. Tappei Takada, Akiyoshi Nakayama, Hirotaka Matsuo, Hirofumi Nakaoka, Masayuki Sakiyama, Nobuyuki Hamajima, Hiroshi Suzuki, Tatsuhiro Hosoya, Kimiyoshi Ichida, Nariyoshi Shinomiya “Comparison for the effects on hyperuricemia progression between genetic and environmental factors”
49. Shunya Uchida, Wenxiu Chang, Takeshi Shiraiishi, Yoshifuru Tamura, Tatsuru Ota, Shigeru Shibata, Yoshihide Fujigaki, Kiyoko Kaneko, Makoto Hosoyamada, Shin Fujimori “Serum uric acid has a significant impact on renal outcomes using propensity score method”
50. Puig JG, de Miguel E, Torres RJ, Sánchez A, Beltrán LM “Ultrasound-based classification and treatment of asymptomatic hyperuricemia and gout”
ABSTRACT

SATURDAY, JUNE 6, 2015 - (Seminar Room 1,2,3,4 & Reception)

16:00-16:25: I-1: Rosa Torres, PhD "Hypoxanthine deregulates genes involved in early neuronal development. Implications in Lesch-Nyhan disease pathogenesis"

Torres RJ and Puig JG.
Metabolic-Vascular Unit, La Paz University Hospital, IdiPAZ, Madrid, Spain.

Neurological manifestations in Lesch-Nyhan disease (LND) are attributed to the effect of hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficiency on the nervous system development. HPRT deficiency causes the excretion of increased amounts of hypoxanthine into the extracellular medium. We hypothesized that HPRT deficiency related hypoxanthine excess may lead, directly or indirectly, to transcriptional aberrations in a variety of genes essential for the development and function of striatal progenitor cells.

We have examined the effect of hypoxanthine excess on the differentiation of neurons in the well-established human NTERA-2 cl.D1 (NT2/D1) embryonic carcinoma neurogenesis model. NT2/D1 cells differentiate along neuroectodermal lineages after exposure to retinoic acid (RA). Hypoxanthine effect on RA-differentiation was examined by the changes in the expression of various transcription factor genes essential to neuronal differentiation and by the changes in tyrosine hydroxylase (TH), dopamine, adenosine and serotonin receptors (DRD, ADORA, HTR).

We report that hypoxanthine excess deregulates WNT4, from Wnt/β-catenin pathway, and engrailed homeobox 1 gene and increased TH and dopamine DRD1, adenosine ADORA2A and serotonin HTR7 receptors, whose over expression characterize early neuro-developmental processes.


Claus Desler1,2, Jane H. Frederiksen1,2, Maria Angleys1,2, Scott Maynard1,2, Guido Keijzers1,2, Birgitte Fagerlund1,3, Erik Lykke Mortensen1,4, Merete Osler4,5, Martin Lauritzen3,6,7, Vilhelm A. Bohr3,8, Lene Juel Rasmussen1,2

1Center for Healthy Aging; 2Department of Cellular and Molecular Medicine, University of Copenhagen, Denmark; 3Lundbeck Foundation Center for Clinical Intervention and Neuropsychiatric Schizophrenia Research (CINS), and Center for Neuropsychiatric Schizophrenia Research (CNSR), Mental Health Centre Glostrup, Denmark; 4Department of Public Health, University of Copenhagen, Denmark; 5Research Center for Prevention and Health, Glostrup Hospital, Glostrup, Denmark; 6Department of Neuroscience and Pharmacology, University of Copenhagen, Copenhagen N, Denmark; 7Department of Clinical Neurophysiology, Glostrup Hospital, Glostrup, Denmark; 8Laboratory of Molecular Gerontology, National Institutes on Aging, NIH

Mitochondria are the powerhouse of the cell and where cellular energy supplies in the form of ATP are generated. Because of this pivotal role, mitochondrial dysfunction is very damaging for the cell and can lead to numerous pathological conditions in humans.

Most notably, mitochondrial dysfunction is associated with cognitive decline, neurological abnormalities and aging. Balanced levels of dNTP are important for genomic stability. Accordingly, imbalance of the cytosolic dNTP pool has been demonstrated to decrease the genetic stability. We have previously shown that depletion of mtDNA of human cell lines results in an imbalance of the cytosolic dNTP pools and a decrease of chromosomal stability. MtDNA primarily encodes peptides essential for the activity of the mitochondrial electron transport chain and, therefore, also ATP produced by oxidative phosphorylation. The ETC is also linked to the de novo synthesis of pyrimidines through the enzyme dihydroorotate dehydrogenase (DHODHase) located in the inner membrane of the mitochondria. Our findings support a model for the initiation of genome instability through a mitochondrial dysfunction and resulting imbalance of the cytosolic dNTP levels. This places fitness of mitochondria as an important determinant of genomic instability.

Cognitive impairment in adults may be an early indicator of later life dementia. Therefore, it is important to search for early biomarkers of cognitive decline. We have tested peripheral blood mononuclear cells (PBMCs) isolated from low- and high-cognitive score subjects, for correlates to cognitive function that could potentially serve as biomarkers. Cognitive score is assessed by the Isaacs Set Test (IST) (1) and the Danish Military Draft Board Intelligence Test (Børge Priens Prøve, BPP). The population is drawn from the Metropolit Cohort of men born in 1953 as part of the Copenhagen Aging and Midlife Biobank (CAMB) data collection. The extremes of high and low cognitive measure are being used in this study. We have measured oxygen consumption rate (OCR), dNTP ratios, reactive oxygen species (ROS) levels, and DNA damage for the all cohort participants who participate. Our data promote investigation into mitochondrial activities, dNTP levels, and DNA damage as potential correlates or predictors of cognitive decline, which may lead to early treatment initiatives in order to delay or prevent later life dementia.
Gout is a common disease caused by the deposition of monosodium urate crystals due to hyperuricemia. Genetic and environmental factors each play a role in hyperuricemia pathogenesis. Considering genetic factors, a number of single nucleotide polymorphisms in urate transporter genes have been associated with hyperuricemia.

To further understand this correlation, we investigated the relationship between ABCG2 dysfunction and urate excretion in hyperuricemic Japanese male outpatients. The severity of their ABCG2 dysfunction was estimated by genotype combination of two common ABCG2 variants, nonfunctional Q126X (rs72552713) and half-functional Q141K (rs2231142). The analysis revealed that ABCG2 dysfunction significantly increased the risk of overproduction type hyperuricemia as well as underexcretion type, and that the level of risk corresponds with the severity of ABCG2 impairment. In another experiment using Abcg2-knockout mice, we confirmed that serum urate levels and renal urate excretion increased, while intestinal urate excretion decreased. These data suggest that the “overproduction type” in the conventional hyperuricemia classification actually consists of two subtypes, “extra-renal urate underexcretion” and genuine “urate overproduction.” In this talk, new classifications of hyperuricemia and the significance of genetic factors in causing hyperuricemia will be discussed.

Purine and pyrimidine nucleotides are essential for a vast number of biological processes such as the synthesis of RNA, DNA, signal transduction, phospholipids, glycogen and the sialylation and glycosylation of proteins, cofactor synthesis and the supply of high-energy phosphate esters (e.g. ATP) in phosphate transfer reactions. In addition, purines and pyrimidines play an important role in the regulation of the central nervous system and metabolic changes affecting the levels of purines and pyrimidines might lead to abnormal neurological activity.

Both purines and pyrimidines can be synthesized de novo in mammalian cells through multistep processes. In addition to the de novo synthesis, purine nucleotides can also be synthesized via the salvage of the purine bases adenosine, guanosine and hypoxanthine. In contrast, the salvage pathway for pyrimidine nucleotides occurs at the level of the nucleosides uridine and cytidine. Ribonucleosides and deoxyribonucleosides are imported into the cells by nucleoside transport proteins that facilitate diffusion or actively transport nucleosides across the membrane. The de novo and salvage pathways are present in all nucleated cells and particularly active in proliferating cells. However, the majority of the ribonucleotides is preferentially synthesized via the energetically favorable salvage pathway. In contrast, the majority of the deoxyribonucleotides are synthesized by the de novo pathway. In proliferating cells, this pathway is the main provider of dNTPs needed for DNA replication. Opposing the action of the enzymes involved in anabolism of purines and pyrimidines are those facilitating the degradation. The degradation of purines ultimately leads to the production of uric acid while the pyrimidine catabolism results in the synthesis of β-alanine and β-aminoisobutyric acid.

To date, more than 30 defects of the metabolism of purines and pyrimidines have been described. Inherited disorders of purine and pyrimidine metabolism have a wide variety of clinical presentations which include, among others, anaemia, immunodeficiency, kidney stones, convulsions, mental retardation, autism and growth retardation. So far, therapeutic options such as bone marrow transplantation, enzyme replacement therapy, gene therapy, substrate reduction and administration of nucleoside (analogues) are available for only a few inborn errors of purine and pyrimidine metabolism.

Patients with a defect in the pyrimidine degradation pathway present mainly with neurological symptoms. However, the identification of individuals with a complete deficiency in one of the enzymes of the pyrimidine degradation pathway without any clinical manifestations and the marked intrafamilial variability in phenotype, indicate that additional (epi)genetic and environmental factors are likely to be involved. The pathogenesis underlying the various clinical manifestations remains as yet unknown. Biochemically, patients with a deficiency in one of the three enzymes of the pyrimidine degradation pathway show moderately decreased levels of β-alanine and strongly decreased levels of β-aminoisobutyric acid. β-Alanine is a structural homologue of γ-aminoobutyric acid and glycine, which are the major inhibitory neurotransmitters in the central nervous system. β-Alanine itself has been suggested to be involved in synaptic transmission and has been shown to regulate dopamine levels. In addition, β-alanine is the rate-limiting substrate for the synthesis of carnosine. Oral administration of β-alanine increases the carnosine content in muscles and delays the onset of neuromuscular fatigue while improving endurance performance and lean body mass. β-Aminoisobutyric acid is a partial agonist of the glycine receptor. Recently, it was demonstrated that β-aminoisobutyric acid is able to increase the secretion of leptin and stimulate fatty acid oxidation. Leptin itself has been shown to have neuroprotective, cognitive and anticonvulsant effects. Thus, it is conceivable that the altered homeostasis of β-aminoisobutyric acid in patients with a pyrimidine degradation defect might affect leptin levels and fatty acid oxidation and contribute to the development of mental retardation and hypotonia in these patients.
08:30-10:30 am. Session 1: “Advances in gout and hyperuricemia”. Becker MA (USA) and Tappei Takada (Japan)

08:30-09:00: I-5: Tappei Takada. “Comparison for the effects on hyperuricemia progression between genetic and environmental factors”

Tappei Takada, Akiyoshi Nakayama, Hirotaka Matsuo, Hirofumi Nakaoka, Masayuki Sakayama, Nobuyuki Hamajima, Hiroshi Suzuki, Tatsuho Hosoya, Kimiyoshi Ichida, Nariyoshi Shinomiya

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OBJECTIVES: Gout and hyperuricemia is a common multifactorial disease and known to have typical environmental risk factors including overweight/obesity, heavy drinking, and aging. Recently, common dysfunctional variants of urate exporter gene ABCG2/BCRP are revealed to be a major cause of gout and hyperuricemia.

MATERIAL AND METHOD: In a cohort of 5,005 Japanese participants, we compared the influence of ABCG2 dysfunction and other typical risk factors on hyperuricemia using population-attributable risk percent (PAR%), as well as on serum uric acid (SUA) levels through linear regression analyses.

RESULTS: ABCG2 dysfunction was detected in 53.3% of the population investigated. The PAR% for hyperuricemia was 29.2%, which was much higher than those for the other typical environmental risks, i.e. overweight/obesity (BMI ≥ 25.0; PAR = 18.7%), heavy drinking (> 196 g/week (male) or > 98 g/week (female) of pure alcohol; PAR% = 15.4%), and aging (≥ 60 years old; PAR% = 5.74%), while PAR% of sex (male) was 91.7%. SUA significantly increased as the predicted function of ABCG2 decreased (P = 5.99 × 10^{-19}). A regression analysis revealed that ABCG2 dysfunction had a stronger effect than other factors; a 25% decrease in the predicted ABCG2 function was equivalent to “an increase of BMI by 1.97-point” or “552.1 g/week alcohol intake as pure ethanol” in terms of ability to increase SUA.

DISCUSSION: Common dysfunctional variants of ABCG2 have a much stronger impact on the progression of hyperuricemia than other well-known risks. Our study provides a better understanding of common genetic and environmental factors for common diseases.

9:15-9:30: O-2: Shunya Uchida. “Serum uric acid has a significant impact on renal outcomes using propensity score method”

Shunya Uchida, Wenxiu Chang, Takeshi Shiraishi, Yoshifuru Tamura, Tatsuru Ota, Shigeru Shibata, Yoshihide Fujigaki, Kiyoko Kaneko, Makoto Hosoyamada, Shin Fujimori

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BACKGROUND: Hyperuricemia has been advocated as a possible risk factor for CKD progression. However, evidence still remains scarce and several randomized clinical trials are under way. By using propensity score (PS) method, the present study aimed to examine the impact of serum uric acid (UA) on renal outcomes using retrospective CKD cohort in our institution.

METHODS: 770 CKD patients at the stage of 3 to 4 were enrolled from January 2008 to January 2014 (61.9 ± 13.1 years: M : F = 481 : 289), excluding nephrotic syndrome, malignancy, acute kidney injury, obstructive nephropathy and so on. Follow-up serum UA was calculated as time-averaged value by a trapezoidal rule. Binary multivariate logistic regression was done by separating at the median of the time averaged UA (TA-UA) values together with other baseline covariates such as sex, age, BMI, blood pressure, diabetic nephropathy, eGFR, proteinuria, hematuria, UA, etc. For PS matching, a caliper size was set at 0.25 x SD of PS. Renal outcomes were defined as dialysis inception and decline of eGFR.

RESULTS: After matching, the number of participants and the median of TA-UA turned out to be n = 338 and 6.65 mg/dl for dialysis inception, n = 358 and 6.62 mg/dl for 50% decline of eGFR and n = 350 and 6.54 mg/dl for 30% decline of eGFR. None of the 20 confounders showed the significant difference between two subgroups. Kaplan-Meier analyses revealed that the higher level of TA-UA resulted in worse renal outcomes. Moreover, Cox regression analyses confirmed the independent effects of TA-UA on the renal survival with respect to ESRD in the full cohort.
CONCLUSION: PS matching method can mimic the prospective randomized clinical trial using the retrospective observation cohort. Our results show that hyperuricemia per se has a significant impact on the renal outcomes.


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OBJECTIVES: Gout is a common disease resulting from hyperuricemia. Recently, genome-wide association studies (GWASs) of gout have been reported; however, they included self-reported gout cases in which clinical information was not sufficient. Therefore, the relationship between genetic variation and clinical subtypes of gout remains to be clarified. Thus, we first performed gout GWAS using clinically-defined cases only in a Japanese population.

MATERIAL AND METHOD: GWAS was conducted with 945 male patients with clinically-defined gout cases and 1,213 male controls. Additionally, replication study of 1,048 clinically-defined cases and 1,334 controls was performed.

RESULTS: Five gout susceptibility loci were identified at the genome-wide significance level (p < 5.0 × 10⁻⁸), which contained well-known urate transporter genes (ABCG2 and SLC2A9) and additional genes reported to have relationships with metabolic pathways: rs1260326 (p = 1.9 × 10⁻¹²; OR = 1.36) of GCKR (a gene for glucose and lipid metabolism), rs2188380 (p = 1.6 × 10⁻²³; OR = 1.75) of MYL2-CUX2 (genes associated with cholesterol and diabetes mellitus) and rs4073582 (p = 6.4 × 10⁻⁹; OR = 1.66) of CNIH-2 (a gene for regulation of glutamate signaling). The latter two are identified as novel loci for gout. Furthermore, among the identified SNPs, we demonstrated that the SNPs of ABCG2 and SLC2A9 were differentially associated with subtypes of gout and clinical parameters underlying specific subtypes (renal underexcretion type and renal overload type). The effect of the risk allele of each SNP on clinical parameters showed significant linear relationships with the ratio of the case-control ORs for two distinct types of gout (r = 0.96 [p = 4.8 × 10⁻⁴] for urate clearance and r = 0.96 [p = 5.0 × 10⁻⁴] for urinary urate excretion).

CONCLUSION: Our findings provide clues to better understand the pathogenesis of gout and will be useful for development of companion diagnostics.

9:45-10:30: I-6: Juan Puig. "Ultrasound-based classification and treatment of asymptomatic hyperuricemia and gout"

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Sensitive imaging techniques, such as ultrasound and dual-energy computed tomography have demonstrated that a large percentage of patients with asymptomatic hyperuricemia (AH) or gout show monosodium urate deposits (tophi) and that these deposits elicit inflammation. Accordingly, a new clinical staging system for AH and gout has been proposed.

A. Patients with increased serum urate concentrations but without evidence of urate deposits (patients with heart failure, COPD, metabolic syndrome ...).
B. Patients with increased serum urate concentrations and evidence of urate deposits (same patients as in A in whom image techniques demonstrate urate deposits).
C. Patients with previous gout flares but no evidence of urate deposits (patients with gout).
D. Patients with tophaceous gout (same patients as in C in whom image techniques demonstrate urate deposits).

In 2008 we published for the first time that about third of the patients with AH do have ultrasound findings of urate deposits ("microtophi") and about a fourth of these patients show Doppler signal (inflammation). Thereafter three additional studies have show similar results (urate deposits in AH, 34-42%). These observations prompt two important questions: (1) If a given patient with AH has tophi, should the subject be diagnosed as having AH (type A) or should the subject be diagnosed as having asymptomatic...
gout (type B)?, and (2) Should this patients with asymptomatic tophi and Doppler signal be treated with hypouricemic agents? If both questions are answered in an affirmative way, we may well envision an important change concerning AH.

Close to 50% of the patients with gout and no clinical tophi do have microtophi when examined with image techniques. Three apparent beneficial consequences could be envisioned if an ultrasound study is performed in every gout patient. First, patient classification into C and D would be more precise. Second, hypouricemic treatment in patients with tophi would be more intensive (<5.0 mg/dL) to achieve resolution. And third, prophylactic therapy to prevent acute gout flares, in addition to hypouricemic treatment, would be guided by ultrasound findings. All together, we firmly believe that sonography will markedly change nowadays clinical classification of asymptomatic hyperuricemia and gout with an impressive impact on therapy.

11:00-11:30: I-7: Monika Loeffler “Orotic acid (1905-2015) - in the cell, in milk: pro-growth and anti-gout”

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The two significant human disorders of pyrimidine de novo synthesis cause strikingly different clinical phenotypes in patients: orotic aciduria (UMP synthase mutations, first patient diagnosed in 1959, overflow in orotate level, global developmental delay, oral uridine therapy) and Miller Syndrome (causative dihydroorotate dehydrogenase mutations known since 2009, putative deficiency of orotate, dysmorphology of newborns, no uridine therapy). No patients with mutants in CAD have been found. This has provoked our re-consideration of the long exciting history of orotic acid: nutritionally valuable milk component, vitamin B13; intermediate of pyrimidine de novo synthesis, product of mitochondria; neuroprotective and uricosuric properties; overdosed in rats: hepatic steatosis and tumors; transcriptional activator; metal ions complexing agent in replacement and antitumor therapies. Since many of these findings were related to rodents but extrapolated to humans, we anticipate that many pending questions may be answered by genetic manipulation of novel / non-mammalian animal models.

11:30-11:45: O-5: Ivan Sebesta “Hereditary xanthinuria is not so rare disorder”

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Hereditary xanthinuria is caused by inherited deficiency of the the xanthine dehydrogenase/ oxidase (XDH/XO) - type I , and is characterized by very low concentration of uric acid in blood and urine and high concentration of xanthine in urine, leading to urolithiasis. Type II results from a combined deficiency of XDH/XO and aldehyde oxidase. Patients present with hematuria, renal colic, urolithiasis or even acute renal failure. A third type, clinically distinct, molybdenum cofactor deficiency is characterized by the lack of sulfite oxidase activity as well as XDH/XO and aldehyde oxidase. The exact prevalence is not known, but about 150 cases have been described so far. An annual incidence has been estimated between 1:6,000 and 1:69,000. This wide variability is due to the fact that half of the affected individuals remains asymptomatic and therefore this condition is underdiagnosed. Moreover, hypouricemia is sometimes overlooked, that’s why we have set up the diagnostic flowchart for this genetic defect of purine metabolism.

This consists of: a) evaluation of uric acid concentrations in serum and urine with exclusion of primary renal hypouricemia as we reported previously, b) estimation of urinary xanthine, c) allopurinol loading test, which enables to distinguish type I and II; and finally in some cases xanthine oxidase activity assay in plasma with molecular genetic analysis. Following this diagnostic procedure we were able to find first patients with hereditary xanthinuria in our Czech population. We have detected nine cases, which is one of the largest group worldwide, in the terms of number of patients. Four patients were asymptomatic. All individuals had profound hypouricemia , which was the first sign and led to referral of these cases to our department. Urinary concentrations of xanthine, were in the range of 170-598 mmol/mol creatinine (normal < 30 mmol/mol creatinine) . Our experience shows that patients with urinary xanthine concentrations in excess of 320 mmol/mol creatinine developed renal calculi. The xanthine oxidase activities in plasma in two cases were 0 and 0.37 pmol/h/mL of plasma (ref.range:3.2-9.2 pmol/h/mL of plasma ). The nonsense heterozygous mutation p.R825X was found in two patients.

Hereditary xanthinuria is still unrecognized disorder and probably not so rare as previously thought. Patients with unexplained hypouricemia need detailed purine metabolic investigation.
11:45-12:00: O-6: Yoko Nakajima  Clinical, biochemical, and molecular analysis of 30 children with β-ureidopropionase deficiency demonstrates high prevalence of the c.977G>A (p.R326Q) mutation"

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β-Ureidopropionase (βUP) deficiency is an autosomal recessive disease characterized by N-carbamyl-β-amino aciduria. Until recently, only 16 genetically confirmed patients with βUP deficiency have been reported. To elucidate the disease state and genetical feature, we studied on the clinical, biochemical and molecular aspects of 30 βUP deficient children.

Pyrimidine analysis by GC/MS or HPLC-MS/MS in urine samples and DNA analysis of UPB1 were performed. Wild-type and mutant βUP protein were expressed in HEK293 cells and used for enzyme activity assay, western blot analysis and native gel electrophoresis.

Highly variable phenotypes ranging from neurological involvement to asymptomatic were observed in diagnosed patients. Three novel missense mutations (p.G31S, p.E271K, and p.I286T) and a recently described mutation (p.R326Q) were identified. The p.R326Q mutation was detected in all 30 patients with 24 patients homozygous for this mutation. The mutant βUP enzymes expressed in HEK293 cells showed that the p.E271K and p.R326Q mutations cause profound decreases in activity. Conversely, the p.G31S and p.I286T mutations possess residual activities of 50 and 70%, respectively. Analysis of a human βUP homology model revealed that the effects of the mutations (p.G31S, p.E271K, and p.R326Q) on enzyme activity are most likely linked to improper oligomer assembly.

In this study, no clear correlation between phenotype and genotype were observed. Identification of the R326Q mutation in both neurologically affected patients and unaffected individuals suggests that additional factors are involved in the clinical outcome. High frequency of the R326Q mutation in our patients indicates that βUP deficiency is not as rare as generally considered.

12:00-12:15 O-7: Juan Puig "Self-injurious behavior following dental extraction in Lesch-Nyhan disease"

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BACKGROUND: Self-injurious behavior (SIB) is the most disturbing manifestation of Lesch-Nyhan disease (LND) and is hardly treatable. Total teeth extraction has been reported in extreme cases with immediate relief. However, we do not know the long term consequences of total dental extraction. We report two LND patients who developed new forms of SIB following total dental extraction.

PATIENTS AND METHODS: MM was diagnosed at age 20 months as having complete HPRT deficiency (c. 384 +1 G>A, exon 4 exclusion, NM_000194.2). At age 6 years he began to bite his lips and hands. At age 11 years he bitted his tongue with tissue loss. A mouth guard was provided but proved to be ineffective. Finally, at age 13 years total teeth extraction was performed under general anesthesia. MAGR was given a diagnosis of LND at age 10 months due to the absence of HPRT activity with a deletion of the HPRT gene including exon 1 and the promoter region. At age 13 months he started to bite his hands. At age two years and due to lip biting an oral prosthesis was provided. His lips cheeks and hands were continuously bitten. At age 4 years the first tooth extraction was performed and this was continued as dentition evolved because self-biting was unavoidable. At age 8 years his last four teeth were removed. Dystonia was assessed according the Fahn-Marsden scale and SIB according to the behavioral problem questionnaire BPI. Quality of life was assessed on a 0 to 100 point visual scale.

RESULTS: Both patient mothers reported that overall and after 12 and 17 month following total dental extraction, they were very satisfied with the radical procedure. Frequency and severity of BPI were markedly reduced in both patients. Their quality of life markedly improved by 50 absolute points in MM and by 80 points in MAGR, on the 100 point scale. However, 2-3 weeks (MM) and 7 months (MAGR) after total dental extraction both patients started to seek other means of self-agression. MM started rubbing one foot against the other and scratching toenails with his hands. MAGR stuck his legs and feet against hard objects. These forms of self-injury behavior could be easily prevented with gloves, socks and protective materials, according to the mothers.

CONCLUSION: Following total dental extraction, we have observed that two LND patients do not bite but develop new forms of SIB which are milder, can be easily prevented, and both patients and families appears relieved with a marked improvement in their quality of life.
12:15-12:30 O-8: Kiyoko Kaneko “Urinary excretion of uric acid, allantoin, and 8-OH-deoxyguanosine in uricase-knockout mice”

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OBJECTIVES: The end product of purine metabolism in humans is uric acid (UA) because of the lack of uricase. But in other mammal, UA is oxidized to allantoin by the action of uricase. Uricase deficient mice are reported to develop uric acid nephropathy, with high mortality rates before weaning. When Uricase-knockout (Uox KO) mice grow up without severe nephropathy, it will be useful as a human model in research of purine metabolism. In this study, we determined urinary excretion of creatinine (Cr), UA and allantoin in Uox KO mice. In human, allantoin is reported to be generated non-enzymatically from UA with an antioxidant reaction of UA. Thus, an oxidative stress marker, 8-hydroxy-2’-deoxyguanosine (8-OHdG) was also determined in urine of Uox KO mice.

MATERIALS AND METHODS Uricase-knockout mice (C57BL/6:129S7-uOXTm/bay/J) were purchased from The Jackson Laboratory and were brought up in animal laboratory of Teikyo University. Wild-type C57BL/6j mice were used as control. 4 males and 4 females around 20 weeks age were used in this study. Urine was collected for 24 hours in metabolic cage with or without feed CRF-1, and was stored at -30 °C until LC-MS analysis. Urine was first eluted on the Monospin SAX to remove impurities. UA and allantoin was determined with liquid chromatography - mass spectrometry (LC-MS). LC-MS was performed with UPLC-micromass ZQ 4000, HSS T3 column (150 mmÅ²2.1 mm i.d.), and 10 mM formic acid /10 mM formic acid ammonium. UA was detected with m/z 169 and allantoin was with m/z 159. Cr and 8-OHdG were measured with a commercial kit.

RESULTS AND DISCUSSION: Urinary excretion of Cr in Uox KO mice was not different from that in wild-type mice in spite of the lack of uricase (17.0 ± 3.1 in Uox KO and 21.8 ± 6.2 μg/g/day in wild-type). But in Uox KO mice, excretion of Cr with food was significantly higher than that without food. Urinary excretion of UA in Uox KO mice was significantly higher than that in wild-type mice. On the other hand, urinary excretion of allantoin was significantly lower than that in wild-type mice. It is interesting that allantoin was detected in urine of Uox KO mice in spite of lack of uricase. Allantoin in Uox KO mice may be generated non-enzymatically from UA. Food intake affected on the urinary excretion. In Uox KO mice with food, the daily excretion of both UA and allantoin increased. Urinary 8-OHdG in Uox KO mice was lower than that in wild-type mice. Low excretion of 8-OHdG means the decrease of oxidative stress. It is possible that high level of UA in Uox KO mice acted as an antioxidant agent. In female Uox KO mice with food, the daily excretion of 8-OHdG increased significantly compared without food.

CONCLUSIONS: Daily excretion of UA in Uox KO mice was higher than that in wild-type mice, and conversely 8-OHdG was lower. These results are considered to show that UA acts as anti-oxidizing agent. Urinary allantoin was detected in Uox KO mice. In order to confirm the origin of allantoin, further examination with the obvious oxidative stress seems to be necessary.

14:30-15:00: I-8: G.J. Peters “Fluoropyrimidines: Beyond inhibition of thymidylate synthase”

GJ Peters
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The classical fluoropyrimidines are based on the uracil analog, 5-fluorouracil (5FU). The development of this compound was based on this similarity but also on that with thymine. Hence already in the early days of 5FU it was recognized that one of the targets of 5FU would be inhibition of thymidylate synthase (TS) by the 5FU metabolite, 5-fluoro-2’-deoxyuridine-monophosphate (FdUMP). This inhibition is mediated by the formation of a binary complex between FdUMP and TS. A breakthrough in the treatment with 5FU would be inhibition of thymidylate synthase (TS) by the 5FU metabolite, 5-fluoro-2’-deoxyuridine-monophosphate (FdUMP).

However, not all patients respond to this treatment, leading to recurrence of disease. In order to select patients for the best treatment and spare them from potential toxicities, several approaches have been taken. These include the evaluation of various polymorphisms/amplification/splicing in the TS gene, in the DPD gene, in genes responsible for activation of 5FU or capecitabine,
in genes important for uptake and metabolism of LV and in genes responsible for breakdown of active metabolites, such as dUTPase. Next to that genomic approaches focused on analysis of the genome, resulting in the characterization of chromosomal regions (either amplified or deleted), that were associated with a poor or good survival. Also gene expression approaches using either classical micro-arrays or a sequencing technology have characterized some unexpected genes that appeared to be associated with survival. Last but not least, recent advances in proteomic technology also led to the characterization of unexpected proteins which are not only associated with the risk for colorectal cancer, but appeared to be increased or decreased upon treatment with 5FU or 5FU-LV. These proteins are currently being investigated as additional targets in combination with 5FU.

In conclusion: despite being used for decades and essential for curing the majority of colorectal cancer patients, we still did not unravel all resistance mechanisms of 5FU.

15:00-15:15: O-9: Lars Petter Jordheim “Downregulation of 5’-nucleotidase cN-II in cancer cells favors tumorigenicity and suggests impact on metabolic adaptability”

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The 5’-nucleotidase cN-II catalyzes nucleoside monophosphates into nucleosides, and is thereby implicated in nucleoside and nucleotide metabolism of cells. To better understand the global function of this enzyme in cancer cells, we inhibited its expression using a specific RNA silencing approach, in human cancer cells of breast (MDA-MB-231), colon (HCT-116), pancreas (MiaPaCa) or lung (NCI-H292) origins. These models showed stable and decreased cN-II protein expression associated with decreased enzymatic activity. Cells with low cN-II expression (pScN-II) as compared to control cells (pScont), did not display altered sensitivity to cancer drugs. However, upon grafting in immuno-deficient mice, tumor growth was enhanced for the four distinct pScN-II models in comparison to their respective pScont cells.

Preliminary metabolic analyses were performed with breast carcinoma MDA-MB-231 lines. Under glucose-limited culture conditions (5-10 mM glucose), pScN-II cells could proliferate more (more biomass), and displayed a better long-term survival than controls as evidenced by AnnexinV/PI staining. This was associated with a lower content of reactive oxygen species (ROS) in pScN-II cells, either due to a lower ROS production or an improved neutralization of ROS. In similar glucose-limiting conditions, pScN-II cells were more sensitive to rotenone, a specific inhibitor of complex I in respiratory chain. Experiments using the xCELLigence system confirmed differences in long term culture. Indeed, while both pScN-II and pScont cells showed comparable initial proliferation, they subsequently manifested a wave of impedance variation that correlated in a time-dependent manner with the initial glucose level. These variations appeared at the same time for both lines but were clearly less marked for pScN-II cells. We hypothesize that such variations may correspond to a forced metabolic shift from glycolysis to oxidative phosphorylation, upon glucose exhaustion, which seems to occur more smoothly for pScN-II cells. Indeed, the same long-term culture experiments performed under hypoxia indicated a higher dependence of pScN-II cells for oxygen since they did not maintain long term survival and died faster under the same glucose limiting conditions.

In summary, our results highlight a connection between the nucleotide metabolism enzyme cN-II and energetic metabolism of glucose in human breast cancer cells MDA-MB-231. In relation with cN-II decrease, tumor cells have modified some bioenergetic parameters allowing improved long term survival under glucose-limiting conditions. Further metabolic studies with other tumor types should enable us to understand the meaning of the relationship between cN-II and energetic metabolism.


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Introduction
Uridine-cytidine kinase (UCK) is a pyrimidine ribonucleoside kinase that catalyzes the phosphorylation of uridine and cytidine to UMP and CMP, respectively. UCK also catalyzes the phosphorylation, and thereby the pharmacological activation, of several cytotoxic pyrimidine ribonucleoside analogues. Two human UCKs have been reported with UCK1 being ubiquitously expressed in several tissues whereas the expression of UCK2 seems to be confined to human placenta and various tumor cells. In this study, we investigated the functional role of UCKs in neuroblastoma.

Materials and Methods:
The expression of UCKs in neuroblastoma cell lines was investigated with quantitative PCR and Western blotting using antibodies generated against purified UCK1 and UCK2. Subcellular localization of UCKs was investigated using UCKs expressed in fusion with green fluorescent protein. Cytotoxicity towards 3-deazauridine was assessed using MTS in neuroblastoma cells, transfected with the pcDNA3.1Zeo(+) vector containing UCK1 or UCK2, or after transfection with siRNA targeting UCK2.
Results:
Analysis of mRNA and protein levels coding for UCK1 and UCK2 showed that UCK2 is by far the most abundantly expressed UCK in a panel of neuroblastoma cell lines. Subcellular localization studies showed that the UCK1-GFP protein was localized in the cell nucleus whereas UCK2-GFP was located in the cytosol. To investigate the role of UCKs in metabolizing pyrimidine analogues we tested the cytotoxicity of 3-deazauridine which is activated primarily by UCK2. Transient overexpression of UCK2 in neuroblastoma cells resulted in an increased cytotoxicity of 3-deazauridine whereas knockdown of endogenous UCK2 protected the neuroblastoma cells from 3-deazauridine-induced toxicity. However, overexpression of UCK1 protected the neuroblastoma cells from 3-deazauridine-induced toxicity. Subcellular localization studies showed that co-expression of UCK1 with UCK2 resulted in a nuclear localization of UCK2 instead of its normal cytosolic localization.

Conclusion:
The discovery that UCK2 is highly expressed in neuroblastoma opens the possibility for selectively targeting neuroblastoma cells using UCK2-dependent pyrimidine analogues, while sparing normal tissues.
08:30-09:00: I-9: Imogen Coe  "The SLC29 family of concentrative purine and pyrimidine nucleoside transporters: New modalities of regulation and therapeutic"

Imogen R. Coe
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Purine and pyrimidine nucleosides rely on membrane transport proteins to cross cell membranes. The SLC28 and SLC29 transporter families are responsible for the uni- or bi-directional flux of nucleosides and nucleoside analog drugs used in a wide variety of clinical settings. Our research focuses on the contributions of the SLC29 family (the ENTs) to purinergic signaling, cellular homeostasis and clinical efficacy of drugs. Factors and mechanisms that regulate the ENTs are still poorly understood and the new roles of ENTs continue to be discovered. We use a variety of biochemical, physiological and pharmacological approaches to investigate the role, function and regulation of ENTs. Our data show that ENTs exist in a continual dynamic flux within the cell and are regulated at the plasma membrane by glycosylation, protein-protein interactions and the formation of homo- and hetero-complexes. Moreover, ENT1 is functionally integrated into, and regulated by, more extensive cellular signaling pathways, including, but not limited to purinergic pathways, while ENT2 splice variants regulate the location and function of ENT2, impacting cellular proliferation in a novel and previously un-described manner. We are also investigating the role of ENTs in novel combination therapies, to address clinical situations where ENT expression is low and therefore drug efficacy is compromised. Taken together, our data suggest that ENTs are involved in basic cellular homeostasis and regulated in complex ways that need to be fully understood in order to optimize nucleoside analog drug therapies.

Funding for the studies presented here is provided by Natural Science & Engineering Council of Canada (NSERC) and by Ryerson University.

09:00-09:30: I-10: Marçal Pastor  "The SLC28 family of concentrative purine and pyrimidine nucleoside transporters: New modalities of regulation and therapeutics"

Marçal Pastor-Anglada
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Human Concentrative Nucleoside Transporter Proteins (hCNT1, hCNT2, and hCNT3) are encoded by the three members of the SLC28 gene family. They show substantial differences in functional properties and are key determinants of nucleoside-derived drug bioavailability and action. They all mediate the Na+-coupled uptake of nucleosides. Along with SLC29 encoded proteins (Equilibrative Nucleoside Transporters, ENTs) they allow transmembrane epithelial fluxes of nucleoside-derived drugs, thereby contributing to drug pharmacokinetics. In target cells, CNTs can also determine drug action, being also suitable targets for therapeutic intervention. CNT trafficking processes might be useful for this purpose. This concept will be addressed showing some examples of transporter regulation relevant to cancer, antiviral and anti-inflammatory therapies. Besides their role as drug transporters, CNT2 and CNT3 are also high affinity concentrative adenosine transporters likely to contribute to purinergic regulation in different epithelia, thereby being members of the purinome. Examples of purinergic regulation of CNT function will be also reviewed. Finally, some CNT proteins appear to behave as “transceptors”, as recently shown for CNT1. The role this protein might play in oncogenesis will be reviewed as well as recent data on CNT1 expression in human tumors will be also discussed. Overall, current knowledge on CNTs strongly support the view that they might play variable functions in a cell-specific manner. This is helping to understand the rationale for the apparent transporter expression redundancy found in most cells.
"ENT1, a novel link between purinergic and calcium signalling"

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Equilibrative nucleoside transporter 1 (ENT1) is an integral membrane protein that transports nucleosides and nucleoside analog drugs across cellular membranes. A relationship between purine nucleoside flux and calcium levels has previously been shown, but the underlying mechanisms have not been identified. We established that calcium regulates ENT1 function by measuring changes in radiolabelled nucleoside flux in HEK293 and RT4 cells in the presence of BAPTA-AM (25 µM), EGTA (50 µM), and thapsigargin (1.5 µM). Chelating extracellular calcium (EGTA) and intracellular and extracellular calcium (BAPTA-AM) decreased ENT1-dependent nucleoside flux by 12% and 39% respectively, while increasing intracellular calcium (thapsigargin) led to a 27% increase in nucleoside flux. We hypothesized that calcium regulates ENT1 through a physical interaction between ENT1 and calmodulin (CaM). To confirm that ENT1 is regulated by direct CaM-ENT1 interaction, we measured nucleoside flux after treatment with the CaM antagonist W7 (50 µM). We observed a 15% decrease in ENT1-dependent nucleoside flux with cells treated with W7 compared to control. Moreover, activation of NMDA receptors by glutamate (1 mM) and glycine (100 µM) in U-87 MG cells stimulated calcium influx (confirmed by live cell calcium imaging) and led to a 61% increase in nucleoside uptake. This increase in nucleoside flux was abolished in the presence of the NMDA receptor antagonist MK-801.
management. The structure of TK1 in serum and its relation to the use of TK1 as a biomarker for cell proliferation and turnover in cancer disease.

The regulation of the TK1 like enzymes by phosphorylation and enzyme concentration-dependent transition from a dimer to tetramer and higher oligomeric forms with altered catalytic efficiencies will be discussed. Results will be reviewed concerning the synthesis will be presented in the session on mitochondrial disorders, but some recent isothermal titration calorimetry (ITC) studies aspects of enzyme regulation in this group of enzymes, as well as structure based design of nucleoside analogs that can serve as translational control of enzyme function in different cells and tissues. Major advances have been made concerning structural which dCK, TK2 and dGK belong can be called the dCK like family. These families differ in biochemical properties such as subunit composition, substrate specificities and kinetics, as well a 3D structure and post-translational modifications. The basic structure-function properties of these enzyme families will be reviewed, focusing on recent finding concerning transcriptional and post mediated urate transport, we performed immunostaining and functional analysis of human NPT1 using Xenopus oocytes. For the measurement of [14C] urate uptake, Xenopus oocytes were incubated in high potassium buffer containing 100 µM [14C] urate for 60 min. In addition, for comparison of NPT1 expression levels of oocytes membrane between 269I and 269T, immunostaining was performed with anti-human NPT1 antibody. As a result, 269T-mediated urate transport is significantly higher than that of 269I while there was no remarkable difference on oocyte expression levels between 269I and 269T. Combined with the previous report that I269T did not induce Km changes but increased the maximum volume (Vmax) of urate transport in a proteoliposome system, our findings suggest that I269T elevates Vmax by increasing the turnover rate of urate transport via NPT1. I269T variant would increase turnover rate of urate transport because 269T is probably more flexible than 269I; i.e., threonine has a smaller and less hydrophobic side chain than that of isoleucine. Thus, 269T, a common missense variant of NPT1, might have faster conformation changes than 269I in terms of the alternating-access model of transporters, and increases renal urate export in humans.

expression of a human NPT1 missense variant which increases the urate export

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Abstract

Human sodium-dependent phosphate cotransporter type 1 (NPT1/SLC17A1) is one of the urate transporters in kidney. Previously, genome-wide association studies have revealed an association between common variants of NPT1 gene and serum uric acid. Also, our recent study revealed that the common missense variant, I269T (rs1165196), of NPT1 decreases the risk of renal underexcretion gout. Moreover, we demonstrated that human NPT1 is localized to the apical membrane of the renal proximal tubule, and that I269T is the gain-of-function variant which increases the NPT1-mediated urate export. Meanwhile, the mechanism by which I269T variant increases the urate export remains to be clarified. In this study, to investigate the mechanism of the NPT1-mediated urate transport, we performed immunostaining and functional analysis of human NPT1 using Xenopus oocytes. For the measurement of [14C] urate uptake, Xenopus oocytes were incubated in high potassium buffer containing 100 µM [14C] urate for 60 min. In addition, for comparison of NPT1 expression levels of oocytes membrane between 269I and 269T, immunostaining was performed with anti-human NPT1 antibody. As a result, 269T-mediated urate transport is significantly higher than that of 269I while there was no remarkable difference on oocyte expression levels between 269I and 269T. Combined with the previous report that I269T did not induce Km changes but increased the maximum volume (Vmax) of urate transport in a proteoliposome system, our findings suggest that I269T elevates Vmax by increasing the turnover rate of urate transport via NPT1. I269T variant would increase turnover rate of urate transport because 269T is probably more flexible than 269I; i.e., threonine has a smaller and less hydrophobic side chain than that of isoleucine. Thus, 269T, a common missense variant of NPT1, might have faster conformation changes than 269I in terms of the alternating-access model of transporters, and increases renal urate export in humans.

An update of the role of deoxynucleoside kinases in purine and pyrimidine DNA precursor metabolism

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DNA synthesis requires a balanced supply of deoxynucleoside triphosphates and there is two ways by which mammalian cells produce these precursors; the de novo and the salvage pathways. Key enzymes for de novo synthesis are ribonucleotide reductase and thymidylate synthase, while the salvage pathway depends on two cytosolic enzymes, TK1 and dCK and two mitochondrial enzymes: TK2 and dGK. These enzymes in mammalian cells belongs to two families; one comprising TK1 like enzymes and one to which dCK, TK2 and dGK belong can be called the dCK like family. These families differ in biochemical properties such as subunit composition, substrate specificities and kinetics, as well a 3D structure and post-translational modifications. The basic structure-function properties of these enzyme families will be reviewed, focusing on recent finding concerning transcriptional and post translational control of enzyme function in different cells and tissues. Major advances have been made concerning structural aspects of enzyme regulation in this group of enzymes, as well as structure based design of nucleoside analogs that can serve as substrate, leading to their activation and pharmacologic effect. This recent important work will be described in other presentations at this symposium as well as evolutionary aspect of these enzyme families. The role of TK2 and dGK in mitochondrial DNA precursor synthesis will be presented in the session on mitochondrial disorders, but some recent isothermal titration calorimetry (ITC) studies with purified recombinant human TK2 to investigate the reaction mechanism and the negative cooperativity of TK2 will be summarized. Results from intrinsic tryptophan fluorescence quenching studies with substrates and products will also be described. The regulation of the TK1 like enzymes by phosphorylation and enzyme concentration-dependent transition from a dimer to tetramer and higher oligomeric forms with altered catalytic efficiencies will be discussed. Results will be reviewed concerning the structure of TK1 in serum and its relation to the use of TK1 as a biomarker for cell proliferation and turnover in cancer disease management.
Recent advances in nuclear medicine have allowed for positron emission tomography (PET) to track transgenes in cell-based therapies using PET reporter gene/probe pairs. A promising example for such reporter gene/probe pairs are engineered nucleoside kinases that effectively phosphorylate isotopically-labeled nucleoside analogs. Upon expression in target cells, the kinase facilitates the intracellular accumulation of radionuclide monophosphate which can be detected by PET imaging.

We have employed computational design and machine-learning algorithms for the semi-rational engineering of human 2’-deoxycytidine kinase (dCK) to create reporter genes with high activity and selectivity for L-nucleosides including L-thymidine and 1-(2’-fluoro-5-methyl-β-L-arabinofuranosyl) uracil (L-FMAU). Our initial design strategy relied on an extended collection of nucleoside kinase sequence information to guide the preparation of small libraries of kinase variants with systematic amino acid changes. Following chemical synthesis of the corresponding gene libraries, the effects of amino acid substitutions in each variant could be assessed by detailed kinetic analysis and functionally beneficial changes combined in subsequent rounds. Three rounds of this semi-rational kinase engineering, involving the evaluation of a total of about 200 enzyme variants, yielded a series of PET reporters with unprecedented high activity and selectivity for the targeted L-nucleosides. Beyond creating effective dCK-based PET reporter genes for L-FMAU, this protein re-design approach is general and can easily be adapted for other combinations of kinase/nucleoside analog.

The 5’-nucleotidase cN-II dephosphorylates nucleoside monophosphates into nucleosides and inorganic phosphate and is able to catalyze the opposite reaction through its phosphotransferase activity. Its expression level or activity in cancer cells has been shown to correlate with the sensitivity to nucleoside analogues or the outcome of patients treated with such drugs. In addition, its inhibition has been shown to induce apoptosis in cancer cells, suggesting that cN-II can be considered as a drug target.

We evaluated the inhibitory effect of clinically used purine nucleoside analogues (fludarabine, cladribine and clofarabine) on recombinant cN-II and observed that fludarabine is a mixed mode inhibitor ($K_m$ increases and $V_{max}$ decreases) with $K_i$ of 0.5 and 9.0 mM. Docking experiments using previously published crystal structure of cN-II suggested a better interaction for fludarabine than adenosine in the effector site II. This site has been shown to be involved in the activation of cN-II activity by adenylic compounds. The interaction was confirmed using mutant cN-II showing a lack of inhibition by fludarabine on mutants in effector site II (F127A) whereas mutations in the active site (F157A) or in effector site I (R144E) were not sensitive to fludarabine inhibition. Finally, we showed that fludarabine was synergistic with the nucleobase 6-mercaptopurin in wild type haematological cancer cells, whereas no synergy was observed in corresponding cN-II deficient cells. This latter observation indicates that fludarabine acts as a cN-II inhibitor in cancer cells, which gives additional information on the mechanism of action of this largely used cancer drug.

Previous work from our laboratory indicates that adenosine, acting at the A2A receptor, promotes wound healing. However, prolonged A2A receptor stimulation leads to overproduction of matrix with scarring. As we have previously shown, adenosine A2A receptors play a critical role in the development of hepatic and dermal fibrosis. Here we discuss the role of adenosine receptors in fibrosis and potential approaches to treating and preventing fibrosis. We discuss the use of specific A2A receptor antagonists and the potential for blockade of adenine nucleotide release by such drugs as tenofovir in the treatment and prevention of fibrosis. Moreover, we discuss the clinical evidence for adenosine receptor antagonists like caffeine and tenofovir in the treatment and prevention of fibrosis in the liver and skin.
The inflammasome is a cytosolic complex of proteins, which upon assembly can activate caspase-1 and result in the activation by cleavage of IL-1β with its subsequent release from the cell. The majority of the data on inflammasome activation has been obtained from the monocyte macrophage cell line. This has revealed that a requirement for a signal for up-regulation of the inflammasome machinery, and a second signal for assembly and activation of the inflammasome. A diverse range of stimuli can provide the second signal, including ATP via the P2x7 receptor. Typical activation results in an acute burst of activity followed by reduction in inflammasome activity in 18-24hrs. This self-limited response can be altered to a more sustained response by adenosine which via up-regulation of HIF-1α can induce increased and sustained production of IL-1β. We hypothesize that the adenosine effect is a signal for tissue injury, resulting in a sustained inflammatory response. This adenosine/HIF-1α response can be reduced by the cardiac glycosidic digitoxin, which can protect from a number of models of tissue injury via down regulation of HIF-1α, and NOX4.

Recent data shows that Ipaf, in addition to its role in innate immunity, can mediate cellular answers to intracellular stress signals. Based on our results, we hypothesize that cN-II can interact with Ipaf in order to regulate its folding and conformation, thus acting as a sensor of global health state of the cell capable of regulating cell death. This clearly shows that cN-II has new roles in cell biology.

The 5′-nucleotidase is involved in intracellular nucleotide metabolism through its action on nucleotide monophosphates. Due to the very strong conservation of its primary sequence through the vertebrata taxon, we hypothesized that this enzyme could interact with other proteins and thus play additional roles in cell biology.

A two-hybrid system screening was set up, and interaction between cN-II and the leucine-rich region (LRR) of the inflammasome protein Ipaf was detected. This interaction was confirmed both in vitro using recombinant proteins and the implication of LRR was confirmed in cell extracts by immunoprecipitation after transfection with various plasmids. Finally, interaction was detected in human cancer cells using proximity ligation assay in cells expressing cN-II but not cells without cN-II expression.

Recent data shows that Ipaf, in addition to its role in innate immunity, can mediate cellular answers to intracellular stress signals. Based on our results, we hypothesize that cN-II can interact with Ipaf in order to regulate its folding and conformation, thus acting as a sensor of global health state of the cell capable of regulating cell death. This clearly shows that cN-II has new roles in human cells which might be independent of its enzymatic activity and nucleotide metabolism.
hypertrophy and inflammation such as MMP-13, collagen-X, fibronectin and osteopontin. Moreover A2AR stimulation regulated chondrocyte responses to IL-1 by inhibiting activation and translocation of NF-kB, and secretion of hypertrophic marker.

CONCLUSION: Mice lacking A2AR suffer from diminished mobility as a result of the spontaneous development of osteoarthritis. These findings are consistent with the hypothesis that local adenosine release maintains cartilage homeostasis by preventing endogenous inflammation and suggest that targeting A2AR may prevent progression of osteoarthritis.
Mitochondrial maintenance disorders are linked to deletions, and somatic point mutations of the mitochondrial genome. So far, mtDNA maintenance disorders have been linked to primary nuclear gene defects causing secondary mtDNA instability leading to depletion, multiple deletions, and site-specific point mutations. Deoxythymidine triphosphate (dTTP) pool imbalances, which in turn cause mtDNA instability. The key regulatory enzymes for the de novo synthesis are ribonucleotide reductase (RRN) and thymidylate synthase (TS), and the de novo synthesis is considered cytosolic. The salvage pathway operates both in the cytosol (key enzymes: TK1 and dCK) and the mitochondria (key enzymes: TK2 and dGK).

Mitochondrial dNTP pools are separated from the cytosolic ones due to the double membrane structure of mitochondria, and are originated in organello by the salvage enzymes e.g. TK2 and dGK together with NMPKs and NDPK in post mitotic tissues, while in proliferating cells the mitochondrial dNTPs are synthesized mainly via the cytosolic pathways and imported into the mitochondria. Recent findings regarding oxidative stress in the regulation of mitochondrial TK2 and dGK will be discussed. Furthermore, there is evidence of interchange of dNTPs between the cytosol and the mitochondria, and dTTP synthesized by the mitochondrial salvage pathway contributes to genome stability. Imbalanced mt dNTP pools lead to mtDNA depletion and/or multiple deletions resulting in mitochondrial diseases with serious clinical manifestations. mtDNA depletion syndrome (MDS) caused not only by deficiencies in enzymes in dNTP synthesis (TK2, dGK, p53R2, and TP) but also enzymes in mtDNA replication (mtDNA polymerase, twinkle helicase, MPV17) and enzymes in the TCA cycle (SUCLA2 and SUCLG1). Why are these enzymes affecting dNTP synthesis and how important is mitochondrial purine and pyrimidine metabolism in the whole cell/organism perspective? This talk will focus on recent advances in the knowledge of purine and pyrimidine metabolism, which have revealed several important links that connect mitochondrial purine and pyrimidine metabolism/mitochondrial function with cytosolic amino acids, glucose and fatty acid metabolism. For example, rho zero cells lacking mtDNA have reduced level of dTTP and nuclear genome instability; MDS causes substantial remodeling of multiple metabolic pathways including up-regulation of glycine and serine metabolism. TK2 deficiency causes abnormal adipose tissues development and defect fatty acid oxidation in the TK2 deficient mouse liver. However, defect fatty acid oxidation had no effect on cellular energy or ribonucleotide (NTPs) levels, but led to dNTP depletion in endothelial cells, which could be reversed by supplement with deoxynucleosides. Twinkle gene mutations cause either mtDNA depletion and/or multiple deletions, leading to altered one-carbon metabolism, i.e. in the folate cycle affecting both purine and pyrimidine nucleotide synthesis. As seen in twinkle gene mutations, serine and glycine metabolism as well as the mitochondrial MTHFD2 is up-regulated in nearly all cancer types, and the elevated serine and glycine levels and the increased mitochondrial folate synthesis support nucleotide synthesis.

Mitochondria are unique mammalian organelles by virtue of having their own DNA, mitochondrial DNA (mtDNA), a 16,569 base-pair circular molecule that encodes only 37 genes that are essential for mitochondrial respiratory chain and oxidative phosphorylation activities. Because maintenance of mtDNA is entirely dependent upon genes encoded by the nuclear DNA (nDNA), it is not surprising that primary nuclear gene defects can cause secondary mtDNA instability leading to depletion, multiple deletions, and somatic point mutations of the mitochondrial genome. To date, mtDNA maintenance disorders have been linked to mutations in 19 nDNA genes, of which, eight encode proteins required for purine and pyrimidine metabolism: TYMP, TK2, DGUOK, ANT1, RRM2B, SUCLA2, SUCLG1, and ABAT. Mutations in these genes are thought to cause mitochondrial deoxynucleotide triphosphate (dNTP) pool imbalances, which, in turn, cause mtDNA instability. The best characterized disorder of mitochondrial maintenance is Mitochondrial NeuroGastrointestinal Encephalomyopathy (MNGIE), an autosomal recessive disease due to TYMP mutations. In this disease, loss of thymidine phosphorylase (TP) activity causes marked increases in plasma and tissue levels of thymidine and deoxyuridine, which, in turn, cause dNTP pool imbalances. Studies of cellular and mouse models of MNGIE have revealed relative increases in deoxothymidine triphosphate (dTTP) and decreases in deoxyctydine triphosphate (dCTP) within mitochondria lead to the depletion, deletions, and site-specific point mutations of mtDNA. Remarkably, this is the first human disease in which dNTP pool imbalances have been associated with somatic DNA mutations. Hematopoietic stem cell transplantation to restore TP activity to MNGIE patients has shown promise, but has caused high morbidity and mortality.
09:30-9:45: O-19: Caterina Garone "Thymidine kinase 2 deficiency: from diagnosis to treatment

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OBJECTIVE: To describe the natural history of thymidine kinase 2 deficiency and analyse efficacy and safety of treatment approaches with nucleoside vs. nucleotides supplementation.

BACKGROUND: Autosomal recessive (AR) TK2 mutations cause severe mtDNA depletion and devastating neuromuscular diseases in infants and children, as well as mtDNA multiple deletions and progressive external ophthalmoplegia (PEO) in adults. Recently, we have demonstrated that molecular bypass therapy with deoxypyrimidine monophosphates is effective in ameliorating Tk2 deficiency in the H126N knockin mouse model while efficacy with nucleosides have been proven in other in vitro disease models.

DESIGN/METHODS: We reviewed clinical history, molecular genetics and biochemical defects of 57 patients that have been reported in literature from 2001-2014 as well as nineteen new patients with TK2 mutations.

RESULTS: Based on the clinical history of our new patients together with a systematic literature review, we identified four disease presentations: i) infantile encephalomyopathy with onset in the first year of life and rapidly progressive to early death; ii) childhood SMA-like myopathy progressing to loss of ambulation over several years; iii) adult myopathy with subclinical or mild myopathy at onset and gradual loss of ambulation, respiratory failure, or both in adulthood; and iv) adult AR-PEO. Molecular genetics defects in muscle range from i) severe mtDNA depletion in the infantile/childhood onset forms, ii) coexisting mtDNA multiple depletion and mild mtDNA depletion in adult-onset myopathy, and iii) isolated mtDNA multiple deletions in AR-PEO. Clinical and molecular data clearly demonstrate that thymidine kinase 2 deficiencies span a disease spectrum and the disease frequency has been underestimated. In addition, Kaplan-Meyer curve showed that 80% of patients died in the first 3 years of life, underlying the importance of early diagnosis for treatment purpose.

CONCLUSION: TK2 deficiency manifests a clinical and molecular genetic disease spectrum. Early and accurate diagnosis is important to initiate timely treatment with dCMP/dTMP, which may modify clinical history of this devastating disorder.

9:45-10:00: O-20: Chiara Rampazzo “Functional interaction of nuclear SAMHD1 and mitochondrial deoxyguanosine kinase in the regulation of mt dNTP pool balance”

Elisa Franzolin, Vera Bianchi and Chiara Rampazzo
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The regulated synthesis of the four dNTPs includes two pathways: (i) de novo synthesis, with reduction of ribonucleotides by ribonucleotide reductase (RNR) as the rate-limiting step and (ii) the salvage pathway, based on phosphorylation of deoxynucleosides by cytosolic and mitochondrial (mt) kinases. In each pathway allosteric regulation of individual enzymes finetunes dNTP synthesis. In addition, a combination of synthesis and degradation sets the intracellular concentrations of each of the four dNTPs. Disturbances in either process may lead to deficiency or surplus of one or more dNTPs and result in genetic diseases through destabilization of DNA synthesis. The deoxyribonucleotide triphosphohydrolase SAMHD1 is a newcomer in this regulatory network. SAMHD1 degrades dNTPs to deoxynucleosides and triphosphate and its impact on the sizes of dNTP pools is particularly strong in quiescent and differentiated cells. The main effect of SAMHD1 is on the cellular concentration of dGTP, as demonstrated by the large expansion of the dGTP pool in cells with SAMHD1 downregulation. The deoxyguanosine (GdR) produced by dGTP hydrolysis is either degraded further by purine nucleoside phosphorylase or recycled via salvage by deoxycytidine kinase (dCK) in the cytosol and deoxyguanosine kinase (dGK) in mitochondria. We have studied to which extent these kinases compensate the degradation of dGTP by SAMHD1 during quiescence. We have used dGK-mutated human fibroblasts, derived from 3 unrelated patients affected by hepatocerebral mtDNA depletion syndrome. When cycling, the mutant cells contained a normal complement of mtDNA. When maintained quiescent for more than a week they developed a progressive mtDNA depletion, whereas under the same conditions quiescent wt fibroblasts maintained a normal mtDNA copy number. We hypothesized that in the quiescent mutant fibroblasts the combination of active SAMHD1 and inactive dGK may disrupt the normal balance of the mitochondrial dNTP pools and inhibit mtDNA synthesis. To test this hypothesis we downregulated SAMHD1 by siRNA transfection during 10 days of quiescence in wt and dGK-deficient fibroblasts. The inactivation of SAMHD1 left the content of mtDNA unchanged in the wt fibroblasts, but prevented mtDNA depletion in the mutant cells. Analysis of the mitochondrial dNTP pools in quiescent mutant fibroblasts showed that dGK deficiency causes mt dNTP pool imbalance without necessarily decreasing mt purine dNTPs. SAMHD1 downregulation restored pool balance and boosted mtDNA copy number compensating the mtDNA depletion to various degrees in the individual mutant lines. Our results indicate that SAMHD1 in the nucleus and dGK in mitochondria functionally interact for the regulation of mitochondrial dNTP pool balance and that the effects of dGK dysfunction are worsened by the unrestrained SAMHD1 activity. (Supported by Grant GGP14005 from Italian Telethon).
Mitochondrial DNA (mtDNA) depletion and deletion syndrome (MDDS) has been associated with mutations affecting an increasing number of human genes. An important group of these genes encodes for proteins directly involved in keeping mitochondrial dNTP homeostasis. Deregelation of dNTP metabolism ultimately leads to limited availability of one or more of the four dNTPs required for normal DNA synthesis. Previous studies by our and other groups have proved that feeding the deoxyribonucleoside salvage pathway by administration of the deoxyadenosine degradation (EHNA), which is otherwise rapidly degraded in cell culture media. Deoxyribonucleoside supply rescues mtDNA depletion in dGK-deficient cells or thymidine-induced mtDNA depleted-fibroblasts (MNGIE, in vitro model) respectively.

%Polymerase gamma is a heterotrimer constituted by one catalytic subunit (encoded by POLG) and two accessory subunits that act as processivity factors and modulators of DNA binding (encoded by POLG2). Mutations affecting the catalytic subunit can interfere with the polymerase affinity for dNTPs and their normal incorporation rate into the nascent DNA-strand and thus theoretically benefit from a higher dNTP concentration at the replication fork. Here, we have studied mtDNA recovery rates after EtBr (ethidium bromide)-forced depletion in quiescent skin fibroblasts derived from three patients harbouring different mutations in POLG: patient 1 (homozygous for the p.R309C mutation); patient 2 (compound heterozygote for the p.W748S and p.G848S mutations); patient 3 (heterozygote for two dominant aminoacid changes in cis p.V1177L and p.E1143G). We treated quiescent fibroblasts obtained from all three patients with 5 ng/ml EtBr for a period of two weeks. All POLG-deficient cells experienced a higher degree of mtDNA depletion compared to that observed in control cells (averaged percent of mtDNA residual levels in patient-derived cells: 16.5 ± 2.3%; in control cells: 37.8 ± 9.1%). We monitored mtDNA recovery after EtBr withdrawal for 12 additional days either in the presence or absence of 200 µM combination of all four dNs plus a specific inhibitor of deoxyadenosine degradation (EHNA), which is otherwise rapidly degraded in cell culture media. MtDNA copy number from control cells reached values above 100% of initial levels 12 days after EtBr withdrawal independently of dNs treatment (139,7 ± 43,0% with treatment; 118,5 ± 33,4% without treatment). Conversely, POLG-deficient cells were unable to recover normal mtDNA levels (26,0 ± 7,0%) unless exposed to dNs. After treatment, residual mtDNA levels of patient cells where comparable to that achieved by control cells (140,7 ± 16,7%).

With this work, we provide evidence that not only dNTP metabolism-related mutations but also those affecting enzymes directly acting at the mitochondrial replication fork may benefit from enhanced nucleotide synthesis provided by dNs administration.

2Camara, Y. et al. Administration of deoxyribonucleosides or inhibition of their catabolism as a pharmacological approach for mitochondrial DNA depletion syndrome. Hum Mol Genet 23, 2459-67 (2014)

10:00-10:15: O-21: Yolanda Camara “Deoxyribonucleoside supply rescues mtDNA depletion in human POLG-deficient fibroblasts”

Yolanda Cámara1,2*, Cora Blázquez-Bermejo1,*, Javier Torres-Torrntereras1,2, Raquel Cabrera1,2, Anne Lombès1, Ramon Martí1,2 *Both authors contributed equally to this work
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Imbalance of the cytosolic deoxyribonucleotide triphosphate (dNTP) pool causes cellular stress, which has been associated with several human age-related diseases. Imbalance of the dNTP pool leads to higher mutation rates, genomic instability and development of cancer. Furthermore, in cohort studies of individuals suffering from declined cognitive function as well as individuals suffering from Alzheimer’s disease, we show that the dNTP levels in blood cells are significant different from matched controls.

Mitochondrial dysfunction has been linked to both Alzheimer’s disease as well as cancer. We have previously shown that depletion of mtDNA in human cell lines results in imbalance of the cytosolic dNTP pools. Therefore, we hypothesize that dysfunctional mitochondria affect the dNTP levels, and thereby causes cellular stress recognized in Alzheimer’s disease and cancer.

The mitochondrial genome primarily encodes peptides essential for the mitochondrial electron transport chain (ETC) and thereby ATP produced by oxidative phosphorylation. The ETC is linked to the de novo synthesis of pyrimidines through the enzyme dihydroorotate dehydrogenase (DHODHase) located in the inner membrane of the mitochondria. The enzyme couples

10:15-10:30: O-22: Claus Desler “Balanced levels of dNTPs links mitochondrial dysfunction to cellular stress”

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dihydroorotate oxidation to respiratory ubiquinone reduction and converts dihydroorotate to orotate. Orotate is further converted to uridine monophosphates, UTP and CTP, and ultimately, dTTP and dCTP.

In this project we show that both an inhibition of ATP synthase and of DHODHase results in a decrease of cytosolic levels of dNTP. We find that inhibition of either ATP synthase or DHODHase leads to increased sensitivity towards UV but not MMS induced DNA damage. Finally we demonstrate that inhibition of DHODHase, but not ATP synthase promotes anchorage independent growth and autophagy. In conclusion our results show that mitochondrial dysfunction can cause cellular phenotypes that are characteristic of common human age-related diseases.

11:00-11:30: I-18: Varsha Gandhi “Vulnerability of LKB1 deficient NSCLC to adenosine analogs”

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MD Anderson Cancer Center, Houston, TX, USA

Nucleoside analogues have played major role as cancer therapeutics and have become standard of care for several malignancies. They are mainly DNA-directed and impact DNA replication/repair. C8-modified ATP congeners are new analogues that inhibit RNA synthesis. Our extensive investigations with 8-chloro-adenosine (8-CI-Ado) demonstrated that this agent is phosphorylated to its mono-, di-, and tri-phosphate forms and incorporates into RNA. Hence, this analogue is distinct in its mechanism of action. In addition to this unique feature, 8-CI-Ado treatment resulted in dramatic decrease in cellular energy (ATP pool). Decline in ATP pool altered ATP/AMP ratio and activated AMP kinase. AMP kinase activation is through LKB1, a tumor suppressor gene. In parallel, high-throughput screening assays for non-small cell lung cancer (NSCLC) cell lines that are mutated for KRAS and LKB1, identified 8-CI-Ado as candidate molecule that was most effective in inducing cytotoxicity. Loss of the tumor suppressor gene LKB1 (STK11) in NSCLC is associated with increased invasiveness and metastatic potential. It commonly cooperates with the KRAS pathway to promote the malignant phenotype. Our (collaboration with Dr. Heymach) preclinical data indicated that LKB1/KRAS mutant NSCLC cells, which are highly resistant to standard chemotherapy as well as EGFR inhibitors, are highly sensitive to 8-CI-Ado. Mechanistic studies demonstrated that wild-type LKB1 protects cells from 8-CI-Ado -induced ATP depletion and cell death. Previously, using a screen of non-small cell lung cancer (NSCLC) cell lines for growth sensitivity against a panel of therapeutic agents, we established that LKB1 mutant NSCLC cells were highly resistant to standard chemotherapy agents but showed high sensitivity to 8-CI-Ado. This sensitivity was diminished in cells made LKB1-proficient [Cancer Res 74s19:5480, 2014]. LKB1 promotes the maintenance of the cellular energy balance by enhancing the activation of AMP activating protein kinase (AMPK) under conditions of reduced ATP levels. Consequently, LKB1 deficient tumors lack proper energy regulation, thus are vulnerable to ATP depletion. To examine the tumoricidal activity of 8-CI-Ado in NSCLC, we assessed isogenic A549 LKB1 proficient and deficient cell lines for 8-CI-Ado-induced death by flow cytometry. 8-CI-Ado was cytotoxic to these cells with greater sensitivity in the LKB1 deficient cells, (38 versus 23% annexin V/propidium iodide positivity with 20 µM 8-CI-Ado for 4d, p = 0.001). HPLC analysis indicated that the endogenous ATP levels are lower in LKB1 deficient cell lines than LKB1 proficient cells (2800 versus 3400 µM, p = 0.011, respectively in the H460 cells and 2556 versus 2812 µM, p = 0.38, respectively in the A549 cells). This was further perturbed by 8-CI-Ado treatment as the ATP levels were depleted to a greater extent in the deficient cells compared with the proficient cells (60 versus 40% reduction, p = 0.002, respectively in the H460 cells and 67 versus 52% reduction, p = 0.023, respectively in the A549 cells). In concert, 8-CI-Ado-induced AMPK phosphorylation was detected in LKB1 proficient cells. Due to AMPK’s ability to promote autophagy for energy restoration and the role of autophagy in chemoresistance, we examined the NSCLC cells for 8-CI-Ado-induced changes in autophagy and demonstrated significantly greater LC3B-lipidation and acridine orange staining (30% higher, p = 0.014) in the LKB1 proficient cells. Our results indicate that LKB1 mutant NSCLC cells are vulnerable to energy altering effects of C8-modified adenosine analogs and that the protective role LKB1 plays during treatment of these cells is due in part to greater induction of autophagy. Collectively, these data identified 8-CI-Ado as a promising molecule for RAS and LKB1 mutant NSCLC.


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Human deoxycytidine kinase (dCK) plays a key role for both the salvage of physiological nucleosides and for the phosphorylation of several clinically-used nucleoside analog prodrugs. This makes dCK a key enzyme for regulating the deoxyribonucleoside triphosphate (dNTP) levels and for providing therapeutic concentrations of activated nucleoside analogs. Previously, we elucidated dCK crystal structures in various complexation states, which together revealed the determinants of substrate specificity and guided the development of more active and promiscuous mutants. Recently we made the discovery that inhibition of dCK activity, in combination with additional agents that perturb the dNTP pools, could potentially play a role in inhibiting the proliferation of some malignancies. Therefore, we set out to develop tight-binding and selective inhibitors of human dCK. Using an iterative structure-guided approach, we improved an initial hit molecule with a micromolar IC50 to our current lead compound that has low nanomolar
Decitabine (5-aza-2’-deoxycytidine, aza-dCyd) is a demethylating agent used clinically in the treatment of myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). The incorporation of azacytosine in DNA promotes the covalent trapping of DNA methyltransferases (DNMTs) and the reactivation of epigenetically repressed genes but also genome-wide DNA damage resulting from inefficient repair of DNMT-DNA cross-links and azadCyd-induced base damage. Here, we report that exposure of cells to decitabine induces the overexpression of several enzymes that participate in pyrimidine metabolism including the nucleotidohydrolase dUTPase, which suggests a role for this enzyme in the metabolism of this cytidine analog. dUTPase converts dUTP into dUMP, thus preventing the incorporation of dUTP by DNA polymerase during replication and contributing this way to the detoxification of the dNTP pool in human cells.Transient depletion of dUTPase by siRNA rendered MRC-5 and HeLa cells hypersensitive to aza-dCyd while overexpression of the enzyme conferred protection against its antiproliferative effect. Moreover, dUTPase-deficient cells exposed to aza-dCyd accumulated a high concentration of dUTP in the nucleotide pool and exhibited increased levels of DNA double-strand breaks as monitored by \( \gamma \)H2AX foci formation. Taken together, these data support an alternative mode of action for decitabine in which this cytidine analog can be partially metabolized into aza-dUMP, which may then act as a thymidylate synthase inhibitor. Especially in the absence of dUTPase, azadCyd cell treatment would cause the accumulation of aza-dUTP/dUTP and subsequent uracil misincorporation with genotoxic consequences. We therefore propose that dUTPase is a key factor in the cellular response to decitabine-based chemotherapy.

Fluorocyclopentenylcytosine (RX-3117) is an orally available novel cytidine analog, which is currently in Phase I clinical trial. RX-3117 has shown promising antitumor activity in various human tumor xenografts including patient derived xenografts resistant to gemcitabine. Initial characterization of RX-3117 indicated that this compound is incorporated into both RNA and DNA, and can downregulate DNA methyltransferase I (DNMT1). RX-3117 is activated by uridine-cytidine kinase2 (UCK2). Because of these properties we investigated whether and how RX-3117 affected cell cycle regulation and cell death in the non-small cell lung cancer (NSCLC) lines SW1573 (alveolar carcinoma), A549 (adenocarcinoma) and H460 (large cell carcinoma), when treated for 24-48 hr at a low fixed (1 µM), concentration and at the higher IC50 and 10 fold the IC50. Cell cycle distribution and cell death were measured using flow cytometry with propidium iodide staining. The mechanism of cell cycle arrest was investigated by measuring cell cycle proteins expression using western blotting. Cell cycle arrest was time, concentration and cell line dependent. In A549, H460 and SW1573 cells 24 hr exposure to 1 µM RX-3117 increased the accumulation of cells in the G1 phase (about 20-40%) and in the S-phase (to a lesser extent), but the accumulation in G2M decreased. At 24 hr exposure no cell kill was observed, but was observed at 48 hr (15% in SW1573 and 8% in A549 cells) accompanied by \( \gamma \)H2AX induction. In A549 cells the effect of RX-3117 on the cell cycle distribution was most pronounced at 48 hr exposure with 45% accumulation in G2 phase. Treatment with RX3117 increased p53 levels, increased cell cycle checkpoint kinase 2 (Chk2) and 1 (Chk1) protein and CDK2, but decreased cdc25C and p-cdc25C. RX-3117 also increased the expression of wee1 mostly after 48 hr. In conclusion, DNA damage induced by RX-3117 triggers apoptosis on one hand and increases Chk1 and Chk2 levels on the other hand. The effect of RX-3117 on cell cycle proteins is time dependent. We suggested that the phosphorylated Chk1 and Chk2 would trigger phosphorylation of cdc-25C and provoke its degradation, which resulted in decreased CDK1 and, thus accumulated cells in S-phase.
Inhibitors of lactate dehydrogenase overcome the resistance towards gemcitabine in hypoxic mesothelioma cells, and modulate the expression of the human equilibrative transporter-1 (hENT1)

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2. University of Pisa, Pisa, Italy
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4. Humanitas Cancer Center, Milano, Italy

Background and aims. Malignant pleural mesothelioma (MPM) is a severely hypoxic malignancy, and hypoxia has been associated with resistance towards several anticancer drugs, including the nucleoside analogue gemcitabine. The muscle-isoform of lactate dehydrogenase (LDH-A) constitutes a major checkpoint for the switch to anaerobic glycolysis, ensuring supply of energy and anabolites in hypoxic environments. Therefore we investigated the molecular mechanisms underlying the pharmacological interaction of a new LDH-A inhibitor in combination with gemcitabine in MPM cells.

Methods. MPM cells (H2052 and H28) were treated for 72 hours with gemcitabine alone or in combination with the LDH-A inhibitor NHI-1, in normoxic and hypoxic (at O2 tension of 1%) conditions, and the inhibition of cell growth was investigated by sulforhodamine-B-assay. The pharmacologic interaction was evaluated using the combination index (CI) method, while cell cycle perturbations were investigated with flow cytometry. Moreover, quantitative PCR analysis was performed to assess whether hypoxia modulated the expression of the gemcitabine activating enzyme deoxycytidine kinase (dCK) and the human equilibrative transporter-1 (hENT1).

Results. Gemcitabine was cytotoxic against H2052 and H28 cells with IC50s of 55.1±6.3, and 103.5±9.2 nM, respectively, but cell growth inhibitory effects were reduced of 5- and 10-fold under hypoxia. The simultaneous addition of NHI-1 analysis showed synergism (CI<1) in both MPM cell lines in hypoxia. Flow cytometric studies demonstrated that hypoxia caused a G1 arrest, whereas cells exposed to the drug combination presented typical apoptotic morphology. Finally, the expression levels of dCK and hENT1 were significantly down-regulated under hypoxic conditions, but treatment with NHI-1 was associated with a rescue of hENT1 expression, which might explain the synergistic interaction with gemcitabine.

Conclusions. Hypoxia is a driving force in MPM chemoresistance. However, apoptosis induction and modulation of the key transporter in gemcitabine uptake may contribute to the synergistic interaction of gemcitabine with the LDH-A inhibitor NHI-1 and support further studies for the rational development of this combination.

Biodiversity of deoxyribonucleoside kinases and their practical use

Deoxyribonucleoside kinases (dNKs) phosphorylate deoxyribonucleosides to their corresponding monophosphate compounds (dNMPs). dNKs also phosphorylate deoxyribonucleoside analogues (NAS). They therefore play a key role in the treatment of cancer and viral diseases by NAS. Mammals have two cytosolic dNKs and two mitochondrial dNKs with distinct substrate specificities. However, the number of dNKs in each organism varies from none in fungi to many and while most eukaryotes can salvage all five natural occurring substrates, many prokaryotes and a few eukaryotes only have the capacity to phosphorylate thymidine.

By the screening of many different species over the last 15 years for potential candidate dNKs for cancer suicide gene therapy, the biodiversity of dNKs has also become a valuable tool to study enzyme evolution and trying to understand the driving biological forces behind enzyme evolution resulting in the four different mammal dNKs. We will present a new characterization of vertebrate dNKs and their evolutionary history.

Some of the newly discovered enzymes have also raised interest as catalysts in the production of dNMPs or analogs thereof.

The lecture will also illustrate the use of insect and plant dNKs for cancer suicide gene therapy and the structure function relationship of dNKs important for nucleoside analog activation. In particular, the tomato thymidine kinase 1 (ToTK1) is a dNK that has been selected for its in vitro kinetic properties and then successfully been tested in vivo in animal models for the treatment of malignant glioma [1, 2].

We have recently selected two improved variants of ToTK1 for suicide gene therapy in combination with AZT by directed evolution. Their selection, recombinant production and a subsequent kinetic and biochemical characterization will
be described. Their improved ability in killing the cells is accompanied by an increase in specificity for AZT over the natural substrate thymidine. Moreover, inhibition by dTTP, the end product of the nucleoside salvage pathway for thymidine, is decreased for these mutations.


15:15-15:35: O-26: María Valente “Balance of dUTP/dTTP ratio in bloodstream forms of Trypanosoma brucei”

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A balanced pool of deoxyribonucleotides is essential for DNA replication and repair and disturbances in the supply of dNTPs may lead to genetic mutations and cell death. Indeed, particularly crucial for cell survival is the maintenance of a low dUTP/dTTP ratio. In most living organisms dUTP may arise as intermediate of both de novo and the salvage pyrimidine pathway and its incorporation into nascent DNA, enhanced under condition of reduced dTTP level, is deleterious to prokaryotic and eukaryotic cells including Tryptosanoma brucei. The removal of dUTP from the nucleotide pool is carried out by a deoxyuridin triphosphatase nucleotidohydrolase (dUTPase) that in trypanosomes converts dUTP and dUDP into dUMP, the unique precursor for de novo synthesis of dTMP, and maintains at the same time a low dUTP/dTTP ratio, thus preventing the incorporation of dUTP during replication. Previous studies demonstrated that dUTPase depletion in T. brucei generates an imbalance in dUTP/dTTP ratio and therefore chromosomal fragmentation and defective parasite growth, which can be partially restored by supplementation of non-physiological concentration of thymidine. Here, we have analyzed the role of salvage in the pyrimidine requirements of dUTPase null mutants in vitro. In particular deoxyuridine, that is substrate of thymidine kinase, and deoxycytidine supplementation were investigated. Surprisingly none of the several nucleosides and nucleobases tested, except for 5-methyl deoxycytidine, can reverse the lethal phenotype and are capable of supporting parasite growth in vitro; not even deoxyuridine for which direct phosphorylation to DUMP was expected. In addition to these observations we present evidence for a central role of thymidine salvage in cell viability, cell cycle progression and the maintenance of the dTTP pool. We propose that: (i) dUTPase plays an essential role in “house-cleaning” more than in thymidilate synthesis; (ii) thymidine salvage is critical for cell survival and modulation of dUTP/dTTP ratio; (iii) an active cytidine deaminase is involved in pyrimidine nucleoside interconversion.


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Rice is a primary food source for the world’s population, and is an important crop in Colombia. Its production is affected by abiotic stress, caused by climate change and other factors. The identification of genes implicated in abiotic stress is key for the development of resistant varieties. Recently, genes of the pyrimidine catabolic pathway have emerged as potential participants in the abiotic stress response of plants.

The degradation of uracil or thymine, to 5,6-dihydrouracil or 5,6-dihydrothymine is initiated by dihydropyrimidine dehydrogenase (DHPD), the product is hydrolytically degraded by dihydropyrimidinase (DHP) to N carbamyl-β-alanine or N carbamyl-β-aminoisobutyrate, which is further metabolized to β-alanine or β-aminoisobutyrate, CO2 and NH3 by β-ureidopropionase (β-UP). Liu and coworkers (2009) found that overexpression of DHPD in rice plants results in tolerance to salt and drought stress, however in that study OsDHPD was misidentified as a type I dihydroorotate dehydrogenase (OsDHODI), the enzyme that catalyzes the fourth step of de novo pyrimidine biosynthesis. DHP appears to be involved in cold stress-response in grass according to proteomic analysis. In addition, the overexpression of β-UP in transgenic tomatoes plants made them resistant to heat during anthesis. The role of pyrimidine catabolism in stress tolerance could be related to this pathway’s contribution to the production of precursors of proline biosynthesis.

Our initial approach to explore the role of this pathway has been to characterize the three enzymes mentioned above. We cloned the first and third enzymes, OsDHPD and Osβ-UP, and expressed them as recombinant proteins in Escherichia coli. The second enzyme of the pathway, DHP, has been amplifie and its cloning process is in progress. Recombinant Osβ-UP of 52.1 kDa was purified with a yield of 7.1 mg per liter of cell culture. Preliminary kinetics of Osβ-UP revealed a Km 0.85 ± 0.11 mM for 3-ureidopropionic acid, and a specific activity apparently 10-fold faster (Vmax 384.3 ± 12.0 nmol/min/mg) than the Arabidopsis thaliana recombinant Osβ-UP (30 nmol/min/mg) (Walsh et al., 2001). Recombinant OsDHPD, although soluble, did not display measurable DHODI or DHPD activity. Comparison
of plant and mammalian DHPD sequences showed that the plant protein lacks the N-terminal domain needed for binding NADH(P), FAD, and Fe/S clusters, suggesting that another, unidentified protein carrying the missing domain could be interacting with OsDHPD in the plastid. Support for this comes from Cornelius and coworkers (2011) who showed that Arabidopsis expressing DHPD in the cytosol, instead of in its normal plastidic location, does not exhibit DHPD activity, and they conclude that this lack of activity is due to the lack of interaction with a plastidic cofactor. In organisms such as Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Shigella sonnei, DHPDs are composed of 2 polypeptides that form the active complex. To identify proteins interacting with OsDHPD, we produced polyclonal antibodies for pull-down assays.

Exploring the dual role of CTP synthase in Toxoplasma gondii.
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Cytidine triphosphate synthase (CTPase, EC 6.3.4.2) catalyzes the production of CTP from UTP, the final step in the production of cytidine nucleotides, and the first step in the formation of phospholipid intermediates. We have identified the CTPase from T. gondii (TgCTP). To achieve its functional characterization, we have expressed and purified his-tagged TgCTP protein from Escherichia coli. We were able to recover a small amount of protein under native conditions which was active as measured spectrophotometrically. In order to maximize the yield of protein, we are working on three different strategies: recovery of protein from inclusion bodies, bacterial expression of an N-terminally truncated TgCTP protein and expression of recombinant TgCTP protein in Saccharomyces cerevisiae. Kinetic characterization is in progress.

CTPases also form large filaments whose functions seem to be species-specific. However the role of those filaments is unknown in most organisms. The TgCTP’s capacity to form filaments in vivo was evaluated using parasites transfected with two different constructs. In the first one, parasites expressing an ectopic copy of TgCTP-c-myc, showed a uniformly cytosolic pattern that co-localized with the known marker TgHsp90 by immunofluorescence assays. In the second construct, parasites expressing TgCTP-c-myc under the control of its native promoter showed a punctuated distribution in the parasite cytosol. The known CTPase inhibitor, 6-diazo-5-oxo-L-norleucine (DON) inhibited parasite growth and caused the formation of TgCTP structures in a concentration-dependent manner. Experiments to explore the dynamics of TgCTP filament formation are in progress.
Demonstration of the importance of sample preparation for the study of endogenous nucleotides in cell cultures

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Quantification of the intracellular pools of endogenous deoxyribonucleotides and ribonucleotides is of major interest for researchers in several fields. We have recently published a LC-MS/MS method coupled with an online extraction that allowed the quantification of nucleoside mono- and triphosphates in cell extracts (Machon C Anal Bioanal Chem 2014, doi 10.1007/s00216-014-7711-1). This method was thoroughly validated from an analytical point of view. Analysis of published papers on quantification of intracellular endogenous nucleotides revealed a high heterogeneity in the preparation of cellular samples. Only few studies have compared different pre-analytical strategies for the quantification of intracellular nucleotides, reaching different conclusions.

Here, we present a recent work concerning the improvement of pre-analytical steps for the quantification of nucleotides in NCI-H292 cells. These steps include cell recovery, the volume and nature of organic solvent used for nucleotide extraction, the use of a chloroform-based clean-up step and the stirring time before freezing in liquid nitrogen. Design of experiments was implemented to substantially decrease the experimental conditions needed to answer all questions. A panel of nucleoside mono-, di and triphosphates derived from four nucleobases (A, U, C, and G) were used to compare different protocols.

Results showed that scraping cells was deleterious probably because of the alteration of cytoplasmic membrane during scraping. Trypsinization followed by nucleotide extraction or nucleotide extraction directly in the flask gave similar results, and thus should be favored for the initial step. Methanol appeared as a better solvent than acetonitrile; however, pure methanol should be avoided due to a decrease of at least 50% of nucleotides extracted, except for UMP which was not affected. Methanol/water (70/30, v/v) seemed preferable to methanol/water (80/20, v/v) for extraction of nucleoside triphosphates, whereas nucleoside mono- and diphosphates were equally recovered. The volume of methanol used did not affect the extraction yield. The clean-up with chloroform either had no effect or was deleterious depending on the other conditions associated. Finally, the stirring time did not influence the levels of nucleotides extracted.

Overall, our study clearly highlights the importance of pre-analytical conditions for the quantification of intracellular nucleotides to be as representative as possible of the real levels in cells. Before initiating any study of deoxyribonucleotides and ribonucleotides pools in cultured cells, sample preparation strategy has to be defined. We believe that the implementation of this step in relevant studies will clearly increase the quality of the results obtained.

Metabolic pathway and effects of 4-pyridone-3-carboxamide-1β-D-ribonucleoside in human endothelial cells.

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We identified that endogenously produced nucleoside 4-pyridone-3-carboxamide-1β-D-ribonucleoside (4PYR), a compound related to nicotinamide give raise to nucleosides phosphate ~ 4PYMP and NAD related derivative 4PYRAD in endothelial human cells. Pathway of 4PYMP and 4PYRAD formation has not yet been identified. Previous studies demonstrated that formation of 4PYMP was mediated by adenosine kinase and there is evidence that 4PYMP is involved in formation of 4PYRAD. Enzymes such as adenosine monophosphate deaminase (AMPD), purine nucleoside phosphorylase (PNP), adenosine deminase (ADA), ecto-nucleoside triphosphate diphosphohydrolase- eNTPD; ecto-5'-nucleotidase- e5NT and ecto-adenosine deaminase- eADA are involved in myopathy, immunodeficiency, controlling of inflammation and platelet aggregation. The aim of this study was to analyze involvement of adenosine kinase (AK), cytosolic 5’nucleotidase type II (NT5C2) and nicotinamide nucleotide adenyltransferase type III (NMNAT3) enzymes in metabolic pathway of 4PYR and influence of 4PYR on activates of extracellular and intracellular enzymes in human endothelial cells (HMEC-1).

Methods:
siRNA for AK, NT5C2, NMNAT3 genes and non-targeting negative control siRNA were purchased (Qiagen). For the experiments HEK 293T cells, were plated in 24-well plates. Cells were transfected according to the manufacturer’s instruction using Lipofectamine RNAiMAX reagent (Invitrogen). Cells were transfected with a final concentration of 6 pmol siRNA for each well. After 24 hours medium was removed and fresh full supplemented DMEM medium with addition of 100µM 4PYR was added. Cells
transfected with AK siRNA were supplemented with 100µM adenine and 2.5mM ribose to provide alternative substrates to maintain adenine nucleotide pool. After 48 hour of incubation, cells and incubation medium were separated and cellular 4PYMP and 4PYRAD were measured. To analyse eNTPD, eSNT, eADA AMPD, PNP and ADA activity, intact HMEC-1 cells and HMEC-1 cell lysates were incubated with appropriate substrates.

Results:
Transient silencing of AK resulted in reduction of 4PYMP and 4PYRAD concentration to less than 25% of control value with negative control siRNA. Silencing of NT5C2 resulted in four fold increases of PYMP compared to control while 4PYRAD level was not different. After transient silencing of NMNAT3 4PYRAD and 4PYMP concentration decreased to half of control level. AMPD and eADA activity decrease after 24 hour of cells treatment with 4PYR as compared to control (22.06±1.52 vs. 37.06±2.28nmol/min/mg protein; 1.55±0.06 vs. 1.92±0.13nmol/min/mg protein, respectively). eNTPD and eSNT activity increase after 72 hour of cell treatment with 4PYR as compared to control (0.40±0.02 vs. 0.29±0.02nmol/min/mg protein; 13.30±0.59 vs. 8.30±0.34 nmol/min/mg protein, respectively).

Conclusion:
Our results suggest that adenosine kinase, cytosolic 5’nucleotidase II and nicotinamide nucleotide adenylyltransferase III are involved in 4PYR metabolism. However other forms of cytosolic 5’nucleotidase and nicotinamide nucleotide adenylyltransferase should be considered as a potential enzymes involved in 4PYR metabolism. Exposure of endothelial cells to 4PYR resulted with decreased AMPD and eADA activities and increased eNTPD and eSNT activities.

**POSTER # 3**

Endogenous and pharmacological factors affecting CD39 and CD73 in human endothelial cells

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Release of adenosine triphosphate (ATP) or adenosine by erythrocytes, endothelium and other cells into extracellular space is involved in regulation of thrombosis, inflammation and immune system. However, its extracellular conversion by ectonucleoside triphosphate diphosphohydrolase 1 (CD39) and ecto-5'-nucleotidase (CD73) plays an equally important role. Endothelial cells (HMEC-1, hAVEC) were exposed to ox-LDL, eplerenone, clopidogrel, laminar and oscillatory shear stress for 24-48 h. Then metabolism of extracellular nucleotides was measured by incubation with ATP or AMP followed by HPLC analysis of media. Results are presented as mean ± SEM.

CD39 activity under oscillatory and laminar shear stress, decreased to 1.02±0.54 nmol/mg prot./ml, 0.93±0.31 nmol/mg prot./min respectively from 1.36±0.26 nmol/mg prot./min in static control. Under oscillatory shear stress activity of CD73 decreased to 0.57±0.12 nmol/mg prot./min, while under laminar shear stress the activity increased to 5.76±0.67 nmol/mg prot./min as compared to static control (3.36±0.51 nmol/mg prot./min). CD73 activity in human valve endothelial cells (hAVEC) (4.04±0.32 nmol/mg prot./min) increased in presence of ox-LDL as compared to control (2.75±0.21 nmol/mg prot./min). There was no effect of ox-LDL on CD39 activity. Incubation with clopidogrel and eplerenone caused increase in CD39 and CD73 activities.

Several external and endogenous factors profoundly affected CD39 and CD73 activities. Ox-LDL, cardiac drugs and laminar shear stress increase activities of CD39 and CD73 which may be considered protective, while, oscillatory shear stress decreased activities, which may be considered deleterious. These changes may contribute to relevant pathologies and treatment.

**POSTER # 4**

Polymorphisms of NT5E gene that alter enzymatic activity and may predispose to calcification of the aortic valve

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Ecto-5'-nucleotidase (eSNT, CD73) a key enzyme of extracellular nucleotide degradation is encoded by the NT5E gene. Particular function of this enzyme protein involves formation of adenosine that via receptor mediated mechanisms and metabolic effects induces wide spectrum of protective effects including inhibition of ectopic calcifications. The aim of this study was evaluation of the effect of polymorphisms of this gene on enzymatic activity of eSNT and analysis of associations of these polymorphisms with calcific aortic valve disease.

Genomic DNA was extracted from blood samples of 95 patients with aortic valve calcification (AVC) and 24 controls using a commercial kit (Blood Mini, A&A Biotechnology). The NT5E gene exon 6 (including the intron-exon boundaries and the proximal flanking regions) was amplified from genomic DNA and the PCR products were analysed by direct sequencing. Activity of eSNT was analysed in valve leaflets collected during valve replacement surgery with HPLC based procedure. Two nonsynonymous, missense single nucleotide polymorphisms (SNPs) were identified in exon 6 of the NT5E gene: c.1126A>G responsible for a threonine to alanine substitution at position 376 (p.T376A) and c.1136T>C responsible for a methionine to threonine substitution at position 379 (p.M379T). Both of them may affect eSNT activity. Genotype frequencies in SNP c.1126 were 0.15 for AA, 0.39 for AG, 0.46 for GG in patients with aortic valve calcification and 0.21 for AA, 0.46 for AG, 0.33 for GG in control patients. As for genotype frequencies in SNP c.1136, they were as follows: 0.80 for TT, 0.18 for TC, 0.02 for CC.
for CC in patients with aortic valve calcification and 0.88 for TT, 0.12 for TC, 0.00 for CC in control patients. In aortic valves from AVC patients, eSNT activity showed a trend toward lower levels in patients with TC and CC genotypes (1.01 ± 0.121 nmol/min/cm², n=15) than in TT genotype (1.23 ± 0.069 nmol/min/cm², n=60), P>0.05. We identified association between polymorphisms of NTSE gene and activity of the enzyme with distribution that was different in patients with valve disease as compared to control population. Polymorphisms of NTSE gene could therefore contribute to pathology of this disease.

**POSTER # 5**

Simultaneous determination of extra- and intra-cellular purines in Caco-2 and HepG2 cells by high-performance liquid chromatography

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OBJECTIVES: To evaluate cellular uptake and transport of purines, we applied our developed HPLC method for quantification of extra- and intra-cellular purines. In particular, we demonstrate the validity of our method by quantifying the substrate and its metabolites simultaneously in these samples.

METHODS: Two cell lines was used for purine uptake and transport assay. Caco-2 cell monolayers mimic the intestinal absorptive epithelium and are a useful tool for studying transepithelial transport. HepG2 cells are used as an in vitro model system for human hepatic cells. A LC-20AD HPLC system (Shimadzu) was used. Separation of analytes was performed on a YMC-TriartC18 column (250 x 4.6 mm, 3 μm; YMC). The mobile phase consisted of buffer A (80 mM ammonium phosphate buffer, pH 4.1) and buffer B (70% buffer A/30% methanol). The following gradient elution program was used: 99% buffer A for 15 min; 1% to 50% linear gradient of buffer B for 20 min. The flow rate was maintained at 0.6 mL/min.

RESULTS AND DISCUSSIONS: To test the uptake of purines, each purine (0, 25, 50, 100, 200 μM) was supplied to Caco-2 and HepG2 cells and the cells were harvested for 2 h. A small percentage of the supplied amount was incorporated into the cells, and many purines were found to be present as a metabolite rather than the original form. Supplying guanosine and guanine to the Caco-2 cells increased xanthine levels in a dose-dependent manner. In the HepG2 cell extract, GTP and uric acid levels were increased. These results suggest that metabolism by guanase enzymes proceeds early in both cells and that hepatocytes have strong xanthine oxidase activity. Supplying adenosine increased hypoxanthine and inosine levels in the Caco-2 cells and increased ATP and IMP levels in the HepG2 cells. Increased intracellular ATP and IMP levels in HepG2 cells were also observed after supplying adenine and inosine. We consider that the salvage pathway involving APRT and HPRT was activated by exposure to adenine, adenosine, and inosine.

To test the transport of purines, Caco-2 cells were cultured on Transwell filter membrane inserts for 21 days before use in each experiment. Purines were added at a concentration of 200 μM, and the apical and basal media were collected after 2 h. In a control with no purine addition, uric acid was present in the largest concentration, 0.3 μM, in the apical and basal media. When various purines were supplied, 0.1% to 5 percent of the supplied amount was detected in the permeate. In this transport study, the transport of IMP and inosine was highest and some uric acid was detected in the permeate.

CONCLUSIONS: Our method had high sensitivity and excellent resolving when applied to complex mixtures, demonstrating that it is useful for studying the overall pattern of purine metabolism.

**NON-MAMMALIAN PURINE AND PYRIMIDINE METABOLISM**

**POSTER # 6**

Balance of dUTP/dTTP ratio in bloodstream forms of Trypanosoma brucei


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A balanced pool of deoxyribonucleotides is essential for DNA replication and repair and disturbances in the supply of dNTPs may lead to genetic mutations and cell death. Indeed, particularly crucial for cell survivor is the maintenance of a low dUTP/dTTP ratio. In most living organisms dUTP may arise as intermediate of both de novo and the salvage pyrimidine pathway and its incorporation into nascent DNA, enhanced under condition of reduced dTTP level, is deleterious to prokaryotic and eukaryotic cells including Trypanosoma brucei. The removal of dUTP from the nucleotide pool is carried out by a deoxyuridine triphosphate nucleotidohydrolase (dUTPase) that in trypanosomes converts dUTP and dUDP into dUMP, the unique precursor for de novo synthesis of dTMP, and maintains at the same time a low dUTP/dTTP ratio, thus preventing the incorporation of dUTP during replication. Previous studies demonstrated that dUTPase depletion in T. brucei generates an imbalance in dUTP/dTTP ratio and therefore chromosomal fragmentation and defective parasite growth, which can be partially restored by supplementation of non-physiological concentration of thymidine. Here, we have analyzed the role of salvage in the pyrimidine requirements of dUTPase null mutants in vitro. In particular deoxyuridine, that is substrate of thymidine kinase, and deoxycytidine supplementation were investigated. Surprisingly none of the several nucleosides and nucleobases tested, except for 5-methyl deoxycytidine, can reverse the lethal phenotype and are capable of supporting parasite growth in vitro;
not even deoxyuridine for which direct phosphorylation to dUMP was expected. In addition to these observations we present evidence for a central role of thymidine salvage in cell viability, cell cycle progression and the maintenance of the dTTP pool. We propose that: (i) dUTPase plays an essential role in “house-cleaning” more than in thymidilate synthesis; (ii) thymidine salvage is critical for cell survival and modulation of dUTP/dTTP ratio; (iii) an active cytidine deaminase is involved in pyrimidine nucleoside interconversion.

**POSTER # 7**

**Pyrimidine catabolism in abiotic stress tolerance in Oryza sativa L.: preliminary characterization of pathway enzymes.**

Andrea J. López Moreno, Heidy Y. Narvaez, and Barbara H. Zimmermann.

Rice is a primary food source for the world’s population, and is an important crop in Colombia. Its production is affected by abiotic stress, caused by climate change and other factors. The identification of genes implicated in abiotic stress is key for the development of resistant varieties. Recently, genes of the pyrimidine catabolic pathway have emerged as potential participants in the abiotic stress response of plants.

The degradation of uracil or thymine, to 5,6-dihydrooracil or 5,6-dihydrothymine is initiated by dihydropyrimidine dehydrogenase (DHPD), the product is hydrolytically degraded by dihydropyrimidinase (DHP) to N carbamyl-β-alanine or N carbamyl-β-aminoisobutyrate, which is further metabolized to β-alanine or β-aminoisobutyrate, CoQ and NH3 by β-ureidopropionase (β-UP). Liu and coworkers (2009) found that overexpression of DHPD in rice plants results in tolerance to salt and drought stress, however, in that study OsDHPD was misidentified as a type I dihydroorotate dehydrogenase (OsDHODI), the enzyme that catalyzes the fourth step of de novo pyrimidine biosynthesis. DHP appears to be involved in cold stress-response in grass according to proteomic analysis. In addition, the overexpression of β-UP in transgenic tomatoes plants made them resistant to heat during anthesis. The role of pyrimidine catabolism in stress tolerance could be related to this pathway’s contribution to the production of precursors of proline biosynthesis.

Our initial approach to explore the role of this pathway has been to characterize the three enzymes mentioned above. We cloned the first and third enzymes, OsDHPD and Osβ-UP, and expressed them as recombinant proteins in Escherichia coli. The second enzyme of the pathway, DHP, has been amplified and its cloning process is in progress. Recombinant Osβ-UP of 52.1 kDa was purified with a yield of 7.1 mg per liter of cell culture. Preliminary kinetics of Osβ-UP revealed a Km 0.85 ± 0.11 mM for 3-ureidopropionic acid, and a specific activity apparently 10-fold faster (Vmax 384.3 ± 12.0 nmol/min/mg) than the Arabidopsis thaliana recombinant Osβ-UP (30 nmol/min/mg) (Walsh et al., 2001). Recombinant OsDHPD, although soluble, did not display measurable DHODI or DHPD activity. Comparison of plant and mammalian DHPD sequences showed that the plant protein lacks the N-terminal domain needed for binding NADH(P), FAD, and Fe/S clusters, suggesting that another, unidentified protein carrying the missing domain could be interacting with OsDHPD in the plastid. Support for this comes from Cornelius and coworkers (2011) who showed that Arabidopsis expressing DHPD in the cytosol, instead of in its normal plastidic location, does not exhibit DHPD activity, and they conclude that this lack of activity is due to the lack of interaction with a plastidic cofactor. In organisms such as Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Shigella sonnei, DHPDs are composed of 2 polypeptides that form the active complex. To identify proteins interacting with OsDHPD, we produced polyclonal antibodies for pull-down assays.

**POSTER # 8**

**Exploring the dual role of CTP synthase in Toxoplasma gondii.**

Heidy Y. Narvaez, Andrea J. López Moreno, Nishith Gupta, and Barbara H. Zimmermann.

Cytidine triphosphate synthase (CTPase, EC 6.3.4.2) catalyzes the production of CTP from UTP, the final step in the production of cytidine nucleotides, and the first step in the formation of phospholipid intermediates. We have identified the CTPase from T. gondii (TgCTP). To achieve its functional characterization, we have expressed and purified his-tagged TgCTP protein from Escherichia coli. We were able to recover a small amount of protein under native conditions which was active as measured spectrophotometrically. In order to maximize the yield of protein, we are working on three different strategies: recovery of protein from inclusion bodies, bacterial expression of an N-terminally truncated TgCTP protein and expression of recombinant TgCTP protein in Saccharomyces cerevisiae. Kinetic characterization is in progress.
CTPases also form large filaments whose functions seem to be species-specific. However the role of those filaments is unknown in most organisms. The TgCTP’s capacity to form filaments in vivo was evaluated using parasites transfected with two different constructs. In the first one, parasites expressing an ectopic copy of TgCTP-c-myc, showed a uniformly cytosolic pattern that co-localized with the known marker TgHsp90 by immunofluorescence assays. In the second construct, parasites expressing TgCTP-c-myc under the control of its native promoter showed a punctuated distribution in the parasite cytosol. The known CTPase inhibitor, 6-diazo-5-oxo-L-norleucine (DON) inhibited parasite growth and caused the formation of TgCTP structures in a concentration-dependent manner. Experiments to explore the dynamics of TgCTP filament formation are in progress.

**PURINE AND PYRIMIDINE ANALOGS**

**POSTER # 9**

Sensitive liquid chromatography mass spectrometry (LC-MS) assay reveals novel insight of antimetabolite incorporation into DNA

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Antimetabolites are used extensively as chemotherapeutic treatments. Due to their chemical similarity they are incorporated into DNA and RNA, causing either inhibition or chain end termination of DNA formation.

Liquid chromatography Mass spectrometry (LC-MS) permits the selective analysis of very low concentrations of metabolites, and can be utilised to determine the % global methyl deoxycytidine within DNA strands, as well as exactly quantify nucleoside analogue incorporation. Established DNA degradation methodology was used in combination with LC-tandem MS (LC-MS-MS) to quantify DNA methylation, mis-incorporation of the endogenous compound deoxyuridine and of nucleoside analogues such as gemcitabine (difluoro-deoxycytidine, dFdC), cladribine (2-chlorodeoxyadenosine) and the novel compound fluorocyclopentenylcytosine (RX-3117). Using Qiagen DNA/RNA mini kits both DNA and RNA were extracted separately in high purity. Subsequent sequential treatment with nuclease S1 (Fermentis, Germany), phosphodiesterase IV and alkaline phosphatase (Sigma/Aldrich, USA) with 2 aqueous 0.1% formic acid and 0.1% formic acid 80% methanol. Mass spectroscopic detection was performed using a Sciex API3000 system with optimised turbo spray ionisation for each component expected within the DNA or RNA matrix.

Quantification was made by regression analysis using nucleoside standard solutions in the same matrix as the prepared samples.

Exposure of CCRF- CEM leukemic cells for 4 hr to either 1 µM dFdC or to 100 µM of its metabolite difluorodeoxyuridine (dFdU) resulted in 3.6 ± 2.8 and 20.5 ± 1.1 pmol dFdC incorporation per µg DNA, respectively. However, in the gemcitabine-resistant CEM-DCK- gemcitabine was not incorporated into DNA (0 pmol / µg DNA) but unaffected when using dFdU (19.6 pmol / µg DNA). At an exposure time of 24 hours the degree of incorporation of dFdC in CEM cells decreased significantly to 0.18 and 0.051 pmol / µg DNA following treatment with 1 µM dFdC and 100 µM dFdU, respectively. Also RX3117 demonstrated detectable evidence of incorporation of RX3117 metabolites into both DNA and RNA (0.0037 and 0.00515 pmol / µg DNA respectively).

All DNA subcomponents could be measured as deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine in all samples. A 9-fold difference in mis-incorporation of deoxyuridine into DNA was observed between 27 cell lines (leukemia, colon, ovarian, lung cancer) being highest in ovarian cancer cells. In paired cell lines the % deoxyuridine misincorporation was increased 2.5-fold under conditions of low folate culture, but much more when thymidylate synthase was inhibited. The rate of global methylation by measuring 5-methyl deoxycytidine showed a modest variation (4.6 to 6.5% global methylation in 27 different cell lines).

Conclusion
We established a sensitive method to exactly quantify the incorporation of gemcitabine, cladribine and deoxyuridine into DNA, as well as the novel nucleoside analogue RX-3117. Interestingly the gemcitabine metabolite dFdU was also incorporated into DNA. Also the level of global methylation could be established.

**POSTER # 10**

Concentration and cell line dependent effects of fluorocyclopentenylcytosine (RX-3117) in non-small cell lung cancer cells.

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Fluorocyclopentenylcytosine (RX-3117) is an orally available novel cytidine analog, which is currently in Phase I clinical trial. RX-3117 has shown promising antitumor activity in various human tumor xenografts including patient derived xenografts resistant to standard chemotherapy. RX-3117 showed a predilection for incorporation into DNA in non-small cell lung cancer cell lines, which correlated with cell line dependent effects, as defined by the percent incorporation of mis-incorporated deoxyuridine into DNA, as well as cell cycle dependent cell growth inhibition of RX-3117 in order to better understand its potential as an antitumor agent.
to gemcitabine. Initial characterization of RX-3117 indicated that this compound is incorporated into both RNA and DNA, and can downregulate DNA methyltransferase I (DNMT1). RX-3117 is activated by uridine-cytidine kinase 2 (UCK). Because of these properties we investigated whether and how RX-3117 affected cell cycle regulation and cell death in the non-small cell lung cancer (NSCLC) lines SW1573 (alveolar carcinoma), A549 (adenocarcinoma) and H460 (large cell carcinoma), when treated for 24-48 hr at a low fixed (1 µM), concentration and at the higher IC50 and 10 fold the IC50. Cell cycle distribution and cell death were measured using flow cytometry with propidium iodide staining. The mechanism of cell cycle arrest was investigated by measuring cell cycle proteins expression using western blotting. Cell cycle arrest was time, concentration and cell line dependent. In A549, H460 and SW1573 cells 24 hr exposure to 1 µM RX-3117 increased the accumulation of cells in the G1 phase (about 20-40%) and in the S-phase (to a lesser extent), but the accumulation in G2M decreased. At 24 hr exposure no cell kill was observed, but was observed at 48 hr (15% in SW1573 and 8% in A549 cells) accompanied by γH2AX induction. In A549 cells the effect of RX-3117 on the cell cycle distribution was most pronounced at 48 hr exposure with 45% accumulation in S phase. Treatment with RX3117 increased pS3 levels, increased cell cycle checkpoint kinase 2 (Chk2) and 1 (Chk1) protein and CDK2, but decreased cdc25C and p-cdc25C. RX-3117 also increased the expression of wee1 mostly after 48 hr. In conclusion, DNA damage induced by RX-3117 triggers apoptosis on one hand and increases Chk1 and Chk2 levels on the other hand. The effect of RX-3117 on cell cycle proteins is time dependent. We suggested that the phosphorylated Chk1 and Chk2 would trigger phosphorylation of cdc-25C and provoke its degradation, which resulted in decreased CDK1 and, thus accumulated cells in S-phase.

**POSTER # 11**

**dUTP nucleotidohydrolase is a major factor in the cellular response to decitabine**

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Decitabine (5-aza-2’-deoxycytidine, aza-dCyd) is a demethylating agent used clinically in the treatment of myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). The incorporation of azacytosine in DNA promotes the covalent trapping of DNA methyltransferases (DNMTs) and the reactivation of epigenetically repressed genes but also genome-wide DNA damage resulting from inefficient repair of DNMT-DNA cross-links and azadCyd-induced base damage. Here, we report that exposure of cells to decitabine induces the overexpression of several enzymes that participate in pyrimidine metabolism including the nucleotidohydrolase dUTPase, which suggests a role for this enzyme in the metabolism of this cytidine analog. dUTPase converts dUTP into dUMP, thus preventing the incorporation of dUTP by DNA polymerase during replication and contributing this way to the detoxification of the dNTP pool in human cells. Transient depletion of dUTPase by siRNA rendered MRC-5 and HeLa cells hypersensitive to aza-dCyd while overexpression of the enzyme conferred protection against its antiproliferative effect. Moreover, dUTPase-deficient cells exposed to aza-dCyd accumulated a high concentration of dUTP in the nucleotide pool and exhibited increased levels of DNA double-strand breaks as monitored by γH2AX foci formation. Taken together, these data support an alternative mode of action for decitabine in which this cytidine analog can be partially metabolized into aza-dUMP, which may then act as a thymidylate synthase inhibitor. Especially in the absence of dUTPase, azadCyd cell treatment would cause the accumulation of aza-dUTP/dUTP and subsequent uracil misincorporation with genotoxic consequences. We therefore propose that dUTPase is a key factor in the cellular response to decitabine-based chemotherapy.

**POSTER # 12**

**Inhibitors of lactate dehydrogenase overcome the resistance towards gemcitabine in hypoxic mesothelioma cells, and modulate the expression of the human equilibrative transporter-1 (hENT1)**

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**Background and aims.** Malignant pleural mesothelioma (MPM) is a severely hypoxic malignancy, and hypoxia has been associated with resistance towards several anticancer drugs, including the nucleoside analogue gemcitabine. The muscle-isofrom of lactate dehydrogenase (LDH-A) constitutes a major checkpoint for the switch to anaerobic glycolysis, ensuring supply of energy and anabolites in hypoxic environments. Therefore we investigated the molecular mechanisms underlying the pharmacological interaction of a new LDH-A inhibitor in combination with gemcitabine in MPM cells.

**Methods.** MPM cells (H2052 and H28) were treated for 72 hours with gemcitabine alone or in combination with the LDH-A inhibitor NHI-1, in normoxic and hypoxic (at O2 tension of 1%) conditions, and the inhibition of cell growth was investigated by sulforhodamine-B-assay. The pharmacologic interaction was evaluated using the combination index (CI) method, while cell cycle perturbations were investigated with flow cytometry. Moreover, quantitative PCR analysis was performed to assess
whether hypoxia modulated the expression of the gemcitabine activating enzyme deoxycytidine kinase (dCK) and the human equilibrative transporter-1 (hENT1).

**Results.** Gemcitabine was cytotoxic against H2052 and H28 cells with IC50s of 55.1±6.3, and 103.5±9.2 nM, respectively, but cell growth inhibitory effects were reduced of 5- and 10-fold under hypoxia. The simultaneous addition of NHI-1 analysis showed synergism (CI<1) in both MPM cell lines in hypoxia. Flow cytometric studies demonstrated that hypoxia caused a G1 arrest, whereas cells exposed to the drug combination presented typical apoptotic morphology. Finally, the expression levels of dCK and hENT1 were significantly down-regulated under hypoxic conditions, but treatment with NHI-1 was associated with a rescue of hENT1 expression, which might explain the synergistic interaction with gemcitabine.

**Conclusions.** Hypoxia is a driving force in MPM chemoresistance. However, apoptosis induction and modulation of the key transporter in gemcitabine uptake may contribute to the synergistic interaction of gemcitabine with the LDH-A inhibitor NHI-1 and support further studies for the rational development of this combination.

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**POSTER # 13**

**Evaluation of Purine utilization by Lactobacillus gasseri strains with potential to decrease the absorption of food derived purines in the human intestine.**

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**OBJECTIVES:** It is known that frequent and heavy intake of purine-rich foods causes elevation of serum uric acid levels, which is a risk factor of hyperuricemia. In order to prevent hyperuricemia, one strategy is to decrease absorption of purines in the human intestine. We hypothesized that lactic acid bacteria ingested with food might utilize purines and contribute to decreasing absorption of them in the intestine. In this study, we investigated the ability of Lactobacillus gasseri strains to utilize purines.

**MATERIALS AND METHODS:** Lactic acid bacteria: In this study, three bacterial strains were used: Lactobacillus gasseri PA-3 (PA-3), Lactobacillus gasseri OLL2851, and Lactobacillus gasseri OLL2996.

**Uptake of purines by Lactobacillus gasseri strains:** Using completely chemically defined growth medium (DM), each bacterial strain was incubated in the presence of 14C-Adenine for 0, 30, and 60 minutes at 37°C. After culture, bacterial cells were collected by filtration and washed with saline three times. Radioactivity of harvested bacterial cells was measured by a liquid scintillation counter.

**Proliferation assay:** Using the DM, PA-3 was cultured in the presence or absence of adenine for 0, 4, and 6 hours at 37°C. After incubation, the optical density was measured at 650nm.

**RESULTS AND DISCUSSION:** PA-3 showed the largest incorporation of 14C-adenine in the tested strains. In additional studies, PA-3 was also proved to uptake 14C-Adenosine 5'-monophosphate (14C-AMP) and 14C-Adenosine. Among the tested purines, adenine was most incorporated by PA-3. When cultured in the presence of AMP, Adenosine, or adenine, PA-3 displayed the most proliferation in response to adenine.

Few reports have shown purine uptake by lactic acid bacteria. No report has demonstrated the uptake of purines by the Lactobacillus gasseri species. We have firstly shown that Lactobacillus gasseri strains can uptake adenine and that PA-3 has the greatest ability to uptake adenine among the three tested strains. Additionally, it was proved that adenine was the most incorporated by PA-3 among AMP, adenosine, and adenine. Because PA-3 has high purine nucleosidase activity outside bacterial cells (data not shown), uptakes of purines bases may be more efficient than of nucleosides. It is likely that incorporated purines are used in the growth of PA-3 because this bacterial strain greatly proliferates in the presence of purines.

**CONCLUSIONS:** It has been clarified that the Lactobacillus gasseri strain PA-3 uptakes and uses purines. By ingesting these bacterial cells with such foods as yogurt, PA-3 might help in decreasing absorption of purines in the human intestine.

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**POSTER # 14**

**Isolation of a novel protein – p12- from adult Drosophila melanogaster that inhibits deoxynucleoside and protein**

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Drosophila melanogaster is known to only have one deoxynucleoside kinase – DmdNK - capable to phosphorylate all four natural deoxynucleosides. DmdNK was originally isolated from an embryonic cell line. We wanted to study the expression of DmdNK during development from embryonic cells to adult flies. We found declining DmdNK activity during development and in adult flies there were no activity. Surprisingly, the extract from adult flies turned out to exhibit a strong inhibitory effect on deoxynucleoside kinase activity. The dNK-inhibitor was precipitable with ammoniums sulfate, and was purified to a high degree by gel-filtration as indicated by LC-MS/MS. Since the inhibitor eluted from G-200 gel-filtration with a size of 10-13 kDa, we named it p12. We tested the purified fraction for specificity towards various enzymes and found that both mammalian and bacterial
dNKs were inhibited, whereas there was no effect on hexokinase and pyruvate kinase. Also, there was no effect on glutathione transferase and acidic phosphatase. However, when tested against cyclin B dependent kinase, we found a strong inhibitory effect. Both with human Cdk1/CycB (20 units) and S. pombe Cdc2/B-type cyclin the purified fraction from Superdex 200 that inhibited Dm-dNK, also inhibited the two protein kinases to the same degree. Furthermore, testing p12 in a DNA polymerase based assay we found that the 3´-5´-exonuclease part of the DNA polymerase (Klenow polymerase) was activated.

POSTER SESSION 2

1) PURINE AND PYRIMIDINE METABOLISM AND MITOCHONDRIAL DISORDER
2) PURINES AND PYRIMIDINES IN INFLAMMATION AND AUTOIMMUNE DISEASE
3) PURINE AND PYRIMIDINE ENZYME REGULATION

PURINE AND PYRIMIDINE METABOLISM AND MITOCHONDRIAL DISORDER

POSTER # 15
Thymidine Kinase 2 deficiency: from diagnosis to treatment

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OBJECTIVE: To describe the natural history of thymidine kinase 2 deficiency and analyse efficacy and safety of treatment approaches with nucleoside vs. nucleotides supplementation.

BACKGROUND: Autosomal recessive (AR) TK2 mutations cause severe mtDNA depletion and devastating neuromuscular diseases in infants and children, as well as mtDNA multiple deletions and progressive external ophthalmoplegia (PEO) in adults. Recently, we have demonstrated that molecular bypass therapy with deoxypyrimidine monophosphates is effective in ameliorating Tk2 deficiency in the H126N knockin mouse model while efficacy with nucleosides have been proven in other in vitro disease models.

DESIGN/METHODS: We reviewed clinical history, molecular genetics and biochemical defects of 57 patients that have been reported in literature from 2001-2014 as well as nineteen new patients with TK2 mutations.

RESULTS: Based on the clinical history of our new patients together with a systematic literature review, we identified four disease presentations: i) infantile encephalomyopathy with onset in the first year of life and rapidly progressive to early death; ii) childhood SMA-like myopathy progressing to loss of ambulation over several years; iii) adult myopathy with subclinical or mild myopathy at onset and gradual loss of ambulation, respiratory failure, or both in adulthood; and iv) adult AR-PEO. Molecular genetics defects in muscle range from i) severe mtDNA depletion in the infantile/childhood onset forms, ii) coexisting mtDNA multiple depletion and mild mtDNA depletion in adult-onset myopathy, and iii) isolated mtDNA multiple deletions in AR-PEO. Clinical and molecular data clearly demonstrate that thymidine kinase 2 deficiencies span a disease spectrum and the disease frequency has been underestimated. In addition, Kaplan-Meyer curve showed that 80% of patients died in the first 3 years of life, underlying the importance of early diagnosis for treatment purpose.

CONCLUSION: TK2 deficiency manifests a clinical and molecular genetic disease spectrum. Early and accurate diagnosis is important to initiate timely treatment with dCMP/dTMP, which may modify clinical history of this devastating disorder.

POSTER # 16
Deoxyribonucleoside supply rescues mtDNA depletion in human POLG-deficient fibroblasts

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Mitochondrial DNA (mtDNA) depletion and deletion syndrome (MDDS) has been associated with mutations affecting an increasing number of human genes. An important group of these genes encodes for proteins directly involved in keeping mitochondrional DNA (mtDNA) integrity. Human POLG, a deoxyribonucleoside triphosphate:DNA polymerase, is highly expressed in brain and is crucial for maintaining mtDNA integrity. Mutations in POLG are associated with MDDS and cause severe brain and muscle defects. We have previously shown that deoxyribonucleosides rescue mtDNA depletion in human POLG-deficient fibroblasts. Here we test the effect of deoxyribonucleosides in three novel POLG-deficient cell lines that were isolated from different patients affected by MDDS. We also evaluate the effect of deoxyribonucleosides in fibroblasts derived from a patient with isolated mtDNA depletion. Our results show that deoxyribonucleosides can rescue mtDNA depletion in POLG-deficient fibroblasts, suggesting that deoxyribonucleosides could be a potential treatment for MDDS. This study also highlights the importance of studying the effect of deoxyribonucleosides in different POLG-deficient cell lines to better understand the underlying mechanisms of MDDS.
mitochondrial dNTP homeostasis. Deregulation of dNTP metabolism ultimately leads to limited availability of one or more of the four dNTPs required for normal DNA synthesis. Previous studies by our and other groups have proved that feeding the deoxyribonucleoside salvage pathway by administration of the defective dNTP substrate precursor in the form of deoxyribonucleosides (dN) may be sufficient for rectifying a given mitochondrial dNTP imbalance and thus prevent mtDNA depletion, the feature associated with the highest severity in MDDS. We have shown that addition to the cell culture media of dNs rescues mtDNA depletion in dGK-deficient cells or thymidine-induced mtDNA depleted-fibroblasts (MNGIE, in vitro model) respectively.

Polymerase gamma is a heterotrimer constituted by one catalytic subunit (encoded by POLG) and two accessory subunits that act as processivity factors and modulators of DNA binding (encoded by POLG2). Mutations affecting the catalytic subunit can interfere with the polymerase affinity for dNTPs and their normal incorporation rate into the nascent DNA-strand and thus theoretically benefit from a higher dNTP concentration at the replication fork. Here, we have studied mtDNA recovery rates after EtBr (ethidium bromide)-forced depletion in quiescent skin fibroblasts derived from three patients harbouiring different mutations in POLG: patient 1 (homozygous for the p.R309C mutation); patient 2 (compound heterozygote for the p.W748S and p.G848S mutations); patient 3 (heterozygote for two dominant aminoacid changes in cis p.V1177L and p.E1143G). We treated quiescent fibroblasts obtained from all three patients with 5 ng/ml EtBr for a period of two weeks. All POLG-deficient cells experienced a higher degree of mtDNA depletion compared to that observed in four fibroblast lines obtained from healthy controls (averaged percent of mtDNA residual levels in patient-derived cells: 16,5 ± 2,3%; in control cells: 37,8 ± 9,1%). We monitored mtDNA recovery after EtBr withdrawal for 12 additional days either in the presence or absence of 200 µM combination of all four dNs plus a specific inhibitor of deoxyadenosine degradation (EHNA), which is otherwise rapidly degraded in cell culture media. MtDNA copy number from control cells reached values above 100% of initial levels 12 days after EtBr withdrawal independently of dNs treatment (139,7 ± 43,0% with treatment; 118,5 ± 33,4% without treatment). Conversely, POLG-deficient cells were unable to recover normal mtDNA levels (26,0 ± 7,0%) unless exposed to dNs. After treatment, residual mtDNA levels of patient cells where comparable to that achieved by control cells (140,7 ± 16,7%).

With this work, we provide evidence that not only dNTP metabolism-related mutations but also those affecting enzymes directly acting at the mitochondrial replication fork may benefit from enhanced nucleotide synthesis provided by dNs administration.


POSTER # 17

Functional interaction of nuclear SAMHD1 and mitochondrial deoxyguanosine kinase in the regulation of mt dNTP pool balance

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The regulated synthesis of the four dNTPs includes two pathways: (i) de novo synthesis, with reduction of ribonucleotides by ribonucleotide reductase (RNR) as the rate-limiting step and (ii) the salvage pathway, based on phosphorylation of deoxyribonucleosides by cytosolic and mitochondrial (mt) kinases. In each pathway allosteric regulation of individual enzymes fine-tunes dNTP synthesis. In addition, a combination of synthesis and degradation sets the intracellular concentrations of each of the four dNTPs. Disturbances in either process may lead to deficiency or surplus of one or more dNTPs and result in genetic diseases through destabilization of DNA synthesis. The deoxyribonucleotide triphosphohydrolase SAMHD1 is a newcomer in this regulatory network. SAMHD1 degrades dNTPs to deoxyribonucleosides and triphosphate and its impact on the sizes of dNTP pools is particularly strong in quiescent and differentiated cells. The main effect of SAMHD1 on the cellular concentration of dGTP, as demonstrated by the large expansion of the dGTP pool in cells with SAMHD1 downregulation. The deoxyguanosine (GdR) produced by dGTP hydrolysis is either degraded further by purine nucleoside phosphorylase or recycled via salvage by deoxyctydine kinase (dCK) in the cytosol and deoxyguanosine kinase (dGK) in mitochondria. We have studied to which extent these kinases compensate the degradation of dGTP by SAMHD1 during quiescence. We have used dGK-mutated human fibroblasts, derived from 3 unrelated patients affected by hepatocerebral mtDNA depletion syndrome. When cycling, the mutant cells contained a normal complement of mtDNA. When maintained quiescent for more than a week they developed a progressive mtDNA depletion, whereas under the same conditions quiescent wt fibroblasts maintained a normal mtDNA copy number. We hypothesized that in the quiescent mutant fibroblasts the combination of active SAMHD1 and inactive dGK may disrupt the normal balance of the mitochondrial dNTP pools and inhibit mtDNA synthesis. To test this hypothesis we downregulated SAMHD1 by siRNA transfection during 10 days of quiescence in wt and dGK-deficient fibroblasts. The inactivation of SAMHD1 left the content of mtDNA unchanged in the wt fibroblasts, but prevented mtDNA depletion in the mutant cells. Analysis of the mitochondrial dNTP pools in quiescent mutant fibroblasts showed that dGK deficiency causes mt dNTP pool imbalance without necessarily decreasing mt purine dNTPs. SAMHD1 downregulation restored pool balance and boosted mtDNA copy number compensating the mtDNA depletion to various degrees in the individual mutant lines. Our results indicate that SAMHD1 in the nucleus and dGK in...
mitochondria functionally interact for the regulation of mitochondrial dNTP pool balance and that the effects of dGK dysfunction are worsened by the unrestrained SAMHD1 activity (Supported by Grant GGP14005 from Italian Telethon).

**POSTER # 18**

**Balanced levels of dNTPs links mitochondrial dysfunction to cellular stress**

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Imbalance of the cytosolic deoxyribonucleotide triphosphate (dNTP) pool causes cellular stress, which has been associated with several human age-related diseases. Imbalance of the dNTP pool leads to higher mutation rates, genomic instability and development of cancer. Furthermore, in cohort studies of individuals suffering from declined cognitive function as well as individuals suffering from Alzheimer’s disease, we show that the dNTP levels in blood cells are significantly different from matched controls.

Mitochondrial dysfunction has been linked to both Alzheimer’s disease as well as cancer. We have previously shown that depletion of mtDNA in human cell lines results in imbalance of the cytosolic dNTP pools. Therefore, we hypothesize that dysfunctional mitochondria affect the dNTP levels, and thereby causes cellular stress recognized in Alzheimer’s disease and cancer.

The mitochondrial genome primarily encodes peptides essential for the mitochondrial electron transport chain (ETC) and thereby ATP produced by oxidative phosphorylation. The ETC is linked to the de novo synthesis of pyrimidines through the enzyme dihydroorotate dehydrogenase (DHODHase) located in the inner membrane of the mitochondria. The enzyme couples dihydroorotate oxidation to respiratory ubiquinone reduction and converts dihydroorotate to orotate. Orotate is further converted to uridine monophosphates, UTP and CTP, and ultimately, dTTP and dCTP.

In this project we show that both an inhibition of ATP synthase and of DHODHase results in a decrease of cytosolic levels of dNTP. We find that inhibition of either ATP synthase or DHODHase leads to increased sensitivity towards UV but not MMS induced DNA damage. Finally, we demonstrate that inhibition of DHODHase, but not ATP synthase promotes anchorage independent growth and autophagy. In conclusion, our results show that mitochondrial dysfunction can cause cellular phenotypes that are characteristic of common human age-related diseases.

**POSTER # 19**

**Oral deoxycytidine treatment of a murine model of MNGIE**

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Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is a rare autosomal recessive disease caused by mutations in the **TYMP** gene encoding thymidine phosphorylase. The loss of thymidine phosphorylase activity is responsible for dramatic elevations of the plasma and tissue pyrimidine nucleosides thymidine and deoxyruridine, which produce mitochondrial deoxynucleotide triphosphate (dNTP) pool imbalances that, in turn, cause somatic multiple deletions, depletion, and site-specific point mutations of mitochondrial DNA (mtDNA). To further define the pathogenesis of MNGIE as well as to study new therapeutic approaches, we generated thymidine phosphorylase /uridine phosphorylase double KO (**Tpp**/−/−/**Upp**/−/−) mice which manifest elevated tissue levels of thymidine and deoxyuridine and recapitulate phenotypic features of the human disease. In previous studies, we have shown that the relative proportion, rather than the absolute amount of dNTPs, is critical for mtDNA replication and maintenance. In vitro studies have indicated that lack of dCTP is responsible for mtDNA depletion in MNGIE. Therefore, we hypothesize that the administration of one or more nucleosides may have a beneficial impact in restoring dNTP pool balance by providing compensatory substrate(s) for enzymes involved in the complex biosynthetic pathways of the mitochondrial nucleotides. To test this hypothesis, we have administrated deoxycytidine (400 mg/kg) for two weeks to 6-9 months-old Thd-dUrd stressed **Tpp**/−/−/**Upp**/−/− knockout. After two weeks of treatment, mutant mice treated with exogenous deoxycytidine showed trends towards reduced levels of mtDNA in brain relative to untreated animals. We also found reduced motor coordination and exercise tolerance, measured by Rotarod test, in treated mutant animals in comparison with untreated. Although preliminary, our data do not support the hypothesized beneficial effect of deoxycytidine supplementation as a treatment for mtDNA depletion in MNGIE.

**POSTER # 20**

**A novel intronic mutation in TK2 affects mRNA expression and causes adult-onset mitochondrial depletion syndrome.**

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Autosomal recessive mutations in the nuclear gene TK2 typically cause mitochondrial depletion syndrome (MDS) in infants, characterized by severe reduction of mitochondrial DNA copy number that causes progressive mitochondrial myopathy [1]. Mutations in TK2 have also been reported to cause mitochondrial myopathy with slow progression in adults [2]. Here, we present a case of a 47 year-old woman who presented with facial muscles weakness, as well as swallowing difficulties and proximal limb weakness. Cytochrome c oxidase (COX) and Gomori trichrome staining revealed 5% COX-negative fibers and several of ragged-red fibers indicating a mitochondrial myopathy. A panel of nuclear gene encoding mitochondrial proteins revealed that this patient carried two heterozygous mutations in TK2: c.469_470insTGGG (p.D157Vfs*11) which creates a frameshift and a premature termination codon, and an intronic mutation, c.156+6T>G, which has not been previously reported. Proximity of the later mutation to exon 2 suggested an involvement in splicing of TK2 mRNA. Reverse transcriptase-PCR using primers spanning exon 1 and exon 5 did not show alternatively spliced transcripts from patient’s mRNA isolated from fibroblast and blood but rather significant lower expression of TK2 mRNA was found in the patient. In addition, radiochemical assay [3] tested on patient’s fibroblasts confirmed that TK2 activity was 35% than those of normal controls.

These results expands our understanding of TK2 related MDS. To our knowledge, this is the first case of a TK2 patient in whom TK2 is not disrupted, but rather reduced, apparently due to reduced mRNA expression. The mechanism by which this intronic mutation reduces expression requires further studies.

References:

PURINE AND PYRIMIDINES IN INFLAMMATION AND AUTOIMMUNE DISEASE

POSTER # 21

Interaction between 5’-nucleotidase cN-II and the inflammasome protein NLRC4/Ipaf suggests new roles of cN-II in cell biology

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The 5’-nucleotidase is involved in intracellular nucleotide metabolism through its action on nucleotide monophosphates. Due to the very strong conservation of its primary sequence through the vertebrata taxon, we hypothesized that this enzyme could interact with other proteins and thus play additional roles in cell biology. A two-hybrid system screening was set up, and interaction between cN-II and the leucine-rich region (LRR) of the inflammasome protein Ipaf was detected. This interaction was confirmed both in vitro using recombinant proteins and the implication of LRR was confirmed in cell extracts by immunoprecipitation after transfection with various plasmids. Finally, interaction was detected in human cancer cells using proximity ligation assay in cells expressing cN-II but not cells without cN-II expression. Recent data shows that Ipaf, in addition to its role in innate immunity, can mediate cellular answers to intracellular stress signals. Based on our results, we hypothesize that cN-II can interact with Ipaf in order to regulate its folding and conformation, thus acting as a sensor of global health state of the cell capable of regulating cell death. This clearly shows that cN-II has new roles in human cells which might be independent of its enzymatic activity and nucleotide metabolism.

POSTER # 22

Mice lacking adenosine A2A receptors develop spontaneous osteoarthritis

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Pain, loss of joint function, social isolation and a broad reduced quality of life, make osteoarthritis a disease with a high medical and social impact. OA is a chronic joint disease characterized by chronic pain, synovial inflammation, and the hallmarks include altered cartilage homeostasis with consequent bony changes: osteophytes and enthesophyte. Chondrocytes are the cell type injured and they play a central role in the pathogenesis of OA by releasing inflammatory mediators and proteolytic enzymes which damage the cartilage. We had observed that mice lacking adenosine A2A receptors (A2AR) have increasing difficulty in grabbing food, walking and mating as they age and to better understand the basis for their difficulties we examined the joints of these mice to determine whether joint pathology could explain their behavioral changes. Knee joint of WT and A2AR-KO mice were studied by histologic, immunohistologic and microcomputed tomography analysis. In vitro studies were performed on primary articular chondrocytes to assed the differences in production and release of hypertrophic markers between WT and A2AR-KO and the effects of the A2AR agonist CGS21680 (1 μM) in chondrocytes stimulated with IL-1β (5ng/ml). The joints in A2AR-KO mice showed progressive loss of cartilage, chondrocyte hypertrophy, osteophytes, and subchondral bone density over time. Chondrocytes isolated from neonatal A2ARKO mice spontaneously expressed markers of chondrocyte hypertrophy and inflammation such as MMP-13, collagen-X, fibronectin and osteopontin. Moreover A2AR stimulation regulated chondrocyte responses to IL-1 by inhibiting activation and translocation of NF-kB, and secretion of hypertrophic marker. CONCLUSION: Mice lacking A2AR suffer from diminished mobility as a result of the spontaneous development of osteoarthritis. These findings are consistent with the hypothesis that local adenosine release maintains cartilage homeostasis by preventing endogenous inflammation and suggest that targeting A2AR may prevent progression of osteoarthritis.

POSTER # 23

CTP synthase 1 deficiency in humans reveals its central role in T lymphocyte proliferation

Emmanuel Martin, Noé Palmic, Christelle Lenoir, Fabian Hauck, Cédric Mongellaz, Mauro Degli Esposti, Naomi Taylor, Robert Wynn, Capucine Picard, Alain Fischer, Peter Arkwright and Sylvain Latour

Effector T lymphocyte functions triggered by antigen recognition and cosignals imply rapid and intense cell division, hence metabolism adaptation. The cytidine nucleotide triphosphate (CTP) is a precursor required for the metabolism of DNA, RNA and phospholipids. CTP originates from two sources: a salvage pathway and a de novo synthesis pathway that depends on two enzymes, the CTP synthase (or synthetase) 1 and 2 (CTPS1 and CTPS2), although their respective roles are not known. CTP synthase activity is a potentially important check point for DNA synthesis in lymphocytes. Here, we report the identification of loss of function mutations in CTPS1 in humans causing a novel and life threatening T-cell immunodeficiency characterized by an impaired capacity of activated T cells to proliferate. Proliferation in response to T-cell antigen receptor (TCR)-CD3 complex-mediated activation is defective in CTPS1-deficient patient cells or in normal T cells knocked-down with shRNA for CTPS1. In contrast, proximal and distal TCR signaling events and responses were only weakly affected by the absence of CTPS1. Normal T-cell proliferation was restored in CTPS1-deficient cells by expressing wild-type CTPS1 or by addition of exogenous CTP or its nucleoside precursor, cytidine. CTPS1 expression was found to be low in resting T cells, but rapidly up regulated following TCR activation. These results highlight a key and specific role of CTPS1 in the immune system by its capacity to sustain the proliferation of activated T cells during the immune response. CTPS1 may therefore represent a therapeutic target of immunosuppressive drugs that could specifically dampen T-cell activation.

PURINE AND PYRIMIDINE ENZYME REGULATION

POSTER # 24

The S'-nucleotidase cN-II is inhibited by fludarabine

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The S'-nucleotidase cN-II dephosphorylates nucleoside monophosphates into nucleosides and inorganic phosphate and is able to catalyze the opposite reaction through its phosphotransferase activity. Its expression level or activity in cancer cells has been shown to correlate with the sensitivity to nucleoside analogues or the outcome of patients treated with such drugs. In addition, its inhibition has been shown to induce apoptosis in cancer cells, suggesting that cN-II can be considered as a drug target.

We evaluated the inhibitory effect of clinically used purine nucleoside analogues (fludarabine, cladribine and clofarabine) on recombinant cN-II and observed that fludarabine is a mixed mode inhibitor (Km increases and Vmax decreases) with Kj of 0.5 and 9.0 mM. Docking experiments using previously published crystal structure of cN-II suggested a better interaction for fludarabine than adenosine in the effector site II. This site has been shown to be involved in the
activation of cN-II activity by adenlyc compounds. The interaction was confirmed using mutant cN-II showing a lack of inhibition by fludarabine on mutants in effector site II (F127A) whereas mutations in the active site (F157A) or in effector site I (R144E) were not sensitive to fludarabine inhibition. Finally, we showed that fludarabine was synergistic with the nucleoside 6-mercaptopurin in wild type haematological cancer cells, whereas no synergy was observed in corresponding cN-II deficient cells. This latter observation indicates that fludarabine acts as a cN-II inhibitor in cancer cells, which gives additional information on the mechanism of action of this largely used cancer drug.

POSTER # 25

INHIBITORY EFFECT OF PLANT EXTRACTS USED IN HERBAL MEDICINE ON XANTHINE OXIDASE ACTIVITY

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Objective: Xanthine oxidase (XO) is involved in the generation of uric acid and is known to be a target enzyme in the treatment of gout and hyperuricemia. Several plant components with XO inhibitory effects have been reported, and efforts to find a related preventive drug are ongoing. The present study was undertaken in order to investigate the effect of daily herbal medicines used as Kampo (Japanese traditional medicine) in purine metabolism through the XO inhibition. In addition, the Kampo medicines are usually extracted from several herbs. The effects of various herbal combinations were examined.

Materials and Methods: The plant samples were selected from the medicinal herbs listed by the Japanese Pharmacopoeia. The shredded herbs were reflux-extracted with 50% ethanol or distilled water. The herbal extracts were obtained by filtration and then concentrated by vacuum centrifuge. The samples were dissolved in dimethyl sulfoxide. Xanthine (34.1µg/800µL) as a substrate, XO (0.04units/20µL) and the herbal extract (100mg/100μL) were incubated in the reaction solution at 25°C, 30min. Generated uric acid was measured by high performance liquid chromatography. The inhibition rate of XO activity was estimated from the difference of the uric acid peak areas between the presence and absence of the herbal extracts.

Results and Discussion: In performing the determination of uric acid, removal of contaminations derived from the plant samples using solid phase extraction at the pretreatment stage was necessary. The inhibition rate of two types RHEI RHIZOMA was more than 90%, and they showed the strongest inhibitory effect in this study. Strong effects were also observed in EPHEDRAE HERBA and the Lamiaceae family of plant. In comparison of the extracted solvent, inhibitory effect of the extract in using 50% ethanol was about twice the distilled water extracts in several of the herbs, such as SCHIZONEPETAE SPICA and MENTHAE HERBA. The synergistic inhibition was not observed in extracts obtained from a combination of two herbs. RHEI RHIZOMA contains anthraquinones, and EPHEDRAE HERBA contains alkaloids, these herbal medicines have a strong effect on humans even in small amounts. Strong inhibitory effect on XO was also observed in these herbal medicines.

POSTER SESSION 3

1) NUCLEOSIDE TRANSPORTERS AND RECEPTORS
2) PURINES, PYRIMIDINES, AND CANCER

NUCLEOSIDE TRANSPORTERS AND RECEPTORS

POSTER # 26

Expression of a human NPT1 missense variant which increases the urate export

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2) Division of Bio-system Pharmacology, Department of Pharmacology, Osaka University
3) Department of Pathophysiology, Tokyo University of Pharmacy and Life Sciences

Human sodium-dependent phosphate cotransporter type 1 (NPT1/SLC17A1) is one of the urate transporters in kidney. Previously, genome-wide association studies have revealed an association between common variants of NPT1 gene and serum uric acid. Also, our recent study revealed that the common missense variant, I269T (rs1165196), of NPT1 decreases the risk of renal underexcretion gout. Moreover, we demonstrated that human NPT1 is localized to the apical membrane of the renal proximal tubule, and that I269T is the gain-of-function variant which increases the NPT1-mediated urate export. Meanwhile, the mechanism by which I269T variant increases the urate export remains to be clarified. In this study, to investigate the mechanism of the NPT1-mediated urate transport, we performed immunostaining and functional analysis of human NPT1 using Xenopus oocytes. For the measurement of [3H] urate uptake, Xenopus oocytes were incubated in high potassium buffer containing 100 µM [3H] urate for 60 min. In addition, for comparison of NPT1 expression levels of oocytes membrane between 269I and 269T, immunostaining was performed with anti-human NPT1 antibody. As a result, 269T-mediated urate transport is
significantly higher than that of 269I while there was no remarkable difference on oocyte expression levels between 269I and 269T. Combined with the previous report that I269T did not induce Km changes but increased the maximum volume (Vmax) of urate transport in a proteoliposome system, our findings suggest that I269T elevates Vmax by increasing the turnover rate of urate transport via NPT1. I269T variant would increase turnover rate of urate transport because 269T is probably more flexible than 269I; i.e., threonine has a smaller and less hydrophobic side chain than that of isoleucine. Thus, 269T, a common missense variant of NPT1, might have faster conformation changes than 269I in terms of the alternating-access model of transporters, and increases renal urate export in humans.

**POSTER # 27**

Expression of Intestinal and Renal Uric Acid Transporters in the 5/6 Nephrectomy Rat Model of Chronic Kidney Disease

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2 Human Physiology and Pathology, Faculty of Pharma-Sciences., Teikyo University, Tokyo, Japan.

**Background:** Recently, we reported that the extra-renal uric acid (UA) excretion might be enhanced in the intestine mediated by the up-regulation of the ATP-binding cassette transporter G2 (ABCG2) in a 5/6 nephrectomy rat model of chronic kidney disease (CKD). In the present study, we examined the expression of other UA transporters, URAT1, GLUT9/URAT1 and NPT4 in the intestine as well as in the renal cortex in the same rat model.

**Methods:** Male Wistar rats (6 weeks old) were randomly assigned to the 5/6 nephrectomy (Nx) group or the sham-operated control group. Urine and blood samples were obtained every 4 weeks and the rats were sacrificed at 8 weeks. Duodenum, jejunum, ileum, and transverse colon in addition to renal cortex were used for investigating the expression of the UA transporters by real time polymerase chain reaction (RT-PCR). Uricase activity was measured in the serum.

**Results:** The Nx group showed significantly decreased creatinine clearance, urine UA excretion/weight and UA clearance as compared with the control group. In contrast, serum UA and uricase activity were not significantly changed. The mRNA expression of GLUT9/URAT1 in the jejunum and ABCG2 in the ileum of the Nx group were significantly increased. The expression of the UA transporters in the renal cortex examined in the Nx group were significantly decreased to the similar degrees.

**Conclusions:** The up-regulation of GLUT9/URAT1 in the jejunum and ABCG2 in the ileum coordinately increase UA excretion from intestine at risk of hyperuricemia induced by CKD. UA transporters in the kidney may not play an important role in the regulation of UA metabolism in CKD.

**POSTER # 28**

Adenosine A2B Receptor Blockade Prevents Methionine-Choline Deficient (MCD) Diet Induced Non-Alcoholic steatohepatitis (NASH) By Regulating MicroRNA Expression

Hailing Liu, Tuere Wilder and Bruce N. Cronstein

Division of Translational Medicine, Department of Medicine, NYU School of Medicine

**Background:** Non-alcoholic fatty liver disease and non-alcoholic steatohepatitis (NASH) are common medical problems affecting more than 5% of the US population and as many as 20% of affected individuals will develop cirrhosis. Prior work has indicated that endogenously released adenosine, acting at A2B receptors, plays a central role in alcohol-induced fatty liver and fibrosis so we asked whether adenosine plays a role in NAFLD as well and whether receptor-mediated regulation of microRNA played a role in the development of NASH in a murine model.

**Methods:** Fatty liver was induced by feeding methionine choline-deficient diet (MCD Diet) (protein 14.6%, fat 22.1%, and CHO 63.2% of total kcal) for four weeks vs standard chow (SC). Hepatic steatosis was graded semi-quantitatively based on percentage of lipid-laden hepatocytes in H&E stained slides and confirmed by oil red O staining and direct measurement of hepatic triglyceride content. miR16, 21, 33b, 34a, 99a/b, 122, 155, 199a/b, and 200a/b (Qiagen) expression levels in livers of A2BR KO mice and wild type mice following MCD diet feeding for 1, 2 and 4 weeks were detected by miScript miRNA PCR Arrays.

**Results:** Wild type (WT) developed severe hepatic steatosis after MCD diet with obvious lipid droplets present in both perportal and pericentral areas of hepatocytes, but A2BKO mice suffered only minimal fatty change (Steatosis grade: 3.70±0.17 vs 1.55±0.3, n=4, p<0.05). Consistent with the histological appearance, the hepatic triglycerides were much lower in A2BKO mice than those in WT (10.75 ± 3.75 mg/g, n=4 vs 51.20 ± 10.64 mg/g, n=4, p<0.05). The inflammation severities were reduced dramatically in livers of A2BKO MCD diet feeding mice (1.25±0.25 vs 2.75±0.25 WT-MCD, n=4, p<0.01). The microRNA expression in livers of WT and A2BKO mice after one, two and four weeks MCD diet feeding is as following table.
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<td>1.14±0.03</td>
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*n=3 for all microRNAs

**Conclusion:** These results indicate that adenosine A2B receptor stimulation plays a role in the development of NASH and this effect is associated with the regulation of microRNAs previously implicated in the pathogenesis of NASH.

**POSTER # 29**

ENT1, a novel link between purinergic & calcium signalling

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Equilibrative nucleoside transporter 1 (ENT1) is an integral membrane protein that transports nucleosides and nucleoside analog drugs across cellular membranes. A relationship between purine nucleoside flux and calcium levels has previously been shown, but the underlying mechanisms have not been identified. We established that calcium regulates ENT1 function by measuring changes in radiolabelled nucleoside flux in HEK293 and RT4 cells in the presence of BAPTA-AM (25 µM), EGTA (50 µM), and thapsigargin (1.5 µM). Chelating extracellular calcium (EGTA) and intracellular and extracellular calcium (BAPTA-AM) decreased ENT1-dependent nucleoside flux by 12% and 39% respectively, while increasing intracellular calcium (thapsigargin) led to a 27% increase in nucleoside flux. We hypothesized that calcium regulates ENT1 through a physical interaction between ENT1 and calmodulin (CaM), a calcium signal transducer. We confirmed the existence of a calcium dependent CaM-ENT1 protein-protein interaction by co-immunoprecipitation of HA-ENT1 and CaM. To confirm that ENT1 is regulated by direct CaM-ENT1 interaction, we measured nucleoside flux after treatment with the CaM antagonist W7 (50 µM). We observed a 15% decrease in ENT1-dependent nucleoside flux with cells treated with W7 compared to control. Moreover, activation of NMDA receptors by glutamate (1 mM) and glycine (100 µM) in U-87 MG cells stimulated calcium influx (confirmed by live cell calcium imaging) and led to a 61% increase in nucleoside uptake. This increase in nucleoside flux was abolished in the presence of the NMDA receptor antagonist MK-801 (50 µM) and the CaM antagonist W7 (50 µM). These findings support our hypothesis that there is crosstalk between purinergic and calcium signalling pathways which represents a novel mechanism of ENT1 regulation. This highlights the role of ENT1 in regulating purinergic nucleoside flux in response to calcium signalling, particularly in the CNS. Future work will elucidate other components involved in the regulation of ENT1 as part of a larger complex of signalling networks and may help to develop innovative approaches to enhance cytotoxicity of nucleoside analog drugs.

**POSTER # 30**

Substrate translocation triggers endocytic regulation of the equilibrative nucleoside transporter 1 (ENT1)

Maliha Zafar, Zlatina Tarmakova, and Imogen Coe

Department of Chemistry and Biology, Faculty of Science, Ryerson University (Toronto)

Although there are many purine and pyrimidine nucleoside analogs available for cancer therapy today, the efficacy of these drugs is limited due to factors that surround their pharmacokinetics (i.e. phosphorylation, efflux and metabolism). Nucleosides enter cells through nucleoside transporters, particularly (although not exclusively) the ubiquitously expressed ENT1. One
mechanism that has been widely studied and which is known to play a role in the life cycle of a transporter is internalization and recycling, which is, in some cases, regulated by substrate itself. We hypothesized that ENT1 is internalized upon continuous exposure to substrate (which occurs during chemotherapy). To confirm this, HA-hENT1 was overexpressed in HEK293 cells, which were then incubated with a bolus treatment of cytidine (40µM) for 6 hours. Fluorescence-activated cell sorting (FACS) analysis of these cells showed a decrease in surface fluorescence from fluorophore conjugated anti-HA antibody, compared to untreated cells. Cells treated in the same manner were fixed and viewed using confocal microscopy. Numerous intracellular punctate structures, suggesting hENT1 internalization, were observed in cytidine treated cells but not in control. Moreover, radio-ligand binding assays with \[^{3}H\]NBTI (a high affinity, non-transportable inhibitor of ENT1) showed a decrease in ENT1 binding sites following cytidine treatment compared to control. While these data support our hypothesis, cytidine pre-treatment had no effect on the subsequent uptake of \[^{3}H\]2-chloroadenosine (10µM) compared to control. We speculate that pre-treatment of cells with substrate results in enhanced endocytosis of “exhausted” ENT1 proteins, leaving behind, at the membrane, transporters with an increased affinity for substrate. To confirm this hypothesis, we will measure V\text{max} and K\text{m} of cytidine-treated and untreated cells. If substrate pre-treatment leads to increased transporter affinity, perhaps “priming” cells with substrate before chemotherapy would enable similar cytotoxic effect at lower concentrations of drug.

**POSTER # 31**

**Ticagrelor regulates osteoblast and osteoclast function and promotes bone formation in vivo by increasing adenosine levels**

Aránzazu Mediero^1, Tuere Wilder^1, Carl Whatling^2, Bruce Cronstein^1.

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**Purpose:** Antiplatelet drugs include ticagrelor, clopidogrel and dipyridamole. Ticagrelor and clopidogrel are both P2Y\textsubscript{12} antagonists but, unlike clopidogrel, ticagrelor also inhibits cellular adenosine uptake via ENT1 and thereby increases extracellular adenosine levels. Dipyridamole induces its antiplatelet effect indirectly via adenosine, an endogenous antiplatelet agent, as it, like ticagrelor, inhibits ENT1. We have previously demonstrated that dipyridamole stimulates bone regeneration in mice by an A2AR-dependent mechanism. Moreover, we have also demonstrated that both adenosine A2AR or A2BR stimulation inhibits osteoclast differentiation. We therefore asked whether the antiplatelet agents ticagrelor and clopidogrel regulated osteoclast differentiation and, if so, whether A2AR or A2BR stimulation played a role in this effect.

**Methods:** Osteoclast and osteoblast differentiation were studied in primary murine bone marrow culture as the number of Tartrate Resistance Acid Phosphatase (TRAP)-positive or Alizarin Red-positive cells, respectively, after challenge with ticagrelor, the active metabolite of clopidogrel or dipyridamole in doses ranging from 1nM to 100µM (n=5 each), in the presence/absence of ZM241385 (A2AR antagonist) or GS6201 (A2BR antagonist) and ADP 10µM. Male C57Bl/6 mice were anesthetized and a trephine defect was created in the calvariae. The defect was covered using a collagen scaffold soaked in saline or 10-100µM ticagrelor or clopidogrel (n=5 each). Animals received the appropriate treatment daily until sacrifice 4 weeks after surgery when calvariae were harvested and prepared for microCT and histology.

**Results:** TRAP staining reveals a dose-dependent reduction in in vitro osteoclast differentiation in the presence of ticagrelor (IC\textsubscript{50}=10µM), clopidogrel (IC\textsubscript{50}=10µM) and dipyridamole (IC\textsubscript{50}=10µM), been these concentrations above the clinical exposures. TRAP staining revealed that ZM241385 1µM blocked the effect of both ticagrelor and dipyridamole, whereas the effect of clopidogrel was reversed by GS6021 (1µM). Alizarin Red staining revealed a slight but non-significant increase by ticagrelor, clopidogrel and dipyridamole treatment. ZM241385 1µM reversed the effect of both ticagrelor and dipyridamole on osteoblast differentiation but the effects of clopidogrel were reversed by GS6021 1µM. microCT showed that blockade of adenosine uptake by treatment with either ticagrelor or clopidogrel markedly enhanced bone regeneration (28±1% and 27±2%, respectively, compared to 15±3% in control, p<0.01). Bone regeneration was similar to that produced by BMP2 treatment. In ticagrelor- and clopidogrel-treated mice there was a decrease in TRAP positive osteoclasts that correlated with a decrease in immunostaining for Cathepsin K. Moreover, we observed an increase in immunostaining for the osteoblast and bone formation marker, Alkaline Phosphatase, in the bony defects treated with ticagrelor or clopidogrel.

**Conclusions:** Ticagrelor inhibits osteoclast differentiation via blockade of adenosine uptake and resulting increases in extracellular adenosine levels which stimulate A2ARs, as we have previously reported for dipyridamole. In contrast, the active metabolite of clopidogrel inhibits osteoclast differentiation via stimulation of A2BRs. Both ticagrelor and the active metabolite of clopidogrel promoted bone regeneration in a murine trephination model. Local treatment with the antiplatelet agents ticagrelor, clopidogrel or dipyridamole may be useful for inhibition of bone destruction or promoting bone growth.
**PURINES, PYRIMIDINE, AND CANCER**

**POSTER # 32**

**Downregulation of 5’-nucleotidase cN-II in cancer cells favors tumorigenicity and suggests impact on metabolic adaptability**

Gabriel Bricard, Emeline Cros-Perrial, Charles Dumontet & Lars Petter Jordheim  
CRCL, INSERM U1052, CNRS UMR 5286, University of Lyon, Lyon, France

The 5’-nucleotidase cN-II catalyzes nucleoside monophosphates into nucleosides, and is thereby implicated in nucleoside and nucleotide metabolism of cells. To better understand the global function of this enzyme in cancer cells, we inhibited its expression using a specific RNA silencing approach, in human cancer cells of breast (MDA-MB-231), colon (HCT-116), pancreas (MiaPaCa) or lung (NCI-H292) origins. These models showed stable and decreased cN-II protein expression associated with decreased enzymatic activity. Cells with low cN-II expression (pScN-II) as compared to control cells (pScont), did not display altered sensitivity to cancer drugs. However, upon grafting in immuno-deficient mice, tumor growth was enhanced for the four distinct pScN-II models in comparison to their respective pScont cells. Preliminary metabolic analyses were performed with breast carcinoma MDA-MB-231 lines. Under glucose-limited culture conditions (5-10 mM glucose), pScN-II cells could proliferate more (more biomass), and displayed a better long-term survival than controls as evidenced by AnnexinV/PI staining. This was associated with a lower content of reactive oxygen species (ROS) in pScN-II cells, either due to a lower ROS production or an improved neutralization of ROS. In similar glucose-limiting conditions, pScN-II cells were also more sensitive to rotenone, a specific inhibitor of complex I in respiratory chain. Experiments using the xCELLigence system confirmed differences in long term culture. Indeed, while both pScN-II and pScont cells showed comparable initial proliferation, they subsequently manifested a wave of impedance variation that correlated in a time-dependent manner with the initial glucose level. These variations appeared at the same time for both lines but were clearly less marked for pScN-II cells. We hypothesize that such variations may correspond to a forced metabolic shift from glycolysis to oxidative phosphorylation, upon glucose exhaustion, which seems to occur more smoothly for pScN-II cells. Indeed, the same long-term culture experiments performed under hypoxia indicated a higher dependence of pScN-II cells for oxygen since they did not maintain long term survival and died faster under the same glucose limiting conditions.

In summary, our results highlight a connection between the nucleotide metabolism enzyme cN-II and energetic metabolism of glucose in human breast cancer cells MDA-MB-231. In relation with cN-II decrease, tumor cells have modified some bioenergetic parameters allowing improved long term survival under glucose-limiting conditions. Further metabolic studies with other tumor types should enable us to understand the meaning of the relationship between cN-II and energetic metabolism.

**POSTER # 33**

**dUTP nucleotidohydrolase is a major factor in the cellular response to decitabine**

Vidal AE, Requena CE, Pérez-Moreno G, Ruíz-Pérez LM and D. González-Pacanowska  
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Decitabine (5-aza-2’-deoxycytidine, aka-dCyd) is a demethylating agent used clinically in the treatment of myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). The incorporation of azacytosine in DNA promotes the covalent trapping of DNA methyltransferases (DNMTs) and the reactivation of epigenetically repressed genes but also genome-wide DNA damage resulting from inefficient repair of DNMT-DNA cross-links and azadCyd-induced base damage. Here, we report that exposure of cells to decitabine induces the overexpression of several enzymes that participate in pyrimidine metabolism including the nucleotidohydrolase dUTPase, which suggests a role for this enzyme in the metabolism of this cytidine analog. dUTPase converts dUTP into dUMP, thus preventing the incorporation of dUTP by DNA polymerase during replication and contributing this way to the detoxification of the dNTP pool in human cells. Transient depletion of dUTPase by siRNA rendered MRC-5 and HeLa cells hypersensitive to aza-dCyd while overexpression of the enzyme conferred protection against its antiproliferative effect. Moreover, dUTPase-deficient cells exposed to aza-dCyd accumulated a high concentration of dNTP in the nucleotide pool and exhibited increased levels of DNA double-strand breaks as monitored by γH2AX foci formation. Taken together, these data support an alternative mode of action for decitabine in which this cytidine analog can be partially metabolized into aza-dUMP, which may then act as a thymidylate synthase inhibitor. Especially in the absence of dUTPase, azaCyd cell treatment would cause the accumulation of aza-dUTP/dUTP and subsequent uracil misincorporation with genotoxic consequences. We therefore propose that dUTPase is a key factor in the cellular response to decitabine-based chemotherapy.
POSTER # 34

Differential expression of uridine-cytidine kinases in neuroblastoma. Implications for development of a targeted therapeutic approach.

André B.P. van Kuilenburg and Rutger Meinsma
Academic Medical Center, Laboratory Genetic Metabolic Diseases, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands.

Introduction
Uridine-cytidine kinase (UCK) is a pyrimidine ribonucleoside kinase that catalyzes the phosphorylation of uridine and cytidine to UMP and CMP, respectively. UCK also catalyzes the phosphorylation, and thereby the pharmacological activation, of several cytotoxic pyrimidine ribonucleoside analogues. Two human UCKs have been reported with UCK1 being ubiquitously expressed in several tissues whereas the expression of UCK2 seems to be confined to human placenta and various tumor cells. In this study, we investigated the functional role of UCKs in neuroblastoma.

Materials and Methods:
The expression of UCKs in neuroblastoma cell lines was investigated with quantitative PCR and Western blotting using antibodies generated against purified UCK1 and UCK2. Subcellular localization of UCKs was investigated using UCKs expressed in fusion with green fluorescent protein. Cytotoxicity towards 3-deazauridine was assessed using MTS in neuroblastoma cells, transfected with the pcDNA3.1Zeo(+) vector containing UCK1 or UCK2, or after transfection with siRNA targeting UCK2.

Results:
Analysis of mRNA and protein levels coding for UCK1 and UCK2 showed that UCK2 is by far the most abundantly expressed UCK in a panel of neuroblastoma cell lines. Subcellular localization studies showed that the UCK1-GFP protein was localized in the cell nucleus whereas UCK2-GFP was located in the cytosol. To investigate the role of UCKs in metabolizing pyrimidine analogues we tested the cytotoxicity of 3-deazauridine which is activated primarily by UCK2. Transient overexpression of UCK2 in neuroblastoma cells resulted in an increased cytotoxicity of 3-deazauridine whereas knockdown of endogenous UCK2 protected the neuroblastoma cells from 3-deazauridine-induced toxicity. However, overexpression of UCK1 protected the neuroblastoma cells from 3-deazauridine-induced toxicity. Subcellular localization studies showed that co-expression of UCK1 with UCK2 resulted in a nuclear localization of UCK2 instead of its normal cytosolic localization.

Conclusion:
The discovery that UCK2 is highly expressed in neuroblastoma opens the possibility for selectively targeting neuroblastoma cells using UCK2-dependent pyrimidine analogues, while sparing normal tissues.

POSTER # 35

Proteomics analysis of the effect of fluorouracil (SFU) and SFU/leucovorin (LV) on colorectal cancer (CRC) in patients

VU University Medical Center, Amsterdam, Netherlands

Background:
SFU is widely used and inhibits thymidylate synthase (TS); LV given in combination with SFU enhances TS inhibition. TS levels and inhibition are associated with survival in advanced disease. The large variation in response between patients indicates that more mechanisms may be important. To make better treatment decisions, several additional biomarkers, next to TS, such as SFU activation, breakdown and incorporation into RNA have been investigated. Several studies on global molecular profiling for SFU activity have been performed on model systems and patients, showing that loss of 18p11-32, which harbors the TS gene, was associated with better survival.

AIM: Identification of new potential mechanisms of action and potential biomarkers of SFU with or without LV treatment using in-depth proteomics analysis of patient CRC tissues.

METHODS: Patients received approximately 48 hours before resection, a test dose of SFU (500 mg/m2) (n=5), SFU with LV (500 mg/m2) (n=7) or no test dose (n=7). For proteomics analysis, samples of CRC resection material were lysed in SDS sample buffer, fractionated by 1D gel electrophoresis, followed by in-gel digestion and nano-liquid chromatography coupled to tandem mass spectrometry (QExactive). The MaxQuant tool was used for protein identification and quantification by spectral counting.

RESULTS: Our CRC proteome analysis identified a total of 6880 proteins and 874 with altered abundance (p< 0.05) upon treatments. Our results indicate that SFU induces upregulation of proteins associated with the extracellular matrix, membrane vesicles, stress and immune responses and downregulates mitochondrial proteins, peroxisomes and (most profoundly) ribosomal proteins. Patients who received SFU-LV also displayed upregulation of extracellular matrix and vesicle/granule proteins in addition to cell adhesion proteins, an extensive downregulation of ribosomal proteins and downregulation of mitochondrial and ubiquitin proteins. In addition, there is an upregulation of the GO-term 'Extracellular region part' in both SFU and SFU/LV treated patients, compared to controls. Top upregulated proteins were isoform NELF-D of negative elongation factor C (NELFCD), AMP deaminase 3 (AMPD) and myeloblastin (PRTN3), while top downregulated were neudesin (NENF), antigen KI-67 (MKI67) and HERC4.
CONCLUSIONS: Proteome analysis revealed strong changes in the CRC proteome upon 48 hrs of 5FU +/- LV treatment, with regulated proteins involved in stress and immune responses, vesicular transport, protein synthesis and metabolism. A number of proteins were not yet associated to 5FU actions. These proteins when correlated in a larger cohort to clinical outcome may function as biomarkers for future treatment decision.

POSTER SESSION 4:
1) INBORN ERRORS OF PURINE AND PYRIMIDINE METABOLISM
2) ADVANCES IN GOUT AND HYPERURICEMIA

INBORN ERRORS OF PURINE AND PYRIMIDINE METABOLISM

POSTER # 36
SEVERE MEGALOBLASTIC ANEMIA IN A PATIENT WITH LESCH NYHAN DISEASE.

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Moderate megaloblastic anaemia has been described in some patients with Lesch Nyhan disease (LND). Ineffective erythropoiesis has been postulated as the cause of anaemia and increased folic acid consumption, due to enhanced de novo purine synthesis may explain megaloblastic features.

We report a LND patient with an uncommonly severe megaloblastic anemia resistant to available symptomatic therapies. Patient APP was first examined at La Paz University Hospital at age 18 months due to psychomotor delay, hyperuricemia, crystalluria, and anaemia. He presented undetectable HGprt activity in the haemolysate with concomitant increased APRT activity (68 nmol/h/mg Hgb). Molecular diagnosis showed a splicing point mutation (c. 533 -2 A>G, NM_000194.2) in genomic DNA which originates exon 8 exclusion in the patient HPRT1 cDNA. He presented severd generalized dystonia and developed self-injurious behaviour at age 9 years. At age 6 years symptomatic anaemia was documented with a red blood cell count (RBC) of 3.43 10^6/µl, a hemoglobin level of 12.1 g/dl and MCV of 110.8 fl. His peripheral blood smear revealed abundant macrocytes, microcytes and hypocromic RBC. Iron metabolism, folic acid and B12 vitamin levels were in the normal range. Despite acid folic and iron supplementation, RBC and hemoglobin levels declined with age. Progressive fatigue limited his daily activities. At age 15 years hemoglobin was of 8.5 g/dl and MCV 110.5 fl. Patient was very symptomatic and therapy with synthetic erythropoietin (EPO), epoetin alpha was instituted. Hemoglobin levels increased to less than 10 g/dl but patient symptomatology was alleviated. No adverse effects were observed. During EPO treatment he presented occasional leucopenia with a WBC count of 3.35 10^3/µl and abnormal RBCs including: poikilocytes, schistocytes, tear drop cells, elliptocytes, and spherocytes, and dimorphic platelets. Erythroblast levels were 1.0% and reticulocyte count 1.9 ± 0.2%. EPO discontinuation for a 6 month period was accompanied by severe anaemia (hemoglobin, 7.2 g/dl) requiring erythrocyte transfusion and re-instauuration of EPO. Nowadays, with EPO therapy, iron, and folic acid supplements, the patient remains oligosymptomatic. His last hemoglobin level was 10.7 g/dl with a MCV of 137.8 fl.

POSTER # 37
HYPOXANTHINE DEREGULATES GENES INVOLVED IN EARLY NEURONAL DEVELOPMENT. IMPLICATIONS IN LESCH-NYHAN DISEASE PATHOGENESIS.

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Neurological manifestations in Lesch-Nyhan disease (LND) are attributed to the effect of hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficiency on the nervous system development. HPRT deficiency causes the excretion of increased amounts of hypoxanthine into the extracellular medium. We hypothesized that HPRT deficiency related hypoxanthine excess may lead, directly or indirectly, to transcriptional aberrations in a variety of genes essential for the development and function of striatal progenitor cells.

We have examined the effect of hypoxanthine excess on the differentiation of neurons in the well-established human NTERA-2 cl.D1 (NT2/D1) embryonic carcinoma neurogenesis model. NT2/D1 cells differentiate along neuroectodermal lineages after exposure to retinoic acid (RA). Hypoxanthine effect on RA-differentiation was examined by the changes on the expression of various transcription factor genes essential to neuronal differentiation and by the changes in tyrosine hydroxylase (TH), dopamine, adenosine and serotonin receptors (DRD, ADORA, HTR).

We report that hypoxanthine excess deregulates WNT4, from Wnt/β-catenin pathway, and engrailed homeobox 1 gene and increased TH and dopamine DRD1, adenosine ADORA2A and serotonin HTR7 receptors, whose over expression characterize early neuro-developmental processes.
Megaloblastic anemia in a hypoxanthine-guanine phosphoribosyltransferase deficiency

Megaloblastic anemia in hypoxanthine-guanine phosphoribosyltransferase deficiency.

Lesch Nyhan disease (LND) is an inborn error of purine metabolism due to deficiency of hypoxanthine-guanine phosphoribosyltransferase (HGprt) activity. Increased uric acid production is common to all HGprt deficient patients and is not related to the severity of the enzyme defect. Patients with complete HGprt deficiency or LND show uric acid overproduction, neurological symptoms, including self-injurious behaviour, and haematological disturbances. Patients who do not show self-injurious behaviour are named LND variants (LNV). These patients are classified into two groups: HGprt-related neurological disease (HRND) and HGprt-related hyperuricemia (HRH), depending on the presence or absence of neurological manifestations, respectively. Megaloblastic anaemia has been described in some LND patients, but we do not know of previous reports on the haematological disturbances of LNV. We have reviewed the haematological disturbances in 31 HGprt deficient patients.

Methods: The following variables were examined: Complete blood count and peripheral smear, serum and erythrocyte folic acid, B12 vitamin levels, iron metabolism, c-reactive protein, thyroid-stimulating hormone, thyroxin, lactate dehydrogenase, total and direct bilirubin, and haptoglobin. Depending on the previous results and clinical data, anti parietal cell antibodies, EPO, reticulocytes, haemoglobin F, A2 and H and haemoglobin electrophoresis and Coombs test were determined in some patients.

Results: According to clinical, enzymatic and genetic tests 18 patients were classified as LND and 13 patients as LNV (8 LNV patients were classified as HRND and 5 as HRH). Complete blood count: LND patients showed a low mean haemoglobin levels (12.9 ± 1.1 g/dL). Haemoglobin levels from LND and HRND patients (13.5 ± 0.8 g/dL) were significantly lower as compared to HRH patients (15.1 ± 0.9 g/dL; p = 0.0002 and p = 0.0109, respectively). However there was no significant difference in haemoglobin levels between LND and HRND. Both LND and HRND patients showed increased mean MCV (97.7 ± 9.2 and 104.0 ± 11.3 fl, respectively). MCV from LND and HRND were significantly higher as compared to HRH patients (85.5 ± 5.2 fl, p= 0.0160 and p = 0.0017, respectively). However mean MCV was similar in LND and HRND. WBC, Platelet count, and platelet volume were similar and normal in LND, HRND and HRH patients. Peripheral blood smear: 100% LND patients presented erythrocyte morphologic abnormalities (78% macrocytosis, 39% anisocytosis, 50% hypochromia and 12.5 % microcytosis). Erythrocyte abnormalities were detected in 5 out of 8 (62.5%) HRND patients. In all HRH patient peripheral blood smear was normal. Folic acid, B12 vitamin, and iron metabolism: Folic acid and B12 vitamin levels were normal in HGprt deficient patients. However, iron deficiency was documented in some HGprt deficient patients, namely in LND patients as the disease evolved. Both iron and ferritin levels were significantly lower in LND than in LNV as a group (p= 0.0216 and p = 0.0021, respectively). Other parameters: All other parameters determined were in the normal range with no statistically significant differences between groups.

Conclusions: Red blood cell abnormalities are common in the most severe phenotypes of HGprt deficiency (LND and HRND).

POSTER # 39

Urinary excretion of uric acid, allantoin and 8-OH-deoxy guanosine in uricase-knockout mice

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OBJECTIVES: The end product of purine metabolism in humans is uric acid (UA) because of the lack of uricase. But in other mammal, UA is oxidized to allantoin by the action of uricase. Uricase-deficient mice are reported to develop uric acid nephropathy, with high mortality rates before weaning. When Uricase-knockout (Uox KO) mice grow up without severe nephropathy, it will be useful as a human model in research of purine metabolism. In this study, we determined urinary excretion of creatinine (Cr), UA and allantoin in Uox KO mice. In human, allantoin is reported to be generated non-enzymatically from UA with an antioxidant reaction of UA. Thus, an oxidative stress marker, 8-hydroxy-2′-deoxyguanosine (8-OHdG) was also determined in urine of Uox KO mice.

MATERIALS AND METHODS: Uricase-knockout mice (C57BL/6J:129S7-Uox tm/bay/J) were purchased from The Jackson Laboratory and were brought up in animal laboratory of Teikyo University. Wild-type C57BL/6j mice were used as control. 4 males and 4 females around 20 weeks age were used in this study. Urine was collected for 24 hours in metabolic cage with or without feed CRF-1, and was stored at -30°C until LC-MS analysis. Urine was first eluted on the Monospin SAX to remove impurities. UA and allantoin were determined with liquid chromatography - mass spectrometry (LC-MS). LC-MS was performed with UPLC-micromass ZQ 4000, HSS T3 column (150 mm×2.1 mm i.d.), and 10 mM formic acid /10 mM formic acid ammonium. UA was detected with m/z 169 and allantoin was with m/z 159. Cr and 8-OHdG were measured with a commercial kit.
RESULTS AND DISCUSSION: Urinary excretion of Cr in Uox KO mice was not different from that in wild-type mice in spite of the lack of uricase (17.0 ± 3.1 in Uox KO and 21.8 ± 6.2 µg/g/day in wild-type). But in Uox KO mice, excretion of Cr with food was significantly higher than that without food. Urinary excretion of UA in Uox KO mice was significantly higher than that in wild-type mice. On the other hand, urinary excretion of allantoin was significantly lower than that in wild-type mice. It is interesting that allantoin was detected in urine of Uox KO mice in spite of lack of uricase. Allantoin in Uox KO mice may be generated non-enzymatically from UA. Food intake affected on the urinary excretion. In Uox KO mice with food, the daily excretion of both UA and allantoin increased.

Urinary 8-OHdG in Uox KO mice was lower than that in wild-type mice. Low excretion of 8-OHdG means the decrease of oxidative stress. It is possible that high level of UA in Uox KO mice acted as an antioxidant agent. In female Uox KO mice with food, the daily excretion of 8-OHdG increased significantly compared without food.

CONCLUSIONS: Daily excretion of UA in Uox KO mice was higher than that in wild-type mice, and conversely 8-OHdG was lower. These results are considered to show that UA acts as an anti-oxidizing agent. Urinary allantoin was detected in Uox KO mice. In order to confirm the origin of allantoin, further examination with the obvious oxidative stress seems to be necessary.

POSTER # 40
Identification of a family with heterozygous pathogenic p.T467M variant for renal hypouricemia type 1

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Renal hypouricemia (RHUC) is a heterogeneous inherited disorder characterized by impaired tubular uric acid (UA) transport, reabsorption insufficiency and/or acceleration of secretion with severe complications, such as acute kidney injury, renal failure and nephrolithiasis. Low UA concentrations in serum and its increased fractional excretion can be used as preliminary biochemical diagnostic markers. So far, more than 100 patients with a loss-of-function mutation in the SLC22A12 gene have been found with most of the described patients being from Asia region (OMIM #220150, RHUC1). More than ten patients with RHUC caused by defects in the SLC2A9 gene (OMIM #612076, RHUC2) have been described in a variety of ethnic groups.

The proband, 9-year-old boy, presented with hematuria and lower abdominal pain. On ultra sound scan of the abdomen a non-obstructive right renal calculus was present. The serum uric acid level in the proband was 97 µmol/L and expressed as an increase in the fractional excretion of UA (33%). The sequencing analysis of SLC22A12 revealed published missense heterozygous transitions p.T467M. The sequencing analysis of SLC2A9 revealed two published variants: homozygous p.V253I and heterozygous p.P350L. Analysis of family relatives identified p.T467M variant in father of proband and two siblings: 41-year-old male (serum uric acid 172 µmol/L, fractional excretion of uric acid 13%), 14-year-old sister (81 µmol/L, fractional excretion of uric acid 15%) and 2-year-old sister (86 µmol/L, fractional excretion of uric acid 25%). The analysis of proband’s mother revealed only heterozygous variants p.V253I and p.P350L in SLC2A9 (179 µmol/L, fractional excretion of uric acid 9%).

Our results confirm an uneven geographical and ethnic distribution of RHUC. Furthermore, this is the first characterization of heterozygous allelic variants in a family with renal hypouricemia type 1.

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POSTER # 41
Extremely high frequency of SLC22A12 variants causing renal hypouricemia 1 in the Czech, Slovak and Spanish Roma population

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Renal hypouricemia is a rare heterogeneous inherited disorder typified by impaired tubular uric acid transport, reabsorption insufficiency, and/or acceleration of secretion with severe complications, e.g. acute kidney injury, renal failure, urolithiasis and nephrolithiasis. As a first step, the diagnosis of renal hypouricemia is determined by hypouricemia with elevated fractional excretion of uric acid. Secondly, RHUC is confirmed by molecular genetic analysis of SLC22A12 and SLC2A9 genes. Finally, the unpublished allelic variants in URAT1 and GLUT9 proteins are usually confirmed as pathogenic RHUC variants through in vitro functional studies. In the present study, we assessed the frequency of previously reported RHUC causal variants in the Roma population.
A retrospective cohort of 945 randomly chosen ethnic Roma (irrespective of their state of health) from two regions in Eastern Slovakia (Prešov and Košice region), two regions in the Czech Republic (Hradec Králové and Central Bohemia region) and region in Spain participated. Genomic DNA was isolated from buccal swabs using the ReliaPrep gDNA Tissue Miniprep System (Promega) and/or from blood samples (Qiagen columns). Exon 7 and 9 of the SLC22A12 gene were amplified using PCR and sequenced directly and/or the c.1245_1253del (p.L415-G417del) and c.1400C>T (p.T467M) genotypes were determined using polymerase chain reaction with allele-specific primers in a multiplex arrangement. Two sequence variants were found: in the cohort of 941 subjects, 25 participants were heterozygous and three were homozygous for deletion c.1245_1253del (in 60 subjects the analysis was failed); 118 participants were heterozygous and two were homozygous for the c.1400C>T substitution. Moreover, two participants were compound heterozygotes. Frequencies of the c.1245_1253del and c.1400C>T variants were 1.87% and 6.59%, respectively. Genotypes were in HWE at the c.1400C>T locus (P = 0.269), but, due to the large number of homozygotes, grossly deviated at the c.1245_1253del locus (P < 0.001). Our finding confirms an uneven geographical and ethnic distribution of SLC22A12 mutant variants that need to be considered in non-Asian patients. We found that the c.1245_1253del and c.1400C>T variants were present in the Czech, Slovak and Spanish Roma population at unexpectedly high frequencies. These variants in the URAT1 transporter may explain the incidence of AKI and nephrolithiasis among the Roma and should be kept in mind during differential diagnostic procedures on patients with low serum uric acid concentrations.

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**POSTER #42**

**Diagnosis of adenylosuccinate lyase deficiency by measuring of succinylpurines in neonatal dried blood spots**

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The current trends for population and selective screening of metabolic disorders are to use methods based on the profiling of a wide spectrum of metabolites in dried blood spots (DBS) using LC-MS/MS. These methods replace classical screening techniques and offer an alternative diagnostic test for many metabolic disorders. One such disorder is adenylosuccinate (ADSL) deficiency, an inherited disorder of purine metabolism. Affected individuals present a variety of non-specific neurological symptoms. The correct diagnosis of ADSL deficiency prevents further costly clinical examination and is essential for predicting the risks associated with a subsequent pregnancy and for the possibility of prenatal diagnosis.

We present methods for the preparation of the isotopically labelled dephophorylated ADSL substrates - succinylaminomidazole carboxamide riboside-13C4 (SAICAr-13C4) and succinyladenosine-13C4 (SAdo-13C4) and demonstrate their use in quantitative LC-MS/MS analysis of accumulated SAdo and SAICAr in neonatal DBS. The concentrations of SAICAr and SAdo were higher in the archived DBS from patients compared to those of the control subjects, even after DBS from patients were stored for 2-23 years.

We developed and validated a method of succinylpurines' analysis in DBS that improves selective screening for ADSL deficiency in the paediatric population and may be used for retrospective diagnosis to aid the genetic counselling of affected families. The isotopically labelled and unlabelled dephosphorylated ADSL substrates are available upon request to other laboratories in the world.

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**POSTER #43**

**Self-injurious behavior following dental extraction in Lesch-Nyhan disease**

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Background: Self-injurious behavior (SIB) is the most disturbing manifestation of Lesch-Nyhan disease (LND) and is hardly treatable. Total teeth extraction has been reported in extreme cases with immediate relief. However, we do not know the long term consequences of total dental extraction. We report two LND patients who developed new forms of SIB following total dental extraction.

Patients and Methods: MM was diagnosed at age 20 months as having complete HPRT deficiency (c. 384 +1 G>A, exon 4 exclusion, NM_000194.2). At age 6 years he began to bite his lips and hands. At age 11 years he bitted his tongue with tissue loss. A mouth guard was provided but proved to be ineffective. Finally, at age 13 years total teeth extraction was performed under total anesthesia.

MAGR was given a diagnosis of LND at age 10 months due to the absence of HPRT activity with a deletion of the HPRT gene including exon 1 and the promoter region. At age 13 months he started to bite his hands. At age two years and due to lip biting an oral prosthesis was provided. His lips cheeks and hands were continuously bitten. At age 4 years the first tooth extraction
was performed and this was continued as dentition evolved because self-biting was unavoidable. At age 8 years his last four teeth were removed. Dystonia was assessed according the Fahn-Marsden scale and SIB according to the behavioral problem questionnaire BPI. Quality of life was assessed on a 0 to 100 point visual scale.

Results: Both patient mothers reported that overall and after 12 and 17 month following total dental extraction, they were very satisfied with the radical procedure. Frequency and severity of BPI were markedly reduced in both patients. Their quality of life markedly improved by 50 absolute points in MM and by 80 points in MAGR, on the 100 point scale. However, 2-3 weeks (MM) and 7 months (MAGR) after total dental extraction both patients started to seek other means of self-aggression. MM started rubbing one foot against the other and scratching toenails with his hands. MAGR stuck his legs and feet against hard objects. These forms of self-injury behavior could be easily prevented with gloves, socks and protective materials, according to the mothers.

Conclusion: Following total dental extraction, we have observed that two LND patients do not bite but develop new forms of SIB which are milder, can be easily prevented, and both patients and families appears relieved with a marked improvement in their quality of life.

POSTER #44

HEREDITARY XANTHINURIA IS NOT SO RARE DISORDER

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Hereditary xanthinuria is caused by inherited deficiency of the the xanthine dehydrogenase/ oxidase (XDH/XO) - type I, and is characterized by very low concentration of uric acid in blood and urine and high concentration of xanthine in urine, leading to urolithiasis. Type II results from a combined deficiency of XDH/XO and aldehyde oxidase. Patients present with hematuria, renal colic, urolithiasis or even acute renal failure. A third type, clinically distinct, molybdenum cofactor deficiency is characterized by the lack of sulfite oxidase activity as well as XDH/XO and aldehyde oxidase. The exact prevalence is not known, but about 150 cases have been described so far. An annual incidence has been estimated between 1:5,000 and 1:69,000. This wide variability is due to the fact that half of the affected individuals remains asymptomatic and therefore this condition is underdiagnosed. Moreover, hypouricemia is sometimes overlooked, that’s why we have set up the diagnostic flowchart for this genetic defect of purine metabolism.

This consists of a) evaluation of uric acid concentrations in serum and urine with exclusion of primary renal hypouricemia as we reported previously, b) estimation of urinary xanthine, c) allopurinol loading test, which enables to distinguish type I and II; and finally in some cases xanthine oxidase activity assay in plasma with molecular genetic analysis. Following this diagnostic procedure we were able to find first patients with hereditary xanthinuria in our Czech population. We have detected nine cases, which is one of the largest group worldwide, in the terms of number of patients. Four patients were asymptomatic. All individuals had profound hypouricemia, which was the first sign and led to referral of these cases to our department. Urinary concentrations of xanthine, were in the range of 170-598 mmol/mol creatinine (normal < 30 mmol/mol creatinine). Our experience shows that patients with urinary xanthine concentrations in excess of 320 mmol/mol creatinine developed renal calculi. The xanthine oxidase activities in plasma in two cases were 0 and 0.37 pmol/h/mL of plasma (ref.range:3.2-9.2 pmol/h/mL of plasma). The nonsense heterozygous mutation p.R825X was found in two patients. Hereditary xanthinuria is still unrecognized disorder and probably not so rare as previously thought. Patients with unexplained hypouricemia need detailed purine metabolic investigation.

POSTER #45

Urat1-Uox double knockout mice are experimental animal model of renal hypouricemia and exercise-induced acute kidney injury

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Renal Hypouricemia (RHUC) is a hereditary disease characterized as low level of plasma urate (UA) and normal urinary excretion of UA. RHUC type 1 and type 2 are caused by mutations of urate transporter URAT1 gene (SLC22A12) and GLUT9 gene (SLC2A9), respectively. However, the plasma UA levels of URAT1 knockout mice were no difference from those of wild type mice, because the difference was canceled by the degradation of urate by hepatic uricase, which was lost in human evolutionally. In this study, URAT1 gene and uricase gene (Uox) double knockout mice (DKO) were studied as an experimental animal model of RHUC type 1 and its complication of excise-induced acute kidney injury (EIAKI).

Uox knockout (Uox-KO) mice are bred with allopurinol until 10-week old and maintained without allopurinol after that. DKO mice are established by mating URAT1 knockout mice and Uox-KO mice and maintained with 27mg allopurinol / 100g feed. One week feeding with variable content of allopurinol was followed by HPLC measurement of UA and creatinine (Cr) concentrations of spot urine and blood from tail. Oxpurinol concentration was also measured from spot urine by HPLC.
The urinary excretion of UA in DKO mice was increased from the urinary excretion of allantoin in wild type mice equivalently, and was about 30 times higher than those of humans. Without allopurinol, plasma Cr and UA levels of DKO mice were higher than those of Uox-KO mice. With allopurinol, plasma Cr levels in DKO mice were normal, however, plasma UA levels were lower than those of Uox-KO mice. There were no difference in the urinary UA excretions between DKO and Uox-KO mice, although allopurinol reduced them dose-dependently. Urinary excretion of oxyprinol was also no difference between DKO and Uox-KO mice.

Thus, hypouricemia and normal urinary UA excretion may indicate that the DKO with allopurinol should be an animal model of RHUC type 1. DKO mice without allopurinol demonstrated acute kidney injury which seems to be caused by over-excretion of urinary UA. Because over-excretion of urinary UA is caused by exercise, DKO mice without allopurinol is likely to be an animal model of EIAKI. Moreover, allopurinol is suggested to be a prophylactic of EIAKI.

**POSTER # 46**

Clinical, biochemical and molecular analysis of 30 children with β-ureidopropionase deficiency demonstrates high prevalence of the c.977G>A (p.R326Q) mutation

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β-Ureidopropionase (βUP) deficiency is an autosomal recessive disease characterized by N-carbamyl-β-amino aciduria. Until recently, only 16 genetically confirmed patients with βUP deficiency have been reported. To elucidate the disease state and genetical feature, we studied on the clinical, biochemical and molecular aspects of 30 βUP deficient children. Pyrimidine analysis by GC/MS or HPLC-MS/MS in urine samples and DNA analysis of UPB1 were performed. Wild-type and mutant βUP protein were expressed in HEK293 cells and used for enzyme activity assay, western blot analysis and native gel electrophoresis.

Highly variable phenotypes ranging from neurological involvement to asymptomatic were observed in diagnosed patients. Three novel missense mutations (p.G31S, p.E271K, and p.I286T) and a recently described mutation (p.R326Q) were identified. The p.R326Q mutation was detected in all 30 patients with 24 patients homozygous for this mutation. The mutant βUP enzymes expressed in HEK293 cells showed that the p.E271K and p.R326Q mutations cause profound decreases in activity. Conversely, the p.G31S and p.I286T mutations possess residual activities of 50 and 70%, respectively. Analysis of a human βUP homology model revealed that the effects of the mutations (p.G31S, p.E271K, and p.R326Q) on enzyme activity are most likely linked to improper oligomer assembly.

In this study, no clear correlation between phenotype and genotype were observed. Identification of the R326Q mutation in both neurologically affected patients and unaffected individuals suggests that additional factors are involved in the clinical outcome. High frequency of the R326Q mutation in our patients indicates that βUP deficiency is not as rare as generally considered.

**ADVANCES IN GOUT AND HYPERURICEMIA**

**POSTER # 47**

GWAS of clinically-ascertained gout identifies multiple risk loci associated with metabolic pathways

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Objectives: Gout is a common disease resulting from hyperuricemia. Recently, genome-wide association studies (GWASs) of gout have been reported; however, they included self-reported gout cases in which clinical information was not sufficient. Therefore, the relationship between genetic variation and clinical subtypes of gout remains to be clarified. Thus, we first performed gout GWAS using clinically-defined cases only in a Japanese population.
Material and method: GWAS was conducted with 945 male patients with clinically-defined gout cases and 1,213 male controls. Additionally, replication study of 1,048 clinically-defined cases and 1,334 controls was performed.

Results: Five gout susceptibility loci were identified at the genome-wide significance level \((p < 5.0 \times 10^{-8})\), which contained well-known urate transporter genes \((ABCG2\) and \(SLC2A9\)) and additional genes reported to have relationships with metabolic pathways: \(rs1260326 (p = 1.9 \times 10^{-12}; OR = 1.36)\) of \(GCKR\) (a gene for glucose and lipid metabolism), \(rs2188380 (p = 6.4 \times 10^{-9}; OR = 1.66)\) of \(CNIH-2\) (a gene for regulation of glutamate signaling). The latter two are identified as novel loci for gout. Furthermore, among the identified SNPs, we demonstrated that the SNPs of \(ABCG2\) and \(SLC2A9\) were differentially associated with subtypes of gout and clinical parameters underlying specific subtypes (renal underexcretion type and renal overload type). The effect of the risk allele of each SNP on clinical parameters showed significant linear relationships with the ratio of the case-control ORs for two distinct types of gout \((r = 0.96 [p = 4.8 \times 10^{-4}]\) for urate clearance and \(r = 0.96 [p = 5.0 \times 10^{-4}]\) for urinary urate excretion).

Discussion: Our findings provide clues to better understand the pathogenesis of gout and will be useful for development of companion diagnostics.

**POSTER # 48**

Comparison for the effects on hiperuricemia progression between genetic and environmental factors

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Objectives: Gout and hyperuricemia is a common multifactorial disease and known to have typical environmental risk factors including overweight/obesity, heavy drinking, and aging. Recently, common dysfunctional variants of urate exporter gene \(ABCG2/BCRP\) are revealed to be a major cause of gout and hyperuricemia.

Material and method: In a cohort of 5,005 Japanese participants, we compared the influence of \(ABCG2\) dysfunction and other typical risk factors on hyperuricemia using population-attributable risk percent (PAR%), as well as on serum uric acid (SUA) levels through linear regression analyses.

Results: \(ABCG2\) dysfunction was detected in 53.3% of the population investigated. The PAR% for hyperuricemia was 29.2%, which was much higher than those for the other typical environmental risks, i.e. overweight/obesity (BMI ≥ 25.0; PAR = 18.7%), heavy drinking (> 196 g/week (male) or > 98 g/week (female) of pure alcohol; PAR% = 15.4%), and aging (≥ 60 years old; PAR% = 5.74%), while PAR% of sex (male) was 91.7%. SUA significantly increased as the predicted function of \(ABCG2\) decreased (\(P = 5.99 \times 10^{-19}\)). A regression analysis revealed that \(ABCG2\) dysfunction had a stronger effect than other factors; a 25% decrease in the predicted \(ABCG2\) function was equivalent to “an increase of BMI by 1.97-point” or “552.1 g/week alcohol intake as pure ethanol” in terms of ability to increase SUA.

Discussion: Common dysfunctional variants of \(ABCG2\) have a much stronger impact on the progression of hyperuricemia than other well-known risks. Our study provides a better understanding of common genetic and environmental factors for common diseases.

**POSTER # 49**

Serum uric acid has a significant impact on renal outcomes using propensity score method

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BACKGROUND: Hyperuricemia has been advocated as a possible risk factor for CKD progression. However, evidence still remains scarce and several randomized clinical trials are under way. By using propensity score (PS) method, the present study aimed to examine the impact of serum uric acid (UA) on renal outcomes using retrospective CKD cohort in our institution.
METHODS: 770 CKD patients at the stage of 3 to 4 were enrolled from January 2008 to January 2014 (61.9 ± 13.1 years: M : F = 481 : 289), excluding nephrotic syndrome, malignancy, acute kidney injury, obstructive nephropathy and so on. Follow-up serum UA was calculated as time-averaged value by a trapezoidal rule. Binary multivariate logistic regression was done by separating at the median of the time averaged UA (TA-UA) values together with other baseline covariates such as sex, age, BMI, blood pressure, diabetic nephropathy, eGFR, proteinuria, hematuria, UA, etc. For PS matching, a caliper size was set at 0.25 x SD of PS. Renal outcomes were defined as dialysis inception and decline of eGFR.

RESULTS: After matching, the number of participants and the median of TA-UA turned out to be n = 338 and 6.65 mg/dL for dialysis inception, n = 358 and 6.62 mg/dL for 50% decline of eGFR and n = 350 and 6.54 mg/dL for 30% decline of eGFR. None of the 20 confounders showed the significant difference between two subgroups. Kaplan-Meier analyses revealed that the higher level of TA-UA resulted in worse renal outcomes. Moreover, Cox regression analyses confirmed the independent effects of TA-UA on the renal survival with respect to ESRD in the full cohort.

CONCLUSION: PS matching method can mimic the prospective randomized clinical trial using the retrospective observation cohort. Our results show that hyperuricemia per se has a significant impact on the renal outcomes.

POSTER # 50

Ultrasound-based classification and treatment of asymptomatic hyperuricemia and gout

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Sensitive imaging techniques, such as ultrasound and dual-energy computed tomography have demonstrated that a large percentage of patients with asymptomatic hyperuricemia (AH) or gout show monosodium urate deposits (tophi) and that these deposits elicit inflammation. Accordingly, a new clinical staging system for AH and gout has been proposed.

- Patients with increased serum urate concentrations but without evidence of urate deposits (patients with heart failure, COPD, metabolic syndrome ...).
- Patients with increased serum urate concentrations and evidence of urate deposits (same patients as in A in whom image techniques demonstrate urate deposits).
- Patients with previous gout flares but no evidence of urate deposits (patients with gout).
- Patients with tophaceous gout (same patients as in C in whom image techniques demonstrate urate deposits).

In 2008 we published for the first time that about third of the patients with AH do have ultrasound findings of urate deposits ("microtophi") and about a fourth of these patients show Doppler signal (inflammation). Thereafter three additional studies have show similar results (urate deposits in AH, 34-42%). These observations prompt two important questions: (1) If a given patient with AH has tophi, should the subject be diagnosed as having AH (type A) or should the subject be diagnosed as having asymptomatic gout (type B)?, and (2) Should this patients with asymptomatic tophi and Doppler signal be treated with hypouricemic agents? If both questions are answered in an affirmative way, we may well envision an important change concerning AH.

Close to 50% of the patients with gout and no clinical tophi do have microtophi when examined with image techniques. Three apparent beneficial consequences could be envisioned if an ultrasound study is performed in every gout patient. First, patient classification into C and D would be more precise. Second, hypouricemic treatment in patients with tophi would be more intensive (<5.0 mg/dL) to achieve resolution. And third, prophylactic therapy to prevent acute gout flares, in addition to hypouricemic treatment, would be guided by ultrasound findings. All together, we firmly believe that sonography will markedly change nowadays clinical classification of asymptomatic hyperuricemia and gout with an impressive impact on therapy.