Medizinische Klinik und Poliklinik IV
Ludwig-Maximilians-Universität
Department of Internal Medicine

Conference Program and Abstracts

ENS@T
14th Scientific Symposium

November 19th-20th, 2015

Carl Friedrich von Siemens Stiftung
Südliches Schlossrondell 23
D-80638 Munich

We gratefully acknowledge the support of this symposium received from:

Carl Friedrich von Siemens Stiftung

DFG Deutsche Forschungsgemeinschaft

Welcome

October 30, 2015

Dear Participants of the 14th ENSAT Scientific Symposium,

the last few years have witnessed significant progress in the understanding of genetics, pathophysiology and molecular mechanisms in adrenal tumorigenesis. Many of these achievements have been made possible through networking efforts including large registries and biobanks and utilization of dedicated technical platforms. Furthermore, these findings have been further refined by mechanistic *in vitro* experiments and studies in preclinical animal models.
The European Network for the Study of Adrenal Tumors has evolved into a pace-maker in international research for adrenal tumors and its yearly scientific meeting is a cornerstone for exchange of recent scientific data and planning of novel studies and projects.
We are therefore very grateful to host the 14th scientific ENSAT symposium at the Carl Friedrich von Siemens Stiftung in Munich. The meeting will be organized following its tradition into the four topics covered by the ENSAT network: adrenal cancer, pheochromocytoma / paraganglioma, and aldosterone and non-aldosterone producing adenomas. In addition to the oral sessions and poster walks four working group sessions will take place to discuss future studies and collaborative projects. Plenty of time for interaction and discussion is reserved during the meeting and the evening program.

We cordially invite you and would be glad if you participate in this prime scientific event, which will take place in Munich for the second time after 2006.

Sincerely,



Felix Beuschlein Constanze Hantel Martin Reincke

for the Local Organizing Committee

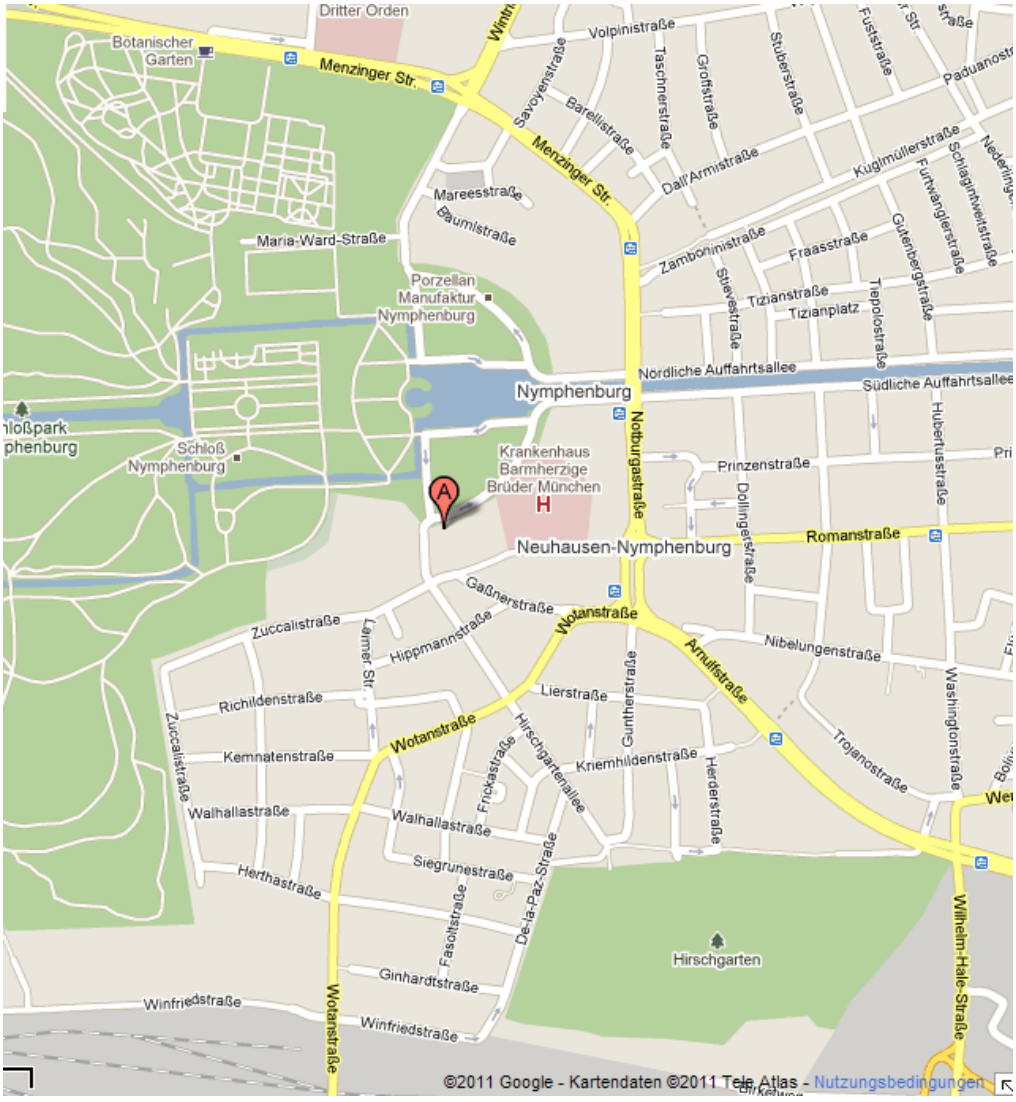
Content

Directions

Conference Venue

A: Carl Friedrich von Siemens Stiftung
Südliches Schlossrondell 23
80638 Munich
Tel: +49 (0)89 / 178 033 0

Free WLAN!



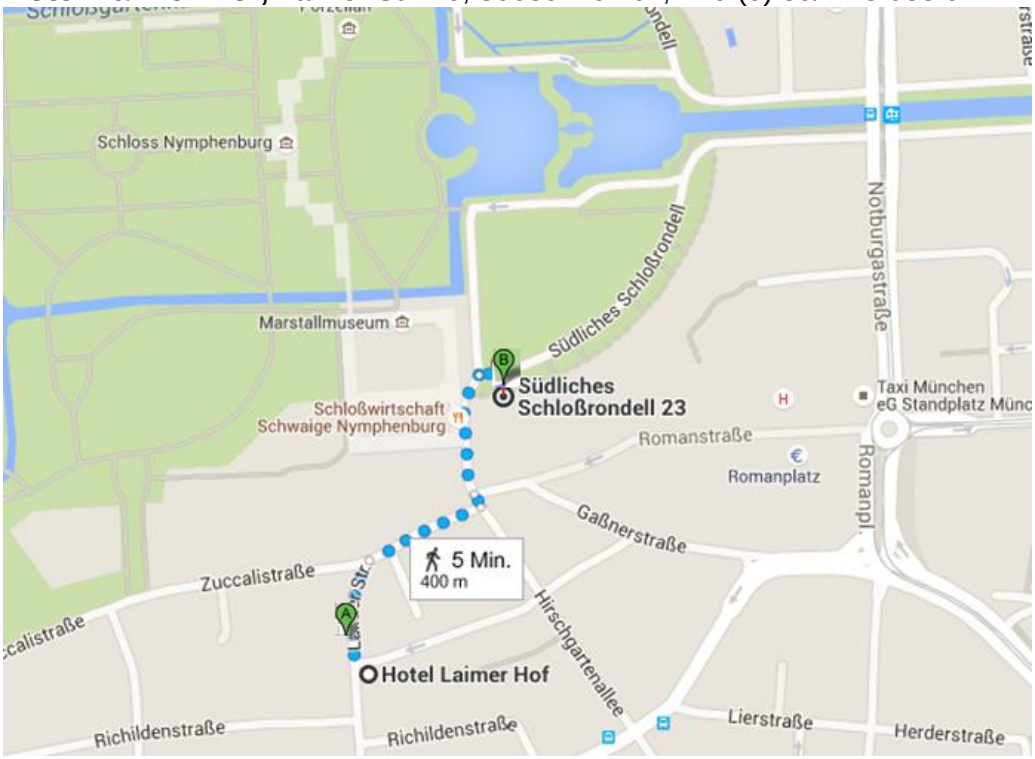
If you are lost, please call:

Christina Brugger: +49 (0) 176/ 6341 5602

Emergency call: 112
Police: 110

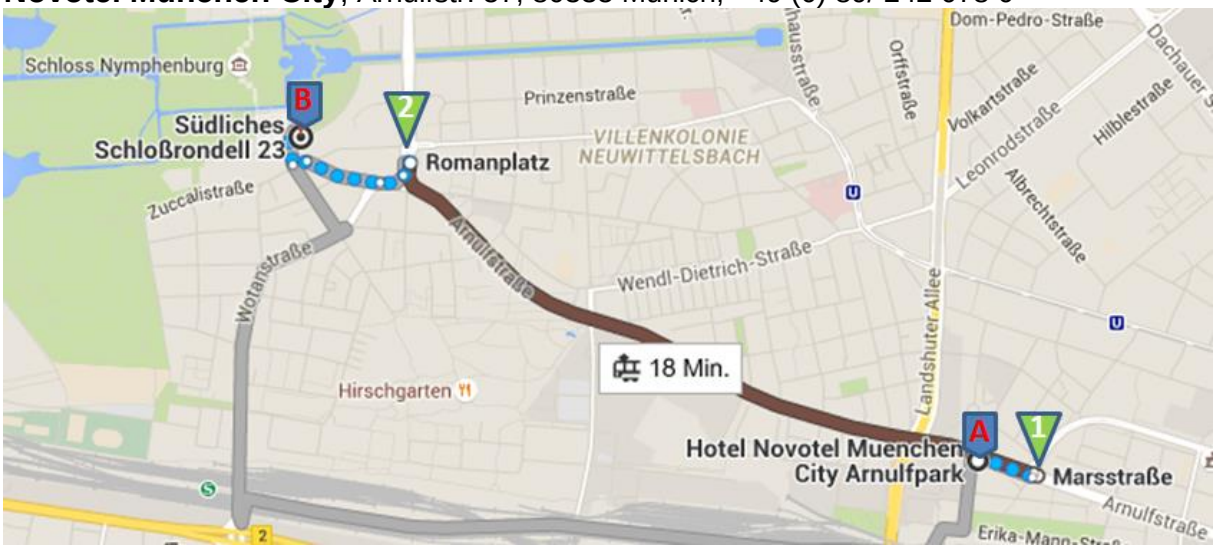
Hotels

Hotel Laimer Hof, Laimer Str. 40, 80639 Munich, +49 (0) 89/ 178 038 0



A: Hotel Laimer Hof
B: Symposium
Walking time: 5 minutes

Novotel München City, Arnulfstr. 57, 80335 Munich, +49 (0) 89/ 242 078 0

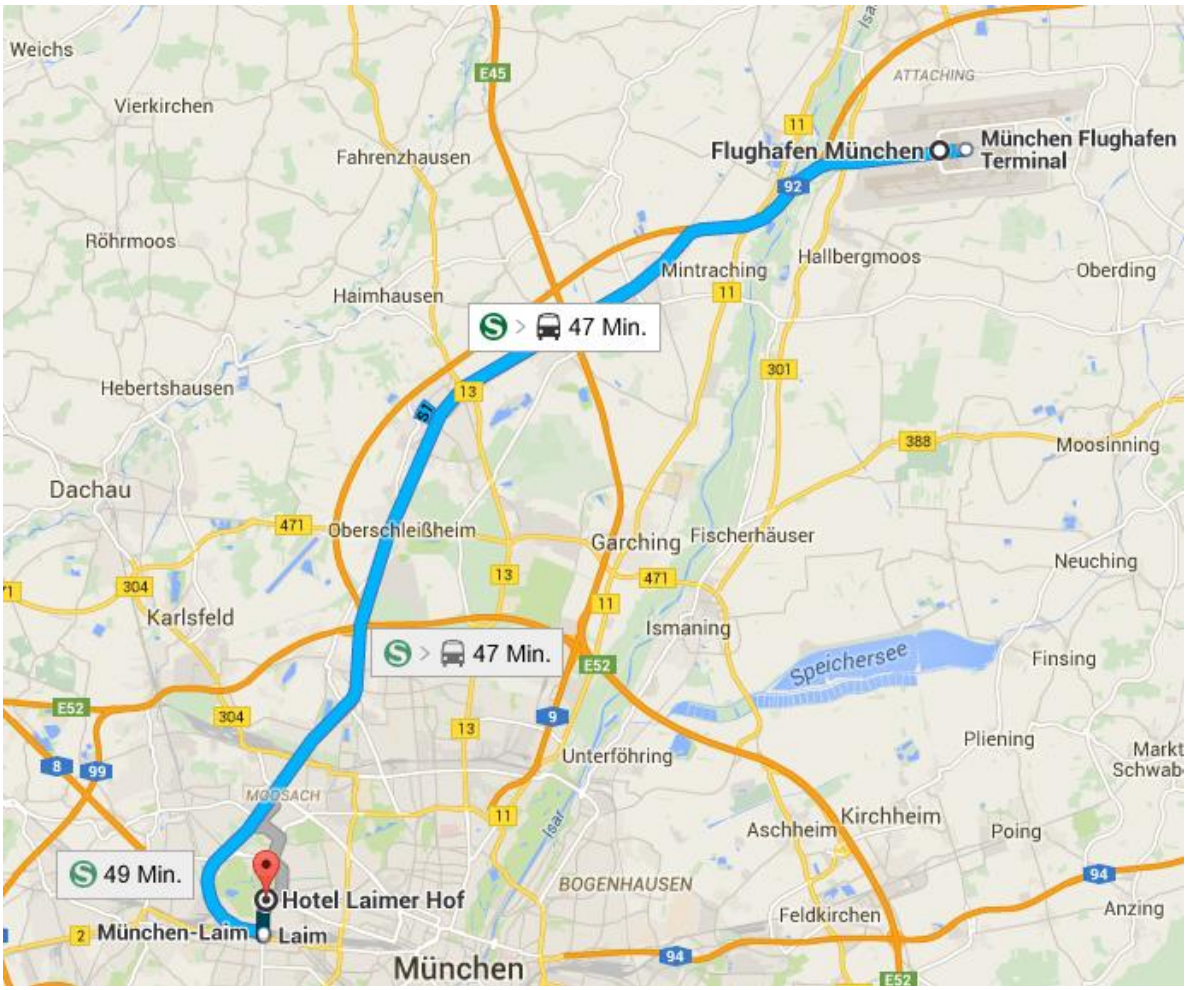


A: Novotel 1: Marsstraße
B: Symposium 2: Romanplatz

Take the tramway „Tram 16“, direction Romanplatz, from the station „Marsstraße“ (1) to the station “Romanplatz” (2); Travel Time: 18 minutes

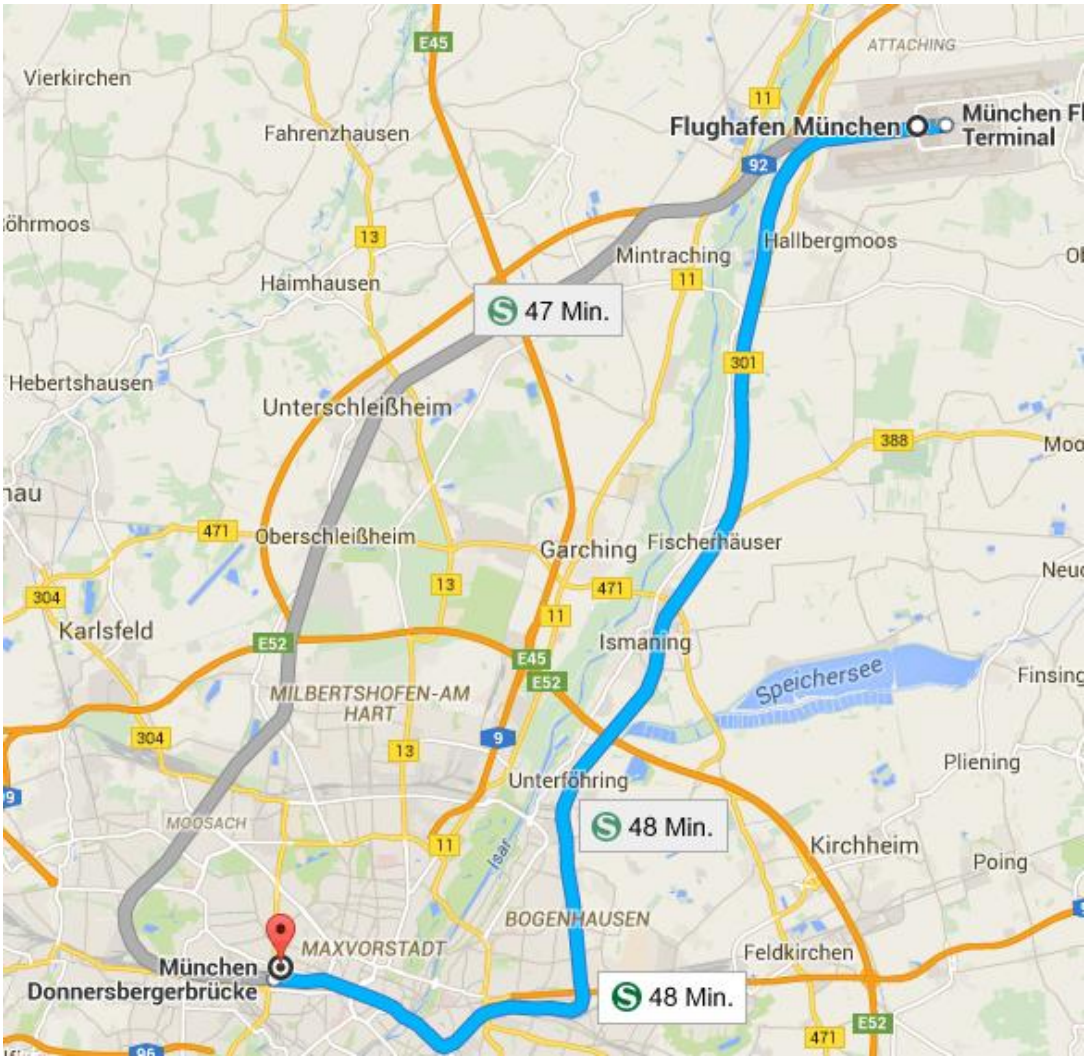
Direction from Airport to your Hotel “Laimer Hof”

Direction from Airport to **Hotel Laimer Hof**
S1 direction “Ostbahnhof” to change at “Laim” to **Bus 151** direction “Westfriedhof”
Get out at stop ”Kemnatenstraße” (duration 42 minutes)
5 minutes walk to Laimer Straße 40



Direction from Airport to your Hotel “Novotel”

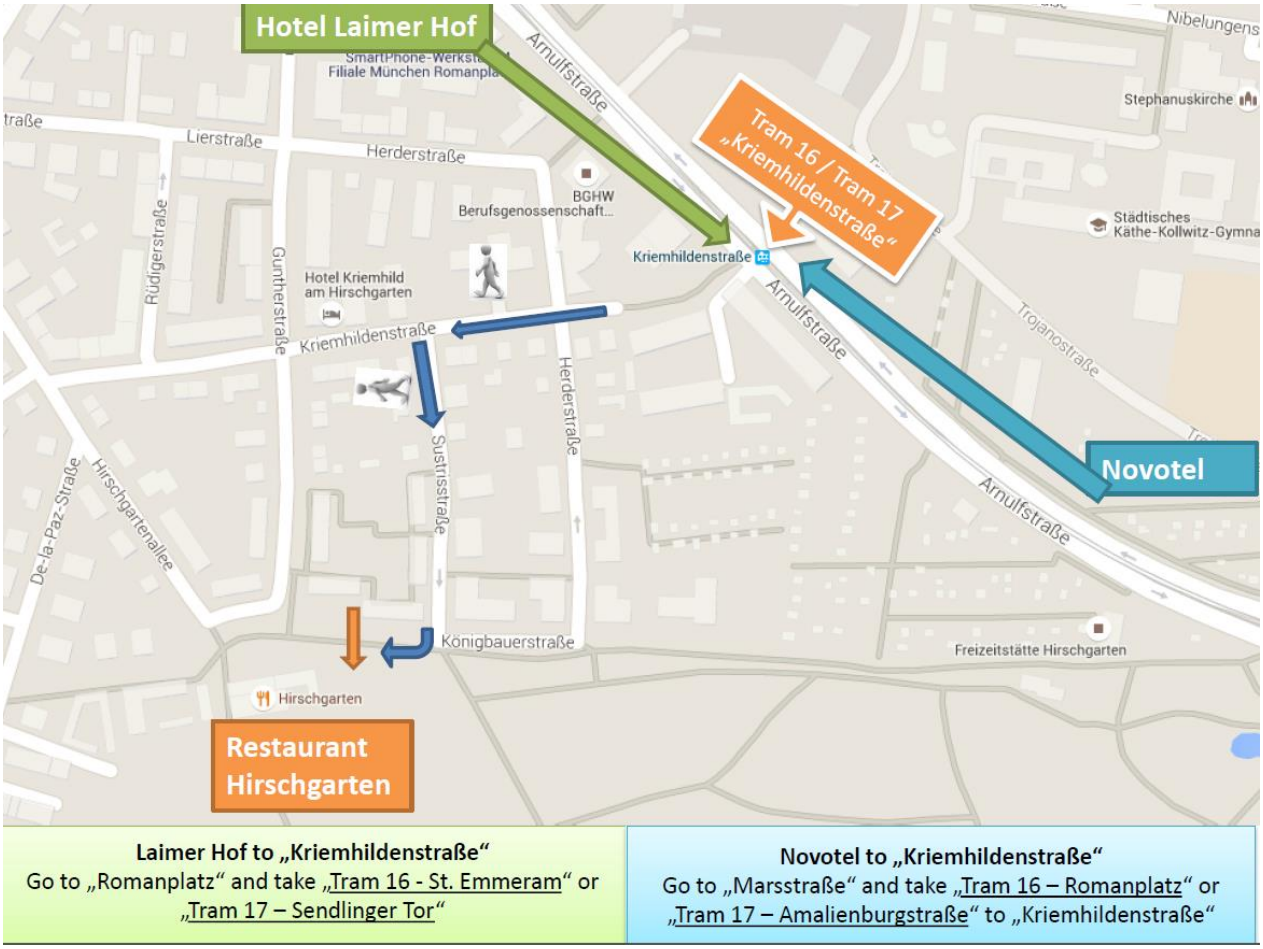
Direction from Airport to **Novotel München City**
S8 direction” Herrsching” **or**
S1 direction „Ostbahnhof“
Get out at stop “Donnersberger Brücke” (duration 44 Minutes)
4 minutes walk to Arnulfstraße 57

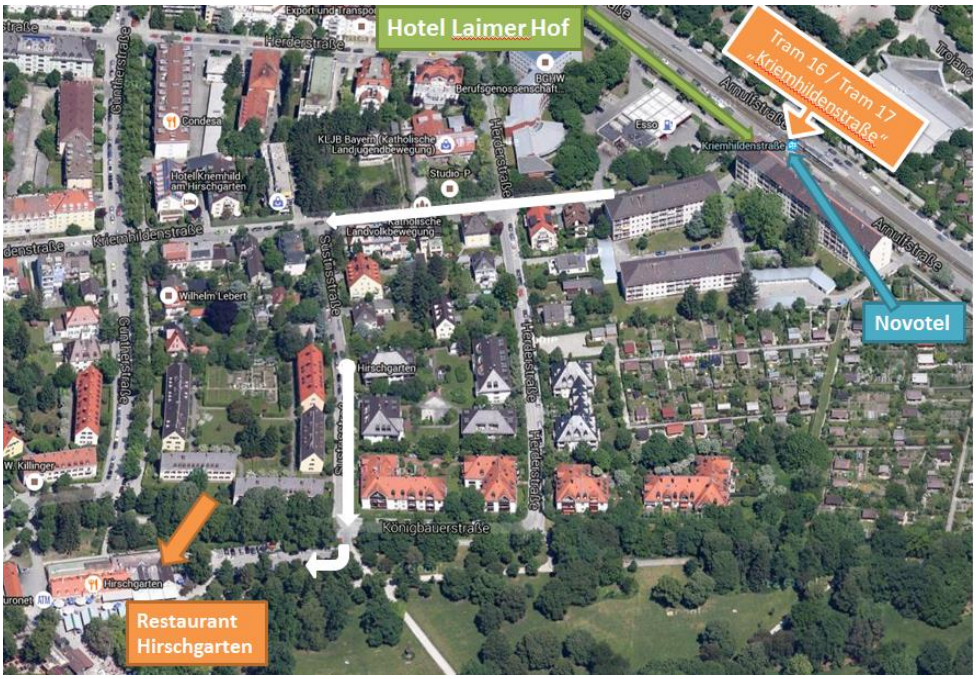


Dinner Locations:

Wednesday, 18th of November 2015

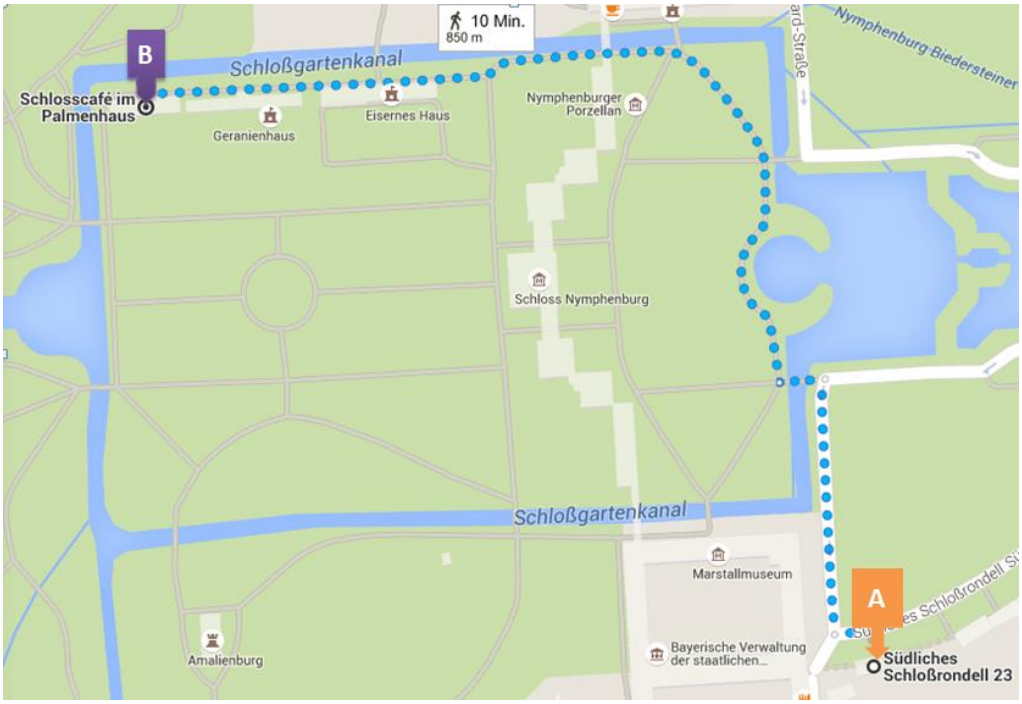
Restaurant Hirschgarten
Hirschgarten 1
80639 München
089 17999119





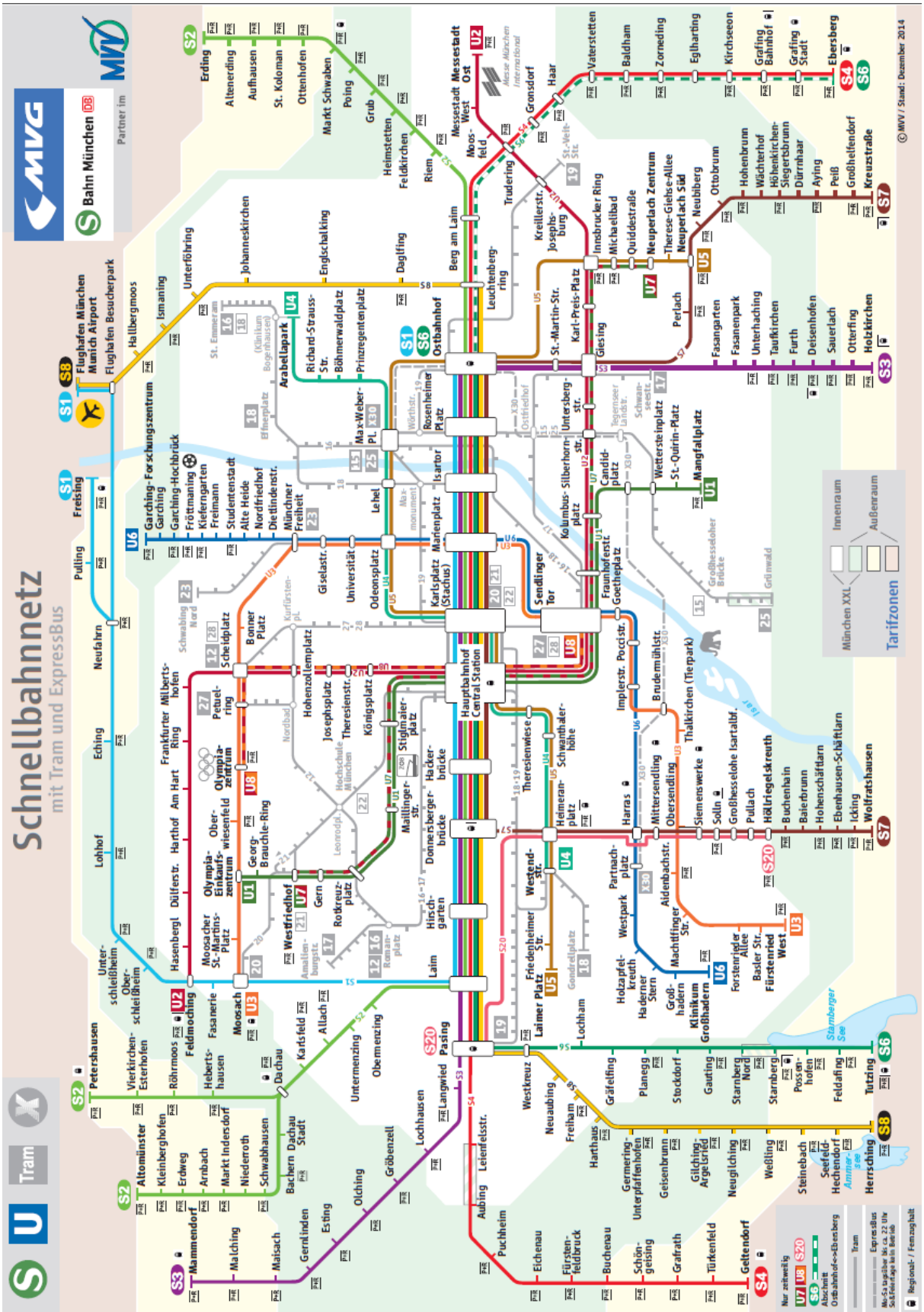
Thursday, 19th of November 2015

Restaurant Schlosscafé im Palmenhaus
Schloss Nymphenburg 43
80639 Munich
+49 (0)89/ 17 53 09



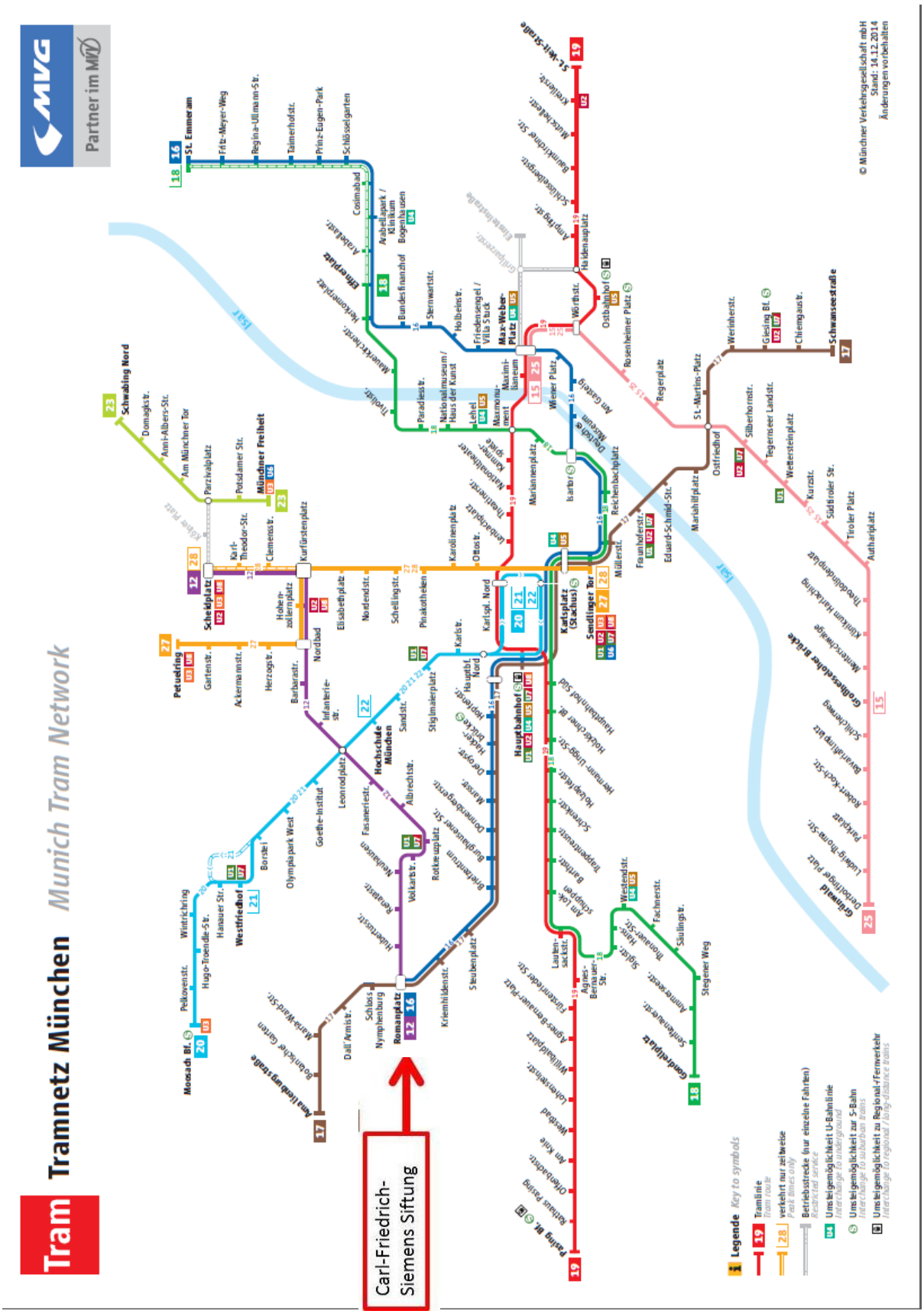
A: Symposium
B: Restaurant Palmenhaus

Urban rail network (U-Bahn (Underground) and S-Bahn Lines)



http://www.mvv-muenchen.de

Tram


Tram
Tramnetz München
Munich Tram Network
MVG
Partner im MVV
Carl-Friedrich-Siemens Siftung
Legende
Key to symbols
Tramlinie
Tram route
verkehrt nur zeitweise
Plätze stehen only
Betriebsstrecke (nur einzelne Fahrten)
Restricted service
Umsteigemöglichkeit U-Bahnlinie
Interchange to underground
Umsteigemöglichkeit zur S-Bahn
Interchange to suburban trains
Umsteigemöglichkeit zu Regional-/Fernverkehr
Interchange to regional / long-distance trains
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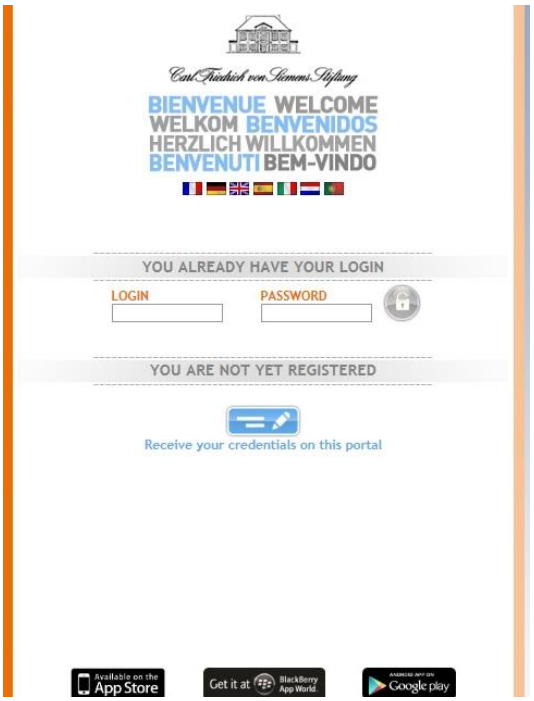
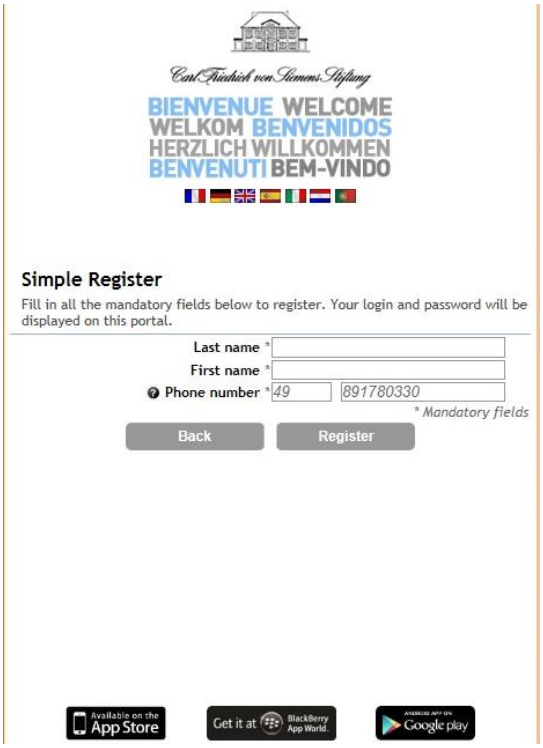
Stand: 14.12.2014
Änderungen vorbehalten

Registration for FREE WLAN at Carl-Friedrich von Siemens Stiftung (Name: “Wissenschaftsfoerderung”)

Step 1



Step 2



Conference Program

Wednesday, November 18th 2015

19.00 ***Welcome and Get Together***
Restaurant Hirschgarten

Thursday, November 19th 2015

08.15 **Registration**

09.00 **Welcome and Introduction**
Felix Beuschlein, Munich

09.10 **ACC Communications I [10 + 2 minutes each]**
Chairs: Jérôme Bertherat and Peter Igaz

SF-1 and its novel dosage-dependent target gene VAV2 promote actin cytoskeleton remodeling and invasion of human adrenocortical cancer cells
Carmen Ruggiero, Aurélie Morin, Mabrouka Doghman, Maddy Parsons, Estelle Robidel, Bruno Ragazzon, Jerome Bertherat, Judith Favier, Enzo Lalli

FATE1 counteracts apoptosis in adrenocortical tumoral cells by uncoupling endoplasmic reticulum and mitochondria
Mabrouka Doghman, Veronica Granatiero, Silviu Sbiera, Frederic Brau, Martin Fassnacht, Rosario Rizzuto, Enzo Lalli

ZNRF3 a new tumor suppressor gene in adrenocortical tumors
Hanin Omeiri, Lucile Lefevre, Ludivine Drougat, Guillaume Assie, Marthe Rizk-Rabin, Jerome Bertherat, Bruno Ragazzon

Suppression of tissue protein SET and circulating hsa-miR-483-5p expression by combined 9-cis retinoic acid + mitotane treatment in an adrenocortical xenograft model
Zoltan Nagy, Kornelia Baghy, Eva Hunyadi-Gulyas, Gabor Nyiro, Henriett Butz, Ilona Kovalszky, Katalin F. Medzihradszky, Karoly Racz, Attila Patocs, Peter Igaz

Adrenocortical carcinoma cell-derived miR-483-5p and miR-139-5p promote angiogenesis

Jonathan Laugier, Claire Agosta, Josiane Denis, Feige Jean-Jacques, Olivier Chabre, Nadia Cherradi

Small non-coding RNAs as marker for therapeutic effects in a preclinical model for adrenocortical carcinoma in vivo
Constanze Hantel, Sara Jung, Martin Reincke, Zoltan Nagy, Peter Igaz, Felix Beuschlein

Investigation of a novel liposomal chemotherapy protocol in different preclinical models for adrenocortical carcinoma in vivo
Sara Jung, Martin Reincke, Gerard Zambetti, Felix Beuschlein, Constanze Hantel

XAV939 and XL147: study of the antiproliferative effect of two new compounds in adrenocortical cell lines
Beatrice Rubin, Raffaele Pezzani, Halenya Monticelli, Filippo Ceccato, Maurizio Iacobone, Ambrogio Fassina, Carla Scaroni, Franco Mantero, Marco Boscaro

11.00 Coffee Break

11.20 ACC Communications II [10 + 2 minutes each]
Chairs: Felix Beuschlein and Enzo Lalli

Intra-Tumour heterogeneity of TERT Promoter Mutations in Adrenocortical Carcinoma
Rajani Maharjan, Joakim Crona, Tobias Åkerström, Per Hellman, Peyman Björklund

Urine steroid metabolomics as a novel diagnostic tool for early detection of recurrence in adrenocortical carcinoma
Vasileios Chortis, Irina Bancos, Katharina Lang, Beverly Hughes, Donna O'Neil, Angela Taylor, Martin Fassnacht, Felix Beuschlein, Jerome Bertherat, Marcus Quinkler, Massimo Terzolo, Massimo Mannelli, Dimitra Vassiliadi, M Conall Dennedy, Urszula Ambroziak, Michael Biehl, Wiebke Arlt

DNA methylation is an independent prognostic marker of survival in adrenocortical cancer (ACC)
Anne Jouinot, Guillaume Assié, Rossella Libé, Martin Fassnacht, Silviu Sbiera, Matthias Kroiss, Cristina Ronchi, Thomas Papathomas, Ronald De Krijger, Lionel Groussin, Xavier Bertagna, Bruno De La Villéon, Olivia Barreau, F. René-Corail, S. Rodriguez K. Perlemoine, M. Neou,

Simon Faillot, Mathilde Sibony, Frédérique Tissier, Antoine Tabarin, Magalie Haissaguerre, Olivier Chabre, Nathalie Sturm, Massimo Mannelli, Michaela Luconi, Jens Waldmann, Marcus Quinkler, Felix Beuschlein, Eric Baudin, Franco Mantero, V. Kerlan, P. Touraine G. Barrande, R. Cohen, L. Amar, Nadim Hamzaoui, Eric Clauser, Joel Coste, Jérôme Bertherat

Relationship between ERCC1 expression and sensitivity to platinum-based chemotherapy in a large series of adrenocortical carcinomas: preliminary data.
Valeria Laufs, Sonja Steinhauer, Silviu Sbiera, Vanessa Wild, Martin Fassnacht, Cristina L. Ronchi

High postoperative circulating miR-483-5p is a prognostic biomarker for adrenocortical cancer
Maurine Oreglia, Silviu Sbiera, Martin Fassnacht, Josiane Denis, Olivier Chabre, Nadia Cherradi

Hyperthermic intraperitoneal chemotherapy for primary and recurrent adrenocortical carcinoma: perioperative and survival analysis.
Leonardo Solaini, Vittorio Ferrari, Silvia Ministrini, Laura Ferrari, Daniele Lomiento, Alfredo Berruti, Guido A.M. Tiberio

[123/131I](R)-1-[1-(4-Iodophenyl)ethyl]-1H-imidazole-5-carboxylic acid azetidinylamide (IMAZA) a novel radiotracer for diagnosis and treatment of adrenocortical tumours - first clinical experience
Stefanie Hahner, Britta Heinze, Ken Herrmann, David Michelmann, Lukas Nannen, Andreas Buck, Christina Blümel, Martin Fassnacht, Bruno Allolio, Andreas Schirbel

ESES (European Society of Endocrine Surgeons) and ENSAT (European Network for the Study of Adrenal Tumors) recommendations on the surgical management of adrenocortical carcinoma
Sebastien Gaujoux, Elisabeth Nieveen van Dijkum, Nada Rayes, Cristian Fiori, Jens Waldmann, Per Hellman, Franck Zinzindohoue, Andrea Valeri, Bruno Carnaille, Hans Langenhuijsen, Frédéric Sébag, Eric Mirallié, Claire Blanchard, Francesco Porpiglia, Frédéric Triponez, Jean-Louis Kraimps, Giovani Donatini, Marco Raffaelli, Kerstin Lorenz, Muriel Mathonnet, Maurizio Iacobone, Bertrand Dousset, Radu Mihai

13.10 Lunch

14.00 **ACC working group meeting**
Chair: Martin Fassnacht

15.30 **Poster walks [3 + 2 minutes for each poster]**
Chairs: **ACC:** Constanze Hantel/ Guillaume Assié
PHEO/PGL: Esther Korpershoek/ Mirko Peitzsch
NAPACA: Rosella Libé/ Guido di Dalmazi
APA: Sheerazed Boulkroun/ Marcus Quinkler

15.30 **Registry tutorial**

17.00 **Coffee Break**

17.20 **General assembly and Prize ceremonies**

18.10 **End of session / Steering committee meeting**

19.30 **Dinner at Restaurant Schlosscafé im Palmenhaus**

Friday, November 20th 2015

08.30 **APA Communications [10 + 2 minutes each]**
Chairs: Martin Reincke and Tracy Williams

Characterization of voltage-gated calcium channels CaV1.3 and CaV3.2 in aldosterone producing adenoma
Christian Gebhard, Yara Rhayem, Anna Dietz, Anna Riester, Tim Strom, Celso Gomez-Sanchez, Martin Reincke, Felix Beuschlein

PRKACA mutations in aldosterone producing adenomas
Yara Rhayem, Luis Gustavo Perez-Rivas, Anna Dietz, Kerstin Bathon, Christian Gebhard, Anna Riester, Brigitte Mauracher, Celso Gomez-Sanchez, Thomas Schwarzmayr, Davide Calebiro, Tim M. Strom, Martin Reincke, Felix Beuschlein

Different somatic mutations in multinodular adrenals with aldosterone producing adenoma
Fabio Fernandes-Rosa, Isabelle Giscos-Douriez, Laurence Amar, Celso Gomez-Sanchez, Tchao Meatchi, Sheerazed Boulkroun, Maria-Christina Zennaro

Aldosterone concentration of the adrenal vein in patients without primary aldosteronism
Hironobu Umakoshi, Mitsuhide Naruse, Norio Wada, Takamasa Ichijo, Kohei Kamemura, Yuichi Matsuda, Yuichi Fujii, Tatsuya Kai, Tomikazu Fukuoka, Ryuichi Sakamoto, Atsushi Ogo, Tomoko Suzuki, Kazutaka Nanba, Mika Tsuiki

A novel CYP11B2-specific positron emission tomography imaging agent for detection of aldosterone-producing adenomas
Tsutomu Abe, Mitsuhide Naruse, William Young, Nobuya Kobashi, Yoshihiro Doi, Akihiro Izawa, Kei Akama, Yuki Okumura, Miho Ikenaga, Hiroyuki Kimura, Hideo Saji, Kuniaki Mukai, Hiroki Matsumoto

09.55 Coffee Break

10.10 APA working group meeting
Chair: Maria-Christina Zennaro

11.40 NAPACA Communications [10 + 2 minutes each]
Chairs: Wiebke Arlt and Franco Mantero

Does measurement of serum-dexamethasone increase diagnostic accuracy of the overnight DXM suppression test in the diagnostic workup of hypercortisolism?
Grethe Åstrøm Ueland, Paal Methlie, Hrafnkell Baldur Thordarson, Gunnar Mellgren, Oskar Kelp, Kristian Løvås, Eystein Sverre Husebye

Effect of the recovery from subclinical hypercortisolism on the risk of vertebral fractures
Antonio Salcuni, Valentina Morelli , Cristina Eller-Vainicher, Serena Palmieri, Elisa Cairoli, Anna Spada, Alfredo Scillitani, Iacopo Chiodini

Preclinical therapy of comorbidities in ACTH-independent Cushing's syndrome: contribution of a murine genetic model
Nathanaëlle Montanier, Isabelle Sahut-Barnola, Yohann Wittrant, Gaël Rochefort, Jean-Christophe Pointud, Hazel Hunt, Typhanie Dumontet, A-Marie Lefrancois-Martinez, Igor Tauveron, Pierre Val, Antoine Martinez

Primary Bilateral Macronodular Adrenal Hyperplasia: ARMC5, a new regulator of the cAMP/PKA pathway

Ludivine Drougat, Stéphanie Espiard, Stéphane Doly, Stéphanie Rodriguez, Marthe Rizk-Rabin, Karine Perlemoine, Rossela Libé, Guillaume Assié, Stefano Marullo, Bruno Ragazzon, Jérôme Bertherat

Genetic landscape of sporadic unilateral adrenocortical adenomas without PRKACA p.Leu206Arg mutation
Cristina L Ronchi, Guido Di Dalmazi, Silviu Sbiera, Guillaume Assie, Isabel Weigand, Davide Calebiro, Silke Appenzeller, Beatrice Rubin, Jens Waldmann, Carla Scaroni, Detlef K Bartsch, Franco Mantero, Massimo Mannelli, Darko Kastelan, Iacopo Chiodini, Jerome Bertherat, Martin Reincke, Tim Strom, Martin Fassnacht, Felix Beuschlein

12.50 Lunch

13.25 NAPACA working group meeting
Chair: Massimo Terzolo

14.55 Coffee Break

15.10 PHEO / PGL Communications [10 + 2 minutes each]
Chairs: Henri Timmers and Mercedes Robledo

Long Term Follow-Up in Patients Operated on a Phaeochromocytoma or a Paraganglioma: compilation of the ENSAT database
Laurence Amar, Olivier Steichen, Timo Deutschbein, Ianthe Piscaer, Christina Brugger, Laetizia Canu, Jerome Bertherat, Anthony Stell, Anne-Paule Gimenez Roqueplo, Martin Fassnacht, Mercedes Robledo, Massimo Mannelli, Jacques Lenders, Henri Timmers, Felix Beuschlein, Pierre-Francois Plouin

Preoperative risk factors of hemodynamic instability during laparoscopic adrenalectomy for pheochromocytoma
Sébastien Gaujoux, Stéphane Bonnet, Claude Lentschener, Jean-Marc Thillois, Denis Duboc, Jérôme Bertherat, Charles Marc Samama, Bertrand Dousset

Role of MDH2 mutations in pheochromocytoma and paraganglioma patients
María Currás-Freixes, Alexandre Buffet, Iñaki Comino-Méndez, Lucía Inglada-Pérez, Esther Korpershoek, Laurent Vroonen, Elena Rapizzi, Ronald R. de Krijger, Martin Fassnacht, Felix Beuschlein, Graeme

Eisenhofer, Massimo Mannelli, Anne-Paule Gimenez-Roqueplo, Alberto Cascón, Mercedes Robledo

In vivo detection of succinate by magnetic resonance spectroscopy as a hallmark of SDHx mutations in paraganglioma
Charlotte Lussey-Lepoutre, Alexandre Bellucci, Aurélie Morin, Alexandre Buffet, Laurence Amar, Maxime Janin, Chris Ottolenghi, Franck Zinzindohoué, Gwennhael Autret, Nelly Burnichon, Estelle Robidel, Paule Bénit, Pierre Rustin, Philippe Halimi, Laure Fournier, Anne-Paule Gimenez-Roqueplo, Bertrand Tavitian, Judith Favier

Genotype-dependent Brown Adipose Tissue Activation in Patients with Pheochromocytoma and Paraganglioma
Troy Puar, Anouk van Berkel, Martin Gotthardt, Bas Havekes, Ad Hermus, Jacques Lenders, Wouter van Marken Lichtenbelt, Ying Xu, Boudewijn Brans, Henri Timmers

16.30 **PHEO / PGL working group meeting**
Chair: Anne-Paule Gimenez-Roqueplo

18.00 **Farewell**
Felix Beuschlein

Poster

19th of November

ACC:

P1 **Cortisol producing adrenocortical carcinoma in a patient with Gardner's syndrome.**

Tobias Åkerström, Rajani Maharjan, Per Hellman, Peyman Björklund

P2 **Adrenocortical Carcinoma: Experience of a tertiary referral center in Greece**

Eleftherios Chatzellis[1], Anna Angelousi[1], Georgios Zografos[2], Gregory Kaltsas[1]

P3 **Effects of temozolomide on human adrenocortical cancer cells and the role of the O6-methylguanine-DNA methyltransferase gene**

SG Creemers[1], PM van Koetsveld[1], ESR van Dungen[1], F Dogan[1], GJH Franssen[2], WW de Herder[1], RA Feelders[1], LJ Hofland[1]

P4 **The methylation pattern of *IGF2* regulatory regions as a novel biomarker to distinguish adrenocortical carcinomas from adenomas**

SG Creemers[1], PM van Koetsveld[1], FJ van Kemenade[2], TG Papathomas[2], GJH Franssen[3], F Dogan[1], WW de Herder[1], JAMJL Janssen[1], RA Feelders[1], LJ Hofland[1]

P5 **Evaluation of the inhibitor of apoptosis protein livin/BIRC7 expression in adrenocortical tumors.**

Barbara Altieri[1,2], Silviu Sbiera[1], Silvia Della Casa[2], Sonja Steinhauer[1], Vanessa Wild[3], Guido Fadda[4], Michaela Bekteshi[1], Andreas Rosenwald[3], Alfredo Pontecorvi[2], Martin Fassnacht[1], Bruno Allolio[1], Cristina L. Ronchi[1]

P6 **Nicotinamide nucleotide transhydrogenase (NNT) as a novel molecular target in adrenocortical carcinoma - impact of NNT knockdown on adrenocortical cell proliferation, redox balance and steroidogenesis**

Vasileios Chortis[1], Angela Taylor[1], Craig Doig[1], Eirini Meimaridou[2], Lou Metherell[2], Wiebke Arlt[1], Paul Foster[1]

P7 **Adrenocortical carcinoma with mixed response to sequential therapy – rational to investigate clonal heterogeneity?**

Joakim Crona, Staffan Welin, Britt Skogseid

P8 **Targeting Tyrosine-Kinase in Adrenocortical Carcinoma cell lines**

Teresa Gagliano, Erica Gentilin, Eleonora Riva, Ettore degli Uberti, Maria Chiara Zatelli

P9 **Investigating the possible role of metformin as anticancer agent in H295R cells.**

Roberta Armignacco, Giada Poli, Giulia Cantini, Letizia Canu, Massimo Mannelli, Michaela Luconi

P10 Patched as a new therapeutic target for adrenocortical cancer

Anida Hasanovic[1,2], Laura Fiorini[1], Carmen Ruggiero[1,2], Mabrouka Doghman[1], Enzo Lalli[1,2], Isabelle Mus-Veteau[1]

APA:

P1 Activating Mutations in *CTNNB1* in aldosterone producing adenomas

Tobias Åkerström, Rajani Maharjan, Per Hellman, Peyman Björklund

P2 Tumor tissue aldosterone content measurement for identification of aldosterone producing adenomas

Tobias Åkerström, Per Hellman, Peyman Björklund

P3 Identification of candidate genes in a mouse model with hyperaldosteronism

Luis Gustavo Perez-Rivas*[1], Yara Rhayem*[1], Sibylle Sabrautzki[2,5], Martin Hrabe de Angelis[2], Tim M Strom[4], Martin Reincke[1], Felix Beuschlein[1], Ariadni Spyroglou[1]

P4 Advancing Care of Primary Aldosteronism in Japan Study (JPAS): A new systematic multicenter Cohort study on the diagnosis and treatment

Mitsuhide Naruse[1], Yoshihiro Ogawa[2], Masanobu Yamada[3], Nobuya Inagaki[4], Osamu Ogawa[4], Hiromi Rakugi[5], Hirotaka Shibata[6], Toshihiko Yanase[7], Takuyuki Katabami[8], Yoshiyu Takeda[9]

P5 High expression of C-X-C chemokine receptor type 4 in the zona glomerulosa and in aldosterone producing adenoma

Carmina Teresa Fuß[1], Britta Heinze[1], Christina Blümel[2], Andreas Schirbel[2], Felix Beuschlein[3], Paolo Mulatero[9], Martin Reincke[3], Christian Gebhard[3], Katja Hirsch[1], Marcus Quinkler[4], Vanessa Wild[5], Celso Gomez-Sanchez[6], Anna-Carinna Reis[7], Stefan Petersenn[8], Timo Deutschbein[1], Andreas K. Buck[2], Bruno Allolio[1], Katharina Lang[1], Ken Herrmann[2], Stefanie Hahner[1]

P6 RAR signaling contributes to adrenal morphology and functional zonation

Sheerazed Boulkroun[1], Amanda Rickard[1], Jose felipe Golib Dzib[2], Benoit Samson-Couterie[1], Celso Gomez-Sanchez[3], Laurence Amar[1,4], Fabio Luiz Fernandes-Rosa[1,5], Norbert Ghyselinck[6], Arndt Benecke[2], Enzo Lalli[7,8], Maria-Christina Zennaro[1,5]

NAPACA:

P1 Dexamethasone test follow-up after two years provide limited information about changes in cortisol secretion in most patients with incidentally detected adrenal adenomas

Ola Lindgren, Albin Kjellbom, Magnus Löndahl, Henrik Olsen

P2 **The role of chronically elevated levels of luteinizing hormone in the cause-effect relationship between the size of adrenal incidentaloma and insulin resistance**

Ljiljana Marina[1], Miomira Ivovic[1], Svetlana Vujovic[1], Milina Tancic-Gajic[1], Zorana Arizanovic[1], Dragana Rakovic[1], Jelena Milin-Lazovic[2], Dragan Micic[1]

P3 **Urinary free cortisol measured by LC-MS/MS to rule out Cushing's Syndrome in patients with adrenal incidentaloma**

Filippo Ceccato[1], Marialuisa Zilio[1], Mattia Barbot[1], Anna Chiara Frigo[3], Giorgia Antonelli[2], Maurizio Iacobone[4], Beatrice Rubin[1], Daniela Regazzo[1], Franco Mantero[1], Mario Plebani[2], Marco Boscaro[1], Carla Scaroni[1]

P4 **Long-term outcome after surgery for incidentally-diagnosed subclinical cortisol-secreting adenomas**

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Susan Richter[1], Barbara Klink[1], Brit Nacke[1], Anastasios Mangelis[1], Elena Rapizzi[2], Aguirre deCubas[3], Matthias Meinhardt[1], Christina Skondra[1], Massimo Mannelli[2], Mercedes Robledo[3], Mario Menschikowski[1], Graeme Eisenhofer[1]

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Anna Angelousi[1], Chrysa Aggeli[2], Pavlos Constantinou[3], Theodora Xoreutaki[2], Dimitra Rontogianni[3], Georges Piaditis[2], Georges Zografos[2], Grigorios Kaltsas[1]

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Abstracts

Oral Communications

from all fields of Adrenal Tumors

Genotype-dependent Brown Adipose Tissue Activation in Patients with Pheochromocytoma and Paraganglioma

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Introduction: Patients with pheochromocytomas and paragangliomas (PGLs) may have brown adipose tissue (BAT) activation induced by catecholamine excess. ^{18}F-fluorodeoxyglucose (^{18}F-FDG) PET/CT detects both PGLs and BAT activity. It is unknown whether BAT is specifically affected by altered cellular energy metabolism seen in *SDHx* and *VHL*-related PGLs. We examined the endocrine and paracrine effects of catecholamine excess on BAT activation in patients with PGLS as detected by ^{18}F-FDG PET/CT, taking into account genetic variation.

Methods: 73 patients with PGLs, age 52.4 ± 15.4 yr, BMI 25.2 ± 4.1 kg/m^2, were fully genetically characterized and underwent pre-surgical ^{18}F-FDG PET/CT imaging for tumor localization and to quantify BAT activation. They were grouped into sporadic, cluster 1 (SDHx, VHL) and cluster 2 (MEN2, NF1, MAX) mutations. ^{18}F-FDG mean standard uptake values (SUV_{mean}) were assessed in predefined BAT locations, including perirenal fat.

Results: 21/73 (28.8%) patients exhibited BAT activation. BAT activation was absent in all six patients with non-secreting PGLs. No difference in ^{18}F-FDG uptake by perirenal fat on the side of the pheochromocytoma and the contralateral side was observed (SUV_{mean} 0.80 *vs*. 0.78 respectively, *P*=0.42). The prevalence of BAT activation did not differ between sporadic (28.9%), cluster 1 (40.0%) and cluster 2 patients (15.4%), *P*=0.36.

Conclusion: Patients with PGLs exhibit a high prevalence of BAT activation on ^{18}F-FDG PET/CT. This is likely due to systemic catecholamine excess. Previous suggestions of 'browning' of peritumoral fat due to paracrine effects of catecholamines could not be confirmed. BAT activation is not associated with specific germline mutations.

In vivo detection of succinate by magnetic resonance spectroscopy as a hallmark of *SDHx* mutations in paraganglioma

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Background: Germline mutations in genes encoding mitochondrial succinate dehydrogenase (SDH) are found in patients with paragangliomas, pheochromocytomas, gastrointestinal stromal tumors, and renal cancers. SDH inactivation leads to a massive accumulation of succinate, acting as an oncometabolite and which levels, assessed on surgically resected tissue are a highly specific biomarker of SDHx-mutated tumors. The aim of this study was to address the feasibility of detecting succinate in vivo by magnetic resonance spectroscopy.

Method: A pulse proton magnetic resonance spectroscopy (^{1}H-MRS) sequence was developed, optimized and applied to image nude mice grafted with *Sdhb*$^{-/-}$ or wild-type chromaffin cells. The method was then applied to paraganglioma patients carrying (n=5) or not (n=4) an *SDHx* gene mutation. Following surgery, succinate was measured using gas chromatography-mass spectrometry and SDH protein expression was assessed by immunohistochemistry in resected tumors.

Results: A succinate peak was observed at 2.44 ppm by ^{1}H-MRS in all *Sdhb*$^{-/-}$-derived tumors in mice and in all paragangliomas of patients carrying an *SDHx* gene mutation, but neither in wild-type mouse tumors nor in patients exempt of *SDHx* mutation. In one patient, ^{1}H-MRS results led to the identification of an unsuspected *SDHA* gene mutation. In another case, it helped defining the pathogenicity of a variant of unknown significance in the *SDHB* gene.

Conclusions: Detection of succinate by ^{1}H-MRS is a highly specific and sensitive hallmark of *SDHx* mutations. This noninvasive approach is a new, simple and robust method allowing *in vivo* detection of the major biomarker of *SDH*-mutated tumors.

Different somatic mutations in multinodular adrenals with aldosterone producing adenoma

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Primary aldosteronism is the most common form of secondary hypertension. Somatic mutations in *KCNJ5*, *ATP1A1*, *ATP2B3* and *CACNA1D* are found in aldosterone producing adenoma. Additionally, adrenals with aldosterone producing adenomas show cortical remodeling and frequently multiple secondary nodules. Our aim was to investigate whether different aldosterone producing nodules from the same adrenal share the same mutational status. Aldosterone synthase expression was assessed in multinodular adrenals from 27 patients. DNA of 37 aldosterone producing secondary nodules was extracted from formalin fixed paraffin embedded tissues and genotyped for *KCNJ5*, *ATP1A1*, *ATP2B3* and *CACNA1D* mutations. Among 17 adrenals with a somatic mutation in the principal nodule, four showed the same mutation in a secondary nodule, while ten had no mutation in any of the known genes. In one adrenal harboring the *KCNJ5* p.Gly151Arg mutation in the principal nodule, the same mutation was present in two secondary nodules, but no mutation was found in a third nodule. Finally, in two adrenals with a *CACNA1D* mutation in the principal nodule, a *KCNJ5* mutation was identified in the secondary nodule. Among ten adrenals without mutations in the principal nodule, one carried a *KCNJ5* mutation in the secondary nodule. No mutations were detected in seven aldosterone producing cell clusters from six adrenals. No association was observed between the presence of mutations in secondary nodules and clinical parameters. In conclusion, different mutations are found in different aldosterone producing nodules from the same adrenal, suggesting that somatic mutations are independent events triggered by mechanisms that remain to be identified.

Preclinical therapy of comorbidities in ACTH-independent Cushing's syndrome : contribution of a murine genetic model

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Our aim is to define the sequence of catabolic and anabolic complications of ACTH independent Cushing's syndrome (CS) developed by a genetic mouse model and to evaluate the efficiency of a GR antagonist approach.

Using $AdKO_{2.0}$ mice, lacking PRKAR1A in the adrenal cortex, we assessed the catabolic effects of CS on the muscle (relative weight of gastrocnemius and soleus) and bone (microarchitecture microtomography and expression of osteoclast/osteoblast markers in RTqPCR). Anabolic disorders were evaluated by insulin resistance tests and analysis of liver gluconeogenic markers (PEPCK, G6Pc). Finally, body composition measurements were performed using an Echo MRI analyser. After development of overt CS, WT and $AdKO_{2.0}$ were divided into three arms of treatments -Vehicle, Mifepristone (GR/PR antagonist, and CT8 (selective GR antagonist)- and received daily intraperitoneal injections for 4 weeks.

From the age of 4 weeks, $AdKO_{2.0}$ male mice showed both a weight and growth delay, an excess of mortality associated with an overt CS, contrasting with a later (10 weeks) and less severe CS in female mice. All comorbidities, except osteoporosis, develop at subclinical CS. Excess fat mass and insulin resistance of $AdKO_{2.0}$ ($p<0.001$) are corrected to varying degrees by both Mifepristone or CT8 treatment. Finally, both compounds were able to counteract bone osteoporosis ($p<0.01$) without improving muscle atrophy.

This work validates $AdKO_{2.0}$ mice as a suitable model for CS complications study, with the ability to provide new therapeutic alternatives for preclinical studies targeting Cushing's syndrome comorbidities.

SF-1 AND ITS NOVEL DOSAGE-DEPENDENT TARGET GENE *VAV2* PROMOTE ACTIN CYTOSKELETON REMODELING AND INVASION OF HUMAN ADRENOCORTICAL CANCER CELLS

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Steroidogenic factor-1 (SF-1) is an essential regulator of adrenogonadal development and steroidogenesis, being critically involved in adrenocortical tumorigenesis.

We have identified the *VAV2* gene as a novel dosage-dependent target of SF-1 in human adrenocortical H295R cancer cells. Clinical evidence from two different ACC cohorts reveals that *SF-1* and *VAV2* expressions are highly significantly correlated.

VAV2 encodes a guanine nucleotide exchange factor (GEF) for the Rho small GTPases, thus participating in actin cytoskeleton reorganization, migration and invasion.

We showed that an increased SF-1 dosage promotes Rac1 and Cdc42 activation, lamellipodia/ruffles and filopodia formation and increased adrenocortical cancer cell invasion through Matrigel. The increased invasive ability correlates with a decreased amount of inactive phosphorylated cofilin. Remarkably, *VAV2* knock-down impairs SF-1 dosage-dependent actin remodeling and cell invasion. VAV2 GEF activity is necessary for adrenocortical cancer cells to invade under increased SF-1 dosage conditions.

Importantly, clinical data from two different cohorts show that *VAV2* expression is significantly associated with poor overall survival in ACC patients.

Altogether, our study shows for the first time that overexpression of SF-1 and of its target VAV2 plays a relevant role in the establishment of an invasive and metastatic phenotype in adrenocortical cancer cells and suggest that VAV2 might represent a potential novel druggable target for ACT therapy.

Experiments are in progress to establish an experimental mouse model for the analysis of the impact of SF1 and VAV2 overexpression on the formation of metastatic ACC lesions.

Effect of the recovery from subclinical hypercortisolism on the risk of vertebral fractures

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Objective. Subclinical hypercortisolism (SH) is associated with increased risk of vertebral fractures (VFx), but the effect on bone of the recovery from SH is unknown.

Design. In this prospective open study, among 605 subjects consecutively referred (January 2008-June 2015) for monolateral AI to our outpatient Clinics, 55 patients with SH were enrolled. We proposed to all patients to undergo adrenalectomy, which was accepted by 32 patients (Group1, age 61.3±8.1 years, 22 postmenopausal females/10 males) and refused by 23 patients, who were followed with a conservative approach (Group2, age 65.4±7.1 years, 10 postmenopausal females/13 males).

Methods. We diagnosed SH on the basis of the presence of ≥2 among urinary free cortisol >70 µg/24h, serum cortisol after 1–mg dexamethasone suppression test (1mg-DST) >3.0 µg/dL and ACTH <10 pg/mL. At baseline and at the end of a variable follow-up (Group 1 39.9±20.9 months, Group2 27.7±11.1 months) we assessed: bone mineral density (BMD) at lumbar spine (LS) and femoral neck (as Z-score) by Dual-energy X-ray Absorptiometry and the VFx presence.

Results. The LS Z-score per year (ΔZ-score/year) tended to increase in Group1 (0.10±0.20) as compared with Group2 (-0.01±0.27, p=0.08). In Group1, the number of new VFx was lower (9.4%) than in Group2 (52.2%, p<0.0001). The recovery from SH was associated with a 30% VFx risk reduction (OR 0.07, 95%CI 0.01-0.50, p=0.008) but not with age, gender, duration of follow-up, 1mg-DST, LS BMD and prevalent VFx.

Conclusions. In AI patients with SH surgery reduces the VFx risk

Does measurement of serum-dexamethasone increase diagnostic accuracy of the overnight DXM suppression test in the diagnostic workup of hypercortisolism?

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Background: 1-mg overnight dexamethasone-suppression test (DST) is commonly used to screen for hypercortisolism. Sensitivity is high (95%), but specificity lower (80%), leading to false positive results. Identifying individuals with abnormal dexamethasone absorption or metabolism could enhance diagnostic accuracy.

Aim: Use s-dexamethasone (DXT) to increase diagnostic accuracy.

Methods: Prospective study of DST for clinical suspicion of Cushing's syndrome (CS) (*n*=49), incidentaloma (*n*=152), and healthy controls (*n*=101). S-cortisol and s-DXT were assayed by LCMSMS. DST results were correlated to the final diagnosis based on current clinical guidelines.

Results: 83/302 persons did not suppress s-cortisol (<50 nmol/L). Of these 11 had CS, and 27 subclinical CS (16% of the incidentalomas). A s-DXT cut-off level of 3.33 nmol/L was established based on the lower 2.5% quantile of DSTs suppressing s-cortisol <50 nmol/L. Applying this cut-off,10/302 (3.3%) DSTs had both inadequate DXM-levels and elevated s-cortisol. Of these, three were misdiagnosed as subclinical-CS. Among non-CS samples, s-cortisol were higher in the incidentaloma-group (median 42.5 nmol/L, range 13-576) (p<0.01) than in those with clinically suspected CS (22.7 nmol/L, 9.9-289) and healthy controls (22.2 nmol/L, 8.4-102). Findings were similar after eliminating patients with lowest levels of s-DXM and estrogen users (*n*=16).

Conclusion: Abnormal absorption or metabolism of DXM is uncommon reasons for false positive 1 mg DXM -tests. Simultaneous measurement of s-DXM increases the accuracy of the test, and helps identify patients with subclinical CS. A minimum s-DXM level of 3.33 nmol/L is needed to suppress S-cortisol <50 nmol/L.

Suppression of tissue protein SET and circulating *hsa-miR-483-5p* expression by combined 9-cis retinoic acid + mitotane treatment in an adrenocortical xenograft model

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Background: The drug treatment repertoire for adrenocortical carcinoma (ACC) is rather narrow and intensive efforts are going on to find novel effective agents. In our previous studies, the in vitro activity of 9-cis retinoic acid (9-cisRA) on adrenocortical NCI-H295R cells was shown along with its antitumoral effects in a small pilot xenograft study.

Objective: To study the antitumoral effects of 9-cisRA and its combination with mitotane on ACC in a large-scale xenograft study.

Methods: 43 male SCID mice xenografted with NCI-H295R cells were treated in four groups (i. control, ii. 9-cisRA, iii. mitotane, iv. 9-cisRA + mitotane) for 28 days. Tumor size follow-up, histological and immunohistochemical (Ki-67) analysis, tissue gene expression microarray (4x44K Agilent Whole Genome Microarray), quantitative real-time-PCR (TaqMan) for the validation of microarray results and to detect microRNAs were performed. Protein expression was studied by proteomics and Western-blot validation.

Results: Mitotane alone and the combination of 9-cisRA and mitotane resulted in significant tumor size reduction. The Ki-67 index was significantly reduced in both 9-cisRA and 9-cisRA+mitotane groups. We have found only modest changes at the mRNA level. The expression of circulating *hsa-miR-483-5p* was significantly reduced in the combined treatment group. The SET protein was validated as being significantly down-regulated in the combined mitotane+9-cisRA group.

Conclusions: 9-cisRA might be a helpful additive agent in the treatment of ACC in combination with mitotane. Circulating *hsa-miR-483-5p* could be utilized for monitoring the treatment efficacy in ACC patients, and the differences in protein SET expression might raise its relevance in ACC biology.

Role of MDH2 mutations in pheochromocytoma and paraganglioma patients.

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Introduction: Pheochromocytomas (PCC) and paragangliomas (PGL), PPGLs, have the highest heritability of all human neoplasms, being a good example of diseases with underlying genetic heterogeneity. Recently, we identified a new PPGL susceptibility gene, MDH2 (malate dehydrogenase 2), by exome sequencing in a patient with multiple metastatic PGLs without family history of the disease. However, its clinical relevance was not addressed.

Objective: This study aimed to examine the prevalence and associated phenotypic features of germline and somatic MDH2 mutations in PCC/PGL.

Material and methods: A total of 588 germline DNAs and 64 tumor DNAs from 652 patients with PPGL from 8 referral centers were screened for MDH2 mutations. All samples were negative for mutations in the major PPGL genes. RBP1 expression was used to assess pathogenicity. This series included 652 index cases: 120 multiple PPGLs (18.4%), 68 metastatic (10.4%), 18 cases with family history (2.8%) and 347 cases diagnosed under the age of 35 years (53.2%). Sixty percent were females and the mean age was 39.7 + 16.7 years.

Results: Seven not previously described variants were found. Among them, 4 missense mutations were detected in germline DNA from patients with a single PCC, and predicted as potentially functional. One of them was discarded as causative by biochemical secretion and RBP1 expression. Conclusions: The prevalence of MDH2 mutations seems to be low, similarly to the last PPGL related-genes described recently. The study will be complemented with the study of gross deletions and results will be presented in the meeting.

Small non-coding RNAs as marker for therapeutic effects in a preclinical model for adrenocortical carcinoma *in vivo*

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The vault complex, consisting of three vault proteins and four small non-coding RNAs is considered the largest intracellular ribonucleoprotein particle. Although in the past vaults were believed to be involved in multidrug resistance, the exact function of this complex has remained unclear. Recently, we investigated the therapeutic applicability of a Tumor-Vascular-Disrupting Agent in preclinical models for endocrine tumors. Subsequent analyses identified vault RNAs 1 to 3 as the most pronounced regulated transcripts in a neuroendocrine tumor model showing therapeutic responsiveness while no changes were detectable in therapy-resistant adrenal NCI-H295R xenografts. Subsequently, we investigated NCI-H295R xenografts, which had been treated and shown to be responsive to two different chemotherapeutic regimens (EDP-M and LEDP-M). In this therapeutic setting treatment-dependent upregulation of vault RNAs in NCI-H295R tumors was also evident (% of controls; vault1: EDP-M 296.3±44%, $p<0.001$; LEDP-M 243.5±16%, $p<0.001$; vault2: EDP-M 157.2±20%, $p>0.05$; LEDP-M 160.7±20%, $p>0.05$; vault3: EDP-M 241.2±55%, $p<0.001$; LEDP-M 118.9±16%, $p>0.05$). Moreover, we investigated expression of various microRNAs in these xenografts. While expression of miR-195, miR-483-3p, miR-483-5p and miR-503 were not significantly altered miR-210 was inhibited by EDP-M (39.69±26%, $p<0.05$) and LEDP-M (37.86±23%, $p<0.05$) compared to controls. miR-210, also known as master hypoxamir, is known to be highly upregulated at hypoxic conditions, contributing to aberrant regulation of cell proliferation, DNA stability and angiogenesis. Moreover, elevated miR-210 levels were recently shown to be associated with tumor aggressiveness and poor prognosis in ACC. In summary, small non-coding RNAs might have potential to improve and monitor efficacy of anticancer treatment against ACC.

Relationship between ERCC1 expression and sensitivity to platinum-based chemotherapy in a large series of adrenocortical carcinomas: preliminary data.

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Platinum-based chemotherapy (PBC) is the most effective cytotoxic treatment in adrenocortical carcinoma (ACC) but response rates remain below 50%. Excision repair cross complementing group 1 (ERCC1) plays a critical role in the repair of platinum-DNA damage and is involved in sensitivity to platinum compounds. Two previous studies on ERCC1 and PBC in small series of ACCs reported contrasting results. The aim was to evaluate ERCC1 protein expression by immunohistochemistry in a large series of ACCs and to correlate it with response to PBC.

Ninety-one ACCs were investigated, 81 of them deriving from first surgery. All patients underwent PBC (median: 4 cycles, range: 1-15). Immunostaining was performed by novel high specific ERCC1-antibody clone 4F9. The relationship between ERCC1 expression and both overall and progression free survival (OS and PFS) and objective response to therapy was evaluated.

High ERCC1 expression (H-score 2-3) was observed in 69% of cases, being more frequent in tumors with low ENSAT-tumor stage (74% in stage 1-2, 70% in stage 3, and 57% in stage 4, *P<0.005*). In this series, ERCC1 expression did not significantly correlate with neither OS (*P=0.64,* HR=1.14) nor PFS (*P=0.44,* HR=1.20) during PBC. Twenty-nine patients presented partial remission or stable disease for >6 months (32%), without any significant relationship with ERCC1 expression.

Conclusions: Our preliminary data shows that high ERCC1 protein expression may be related to early tumor stages, but seems not to be directly associated with resistance to PBC in ACCs. However, we plan to investigate a larger series of ACC samples.

Primary Bilateral Macronodular Adrenal Hyperplasia: ARMC5, a new regulator of the cAMP/PKA pathway

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Primary Bilateral Macronodular Adrenal Hyperplasia (PBMAH) are adrenocortical tumors leading to adrenal Cushing's syndrome. Recently, our laboratory has identified the first gene predisposing to PBMAH in adults, named ARMC5 (Armadillo Repeat Containing 5)[1]. The ARMC5-inactivating mutations at germline and somatic levels identified in PBMAH suggest that ARMC5 acts as a tumor suppressor gene. The ARMC5 protein contains two domains, Armadillo repeat and the BTB/POZ domains, involved in proteins-proteins interactions; however its mechanism of action remains unknown. The aim of this work is to identify ARMC5 protein partners and the involved signalling pathways.
The screening of ARMC5 partners by mass spectrometry, we allowed to identify 16 proteins specifically interacting with wild type ARMC5 involved in cAMP/PKA pathway, the degradation of proteins and the redox system. The interaction between ARMC5 and subunits of PKA were then confirmed by co-immunoprecipitations and BRET (Bioluminescence Resonance Energy Transfert) experiments. In parallel, we showed by complementary approaches [PKA activity assay kit, FRET (sensor AKAR3), Western-blot (Phospho PKA Substrate and P-CREB), and CRE Luciferase Reporter System] that ARMC5 silencing leads to downregulation of the PKA activity.
By its binding with actors involved in the cAMP/PKA pathway and the consequence of its invalidation on PKA activity, ARMC5 seems to be a key regulator of the cAMP/PKA pathway. The understanding of biological function of ARMC5 protein will allow to knowledge the impact of identified mutations in the ARMC5 gene in patients with PBMAH.

1- Assié et al., NEJM, 2014

Genetic landscape of sporadic unilateral adrenocortical adenomas without *PRKACA* p.Leu206Arg mutation

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Genetic alterations affecting the PKA/cAMP pathway are commonly found in cortisol-producing adrenocortical adenomas (ACAs), while activating mutations in the gene coding for beta-catenin (*CTNNB1*) have been reported in both adenomas and carcinomas. However, the molecular pathogenesis of adrenocortical adenomas is still largely unclear. Aim of the study was a comprehensive genetic characterization of ACAs and the identification of novel molecular markers involved in adrenal tumorigenesis and steroid autonomy.

Whole-exome sequencing was performed on DNA of adrenocortical tumors and corresponding blood samples of 99 patients with ACAs (39 associated with overt Cushing´s syndrome, 35 with subclinical hypercortisolism and 25 hormonally inactive) negative for the p.Leu206Arg *PRKACA* mutation.

In total, 705 protein-altering somatic mutations were detected in 88/99 ACAs (median: 6 mutations per sample, range: 0-55). Pathway analysis recognized genes involved in extracellular matrix, calcium signaling pathway and collagen formation as the most frequently affected. Three new mutations were identified in the *PRKACA* gene. Several altered genes could be recognized as part of the Wnt/β-catenin pathway (*CTNNB1, APC, APC2, PKP2*, and different members of the cadherin superfamily) associated with larger tumor size and endocrine inactivity, the cAMP/PKA pathway (*GNAS, PRKACA, PRKAR1A, CREB1, CREBBP, ADCY3, GRM3, GRM4, GRM6, RGS14*) associated with female gender and overt Cushing syndrome, and Ca^{2+} signaling (*CACNA1C, RYR1, RYR2, RYR3, CACNA1E, GRIA1, GRID1, GRIK2, GRIN1, GRIN3B, GRIP1, GRIA2*).

In conclusion, this study represents the most comprehensive genetic characterization of unilateral ACAs, including inactive adenomas. We thereby identified somatic alterations affecting pathways known or potentially involved in the adrenal tumorigenesis.

A novel CYP11B2-specific positron emission tomography imaging agent for detection of aldosterone-producing adenomas

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Context: Although adrenal vein sampling (AVS) is the standard method to distinguish unilateral from bilateral forms of primary aldosteronism (PA), it is an invasive and technically difficult procedure. ^{11}C-metomidate (MTO)-positron emission tomography (PET) was reported as a potential replacement for AVS. However, MTO has low selectivity for CYP11B2 over CYP11B1. In addition, PET imaging agents labeled with carbon-11 are limited because of the very short half-life of carbon-11. Therefore, a longer half-life fluorine-18-labeled imaging agent with a higher selectivity for CYP11B2 over CYP11B1 is desirable for APA imaging.

Objective: This study aimed to determine the selectivity of a new fluorine-18-labeled imaging agent for CYP11B2 over CYP11B1 and determine whether the biodistribution profile is favorable for imaging CYP11B2.

Methods: The half maximal inhibitory concentration for the enzymatic activities of CYP11B2 and CYP11B1 was determined using cells with stable expression of either enzyme. *In vitro* autoradiography of human adrenal sections with aldosterone-producing adenomas was performed to confirm the specific binding ability to CYP11B2-expressing regions. Furthermore, PET and magnetic resonance imaging were performed in rats.

Results: Our study showed that the new imaging agent had high selectivity for CYP11B2 over CYP11B1 compared to a MTO analog and it had a favorable biodistribution for imaging CYP11B2.

Conclusion: Our new fluorine-18-labeled PET imaging agent holds promise to be an alternative to AVS for the subtype diagnosis of PA.

Aldosterone concentration of the adrenal vein in patients without primary aldosteronism

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Adrenal venous sampling (AVS) is considered to be the most reliable diagnostic procedure to lateralize aldosterone excess in primary aldosteronism (PA). However, diagnostic criteria have not been established mainly because of a lack of data in non-PA hypertensive patients. Aim of the study was to investigate aldosterone concentration and its gradient in the adrenal vein of non-PA hypertensive patients. We retrospectively studied the results of cosyntropin-stimulated AVS in 41 hypertensive patients who showed negative results in 2 confirmatory testing, captopril challenge test and saline infusion test, recommended by the Endocrine Society Guideline. Plasma aldosterone concentration (PAC), aldosterone to cortisol (A/C) ratio, and its higher to lower ratio (lateralized index: LI) in the adrenal vein were measured. Median PAC in the adrenal vein was 25737 pg/ml (range: 5154-69920) in the higher side and 13347 pg/ml (range: 1866-36190) in the lower side (P<0.001). There was a significant gradient in A/C ratio between the higher and lower sides [27.2 pg/ml/μg/dl (5.4-66.0) vs 17.3 pg/ml/μg/dl (4.0-59.0); P<0.001] with LI ranging from 1.01 to 3.87: 1 to 2 in 33 patients and 2 to 4 in 8 patients. None of the patients showed LI ≥4. The present study clearly demonstrated that PAC in the adrenal vein showed significant variation and gradient even in non-PA hypertensive patients. Interpretation of PAC in the adrenal vein and LI in the grey zone should be carefully examined for the subtype diagnosis of PA before indicating adrenal surgery.

Preoperative risk factors of hemodynamic instability during laparoscopic adrenalectomy for pheochromocytoma

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Background: Adrenalectomy for pheochromocytoma is considered to be a challenging procedure because of the risk of HI, which is poorly defined and unpredictable. The objective of this retrospective study from a prospectively maintained database was to determine the predictive factors for perioperative hemodynamic instability (HI), which is defined as a morbidity-related variable, in patients undergoing unilateral laparoscopic adrenalectomy (LA) for pheochromocytoma.

Methods: A total of 149 patients with unilateral pheochromocytoma undergoing LA were included. First, HI was defined using independent hemodynamic variables associated with perioperative morbidity. Next, a multivariable logistic regression analysis was performed to determine the independent preoperative risk factors for HI.

Results: There was no postoperative mortality; the overall morbidity rate was 10.7%. The use of a cumulative dose of norepinephrine>5mg was the only independent hemodynamic predictive factor for postoperative complications; thus, this variable was used to define HI. A multivariate analysis revealed that a symptomatic high preoperative blood pressure ($p=0.003$) and a ten-fold increase in urinary metanephrine and/or normetanephrine levels ($p<0.0001$) were significant predictors of HI. When no predictive factors were present, the risk of HI and the postoperative morbidity were 1.5% and 4.3%, respectively. However, when two predictive factors were present, the HI risk and the postoperative morbidity were 53.8% and 30.8%, respectively.

Conclusion: Perioperative HI, defined as the need for a cumulative dose of norepinephrine>5mg, is significantly associated with postoperative morbidity and can be predicted by symptomatic preoperative high blood pressure and above a ten-fold increase in urinary metanephrine and/or normetanephrine levels.

ESES (*European Society of Endocrine Surgeons*) and ENSAT (*European Network for the Study of Adrenal Tumors*) recommendations on the surgical management of adrenocortical carcinoma

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Objective: To provide standards for the perioperative surgical care for patients with adrenocortical carcinoma (ACC).

Summary background data: There is a consensus that radical surgery provides the best chance for cure for ACC, but perioperative surgical care for such patients have been poorly standardized.

Methods: A joint initiative from ENSAT (*European Network for the Study of Adrenal Tumors*) and ESES (*European Society of Endocrine Surgeons*) assembled an European working group who produced, evidence-based recommendations for the perioperative surgical care for patients with ACC according to a Delphi methodology. Data were retrieved from electronic databases. Evidence and recommendations were classified according to the Grading of Recommendations, Assessment, Development and Evaluation (GRADE) system and were discussed until consensus was reached within the group. The quality of evidence was rated 'high', 'moderate', 'low' or 'very low'. Recommendations were graded as 'strong' or 'weak'.

Results: The available evidence has been summarized in 27 recommendations for the perioperative surgical care for patients with ACC. The quality of evidence is generally low due to the rarity of the disease and the absence of prospective surgical trial. Multi-institutional prospective cohort and prospective randomized controlled trials are urgently needed and should be strongly encouraged.

Conclusion: The present evidence-based recommendation provides comprehensive advice on optimal perioperative care for the patient undergoing surgery for adrenocortical carcinoma.

Hyperthermic intraperitoneal chemotherapy for primary and recurrent adrenocortical carcinoma: perioperative and survival analysis.

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Background: Local and peritoneal recurrence are a major challenge in surgical management of adrenocortical carcinoma (ACC) as they dramatically worsen the prognosis.

We aim to present the first report on the use of hyperthermic intraperitoneal chemotherapy (HIPEC) for either primary or recurrent ACC and to analyze its impact on survival.

Methods: A total of 9 patients underwent HIPEC (Cisplatin and Doxorubicin at 41.5-42°C) for ACC. Three of them had upfront adrenalectomy plus HIPEC for ACC while the remaining had cytoreductive surgery (CRS) for recurrent disease (2 peritoneal carcinomatosis, 4 local recurrences).

Results: No postoperative complications were registered. Median hospital stay was 15 days. Median follow up was 23 months.

In the upfront group 2 patients are disease-free at 96 and 10 months, respectively. The remaining patient, who had a stage IV ACC (pulmonary metastases) at diagnosis, died at 26 months for extra-abdominal disease progression.

With regards to the cases of peritoneal carcinomatosis, one patients is alive with extra-abdominal disease at 30 months while the other one is disease-free at 6 months.

Among the patients with local recurrence three are disease free at 9, 8 and 4 months respectively. One patient developed a local re-recurrence 12 months after CRS plus HIPEC and was submitted to a re-CRS plus HIPEC; he developed a third local recurrence after 9 months.

Conclusions: Our initial experience suggested that HIPEC may have a role in the management of primary or recurrent ACC.

High postoperative circulating miR-483-5p is a prognostic biomarker for adrenocortical cancer

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Introduction: MicroRNAs (miRs) are promising biomarkers for cancer diagnosis and prognosis. MiR-483-5p and miR-195 have been identified as preoperative circulating diagnostic and prognostic biomarkers for adrenocortical cancer (ACC). In this study, we evaluated their postoperative prognostic value. The primary objective was to determine whether 3 months postoperative circulating miRs levels were different between patients with poor prognosis (relapse or death in the first three years of disease) and patients with good prognosis (at least three years relapse-free survival). **Methods:** We conducted a single center retrospective analysis using sera from patients with non-metastatic ACC sampled at different times of the disease. Circulating miRs levels were measured using real time quantitative PCR (RT-qPCR). **Results:** From 48 patients, we excluded 27 patients because of too short follow-up or the lack of sample in the three months post first surgery. We conducted the primary analysis with 21 patients. We showed that high levels of circulating miR-483-5p were correlated with poor prognosis. No significant changes were found in circulating miR-195 levels. Secondary analyses performed with another patient selection suggested that circulating miR-483-5p is higher after relapse and before death, suggesting its increase with the adverse evolution of the disease. **Conclusion:** We report for the first time that miR-483-5p is a promising postoperative biomarker for ACC prognosis as well as ACC progression. Prospective studies are needed to confirm these observations.

Urine steroid metabolomics as a novel diagnostic tool for early detection of recurrence in adrenocortical carcinoma

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Adrenocortical carcinoma (ACC) is an aggressive malignancy with high recurrence rates. Regular post-operative follow-up imaging is essential, but associated with high radiation exposure and frequent diagnostic ambiguity. Urine steroid metabolomics was recently introduced as a novel diagnostic tool for the detection of adrenocortical malignancy. Here we present the first clinical study assessing the performance of this innovative approach in the follow-up of patients with complete (R0) ACC resection. We included 175 patients from 13 centres registered with the European Network for the Study of Adrenal Tumours. We selected all patients fulfilling the following criteria: 1) recorded as confirmed adrenocortical carcinoma with R0 primary tumour resection; 2) availability of at least two postoperative 24-hour urines, pre- and post- recurrence. 24-hour urines were analysed by gas chromatography-mass spectrometry, with quantification of 38 distinct steroid metabolites. A machine learning-based computational algorithm was employed to detect ACC recurrence. 29 patients developed 30 ACC recurrences during the study period. Steroid metabolomics diagnosed disease recurrence at the time of first abnormal imaging with a sensitivity of 86%. Adjuvant mitotane in 18/30 patients did not affect accuracy. In the subgroup of patients with a diagnostic pre-operative 24-hour urine sample (n=11), we were able to accurately detect all cases of recurrence. In 12 cases, biochemical evidence of disease recurrence pre-dated the first radiological detection by more than two months (range 3-16 months). Our study provides proof-of-principle evidence suggesting a role for urine steroid metabolomics as a potent diagnostic tool in the follow-up monitoring of ACC.

Characterization of voltage-gated calcium channels CaV1.3 and CaV3.2 in aldosterone producing adenoma

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Primary aldosteronism is predominantly caused by unilateral aldosterone producing adenoma (APA). Mutations affecting the voltage-gated calcium channels CaV1.3 encoded by CACNA1D, and more recently CaV3.2 encoded by CACNA1H, were described to be involved in the formation of APAs.

We investigated mutations of CACNA1D, CACNA1H and other yet identified candidate genes (KCNJ5, ATP1A1, ATP2B3 and CTNNB1) in 120 APAs obtained after unilateral adrenalectomy, using exome or direct sequencing. We analysed the α1D subunit of CaV1.3 and the α1H subunit of CaV3.2 using quantitative RT-PCR and studied their immunoexpression in paraffin-embedded APAs. Cellular co-localization of these ion channels with CYP11B2, the rate limiting enzyme for aldosterone production was evaluated using double immunohistochemistry and a recently developed monoclonal antibody.

The results of our study show that CACNA1D mutations affect 8.3% of APA tissues, while CACNA1H mutations were not detected. CaV1.3 and CaV3.2 were immunoexpressed in all APA cells (respective H-Scores mean ± SD: 270,9 ± 19,4 and 225,7 ±37,2). H-Score for CYP11B2 was significantly lower in non-mutated APAs ($P<0.05$). Higher immunoexpression of CaV3.2 was observed in small compact APA cells co-expressing CYP11B2, while no significant difference for CaV1.3 expression was noted between CYP11B2 positive or negative APA cells.

In summary, CACNA1H mutations seem not to be a common somatic alteration in sporadic APA. Furthermore, the relatively stronger immunoexpression of CaV3.2 seen primarily in CYP11B2 positive cells may provide indirect evidence for a functional relevance of this voltage-gated Ca2+ channel in APA.

Investigation of a novel liposomal chemotherapy protocol in different preclinical models for adrenocortical carcinoma *in vivo*

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Recently, we demonstrated for adrenocortical carcinoma (ACC) in NCIh295 xenografts promising antitumoral effects for LEDP-M (etoposide, liposomal doxorubicin, liposomal cisplatin, mitotane) a liposomal variant of the classical EDP-M protocol (etoposide, doxorubicin, cisplatin, mitotane). However, clinical translation of novel therapeutic regimens remains challenging due to high tumor heterogeneity. Thus, to obtain preclinical results with more clinically predictive power we investigated for the first time LEDP-M comparatively in two further xenograft models for ACC *in-vivo*: SW-13 and the novel pediatric tumor model SJ-ACC3. Furthermore, we included liposomal etoposide resulting in a novel treatment scheme called L(l)EDP-M. In short-term experiments, after one treatment cycle, tumors were immunohistochemically [cells/high-power fields (HPF)] analysed regarding total cell number [Ki67 positive and negative/HPF] and apoptosis [TUNEL positive cells/HPF]. The number of tumor cells decreased for SW-13 in all treatment groups with highest therapeutic efficacy for the liposomal variants (EDP-M: 20.5±1.6 $p<0.01$; LEDP-M: 17.2±1.3 $p<0.001$; L(l)EDP-M: 14.7±0.9 $p<0.001$) vs. controls (28.9±2.2). In contrast, in SJ-ACC3 xenografts only EDP-M (30.3±1.2 vs. controls 35.9±1.3, $p<0.05$) led to short-term therapeutic effects. Long-term experiments confirmed for SW-13 antitumoral efficacy upon all treatments compared with controls ($p<0.001$) and for L(l)EDP-M furthermore improved overall survival compared to EDP-M ($p<0.003$). Moreover, while H&E-stainings revealed strong nephrotoxic effects upon repeated treatments with EDP-M, L(l)EDP-M was associated with improved off-target profiles. In summary, liposomal regimens hold promise for clinical translation mainly for adult, but less so for pediatric ACC. However, our results also show that tumor heterogeneity should be taken more into account in preclinical studies.

FATE1 counteracts apoptosis in adrenocortical tumoral cells by uncoupling endoplasmic reticulum and mitochondria

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Steroidogenic Factor-1 dosage has a critical role in regulating the proliferation of human adrenocortical cells and to trigger adrenocortical tumorigenesis in mice. We identified *FATE1* (Fetal and Adult Testis Expressed), a cancer testis antigen, as a new target gene for SF-1.

The aim of our study was to determine the cellular function of FATE1 in human adrenocortical tumoral cells.

We showed that FATE1 is an outer mitochondrial membrane protein and is localized in MAM (Mitochondrial Associated Membranes). MAM are specialized subdomains that have a particular relevance in cancer and play an important role in metabolism, cell survival, cell death and intracellular calcium.

We demonstrated that FATE1 is a new modulator of ER-mitochondria distance and mitochondrial Ca^{2+} uptake in adrenocortical cells. Interestingly, FATE1 decreases sensitivity to mitochondrial Ca^{2+}-dependent proapoptotic stimuli and to mitotane, the chemotherapeutic drug currently used in adrenocortical cancer (ACC). Furthermore, a strong negative correlation exists between FATE1 expression with recurrence-free and overall survival in ACC patients, and high FATE1 expression is associated with poor clinical outcome.

Our findings highlight the novel role of FATE1 on ER-mitochondria distance and calcium influx in mitochondria in adrenocortical cancer cells to resist to apoptotic death and the action of mitotane. Considering the restricted expression pattern of this cancer testis antigen in adrenocortical tumors but not in normal tissues, FATE1 targeting may represent a new strategy to sensitize cancer cells to the effect of chemotherapy.

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XAV939 and XL147: study of the antiproliferative effect of two new compounds in adrenocortical cell lines

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Background. Adrenocortical tumors (ACT) include benign and malignant forms. While benign adrenocortical adenoma (ACA) is the most frequent, ACC (adrenocortical carcinoma) is a rare neoplasm with poor prognosis. Several studies have revealed a wide variety of signaling pathways involved in these tumors, among these Wnt/β-catenin and PI3K/Akt/mTOR pathways resulted often deregulated.

Aim: to evaluate the *in vitro* efficacy of two drugs: **XAV939** and **XL147**, acting on Wnt/β-catenin and on PI3K/Akt/mTOR pathways.

Methods. Cell viability, cell cycle analysis, wound healing assay, colony formation assay were performed. The expression of key regulators of these two pathways were studied by Western blot and by qRT-PCR analysis.

Results. In H295R and SW13 cells, all compounds showed a time and dose-dependent response. Combination of two drugs at 24h did not showed a synergistic effect (CI= 1.2) while at 72h CI=0.88 indicated synergistic effect. Treatments blocked cell growth and decreased the number of colonies and the ability to migrate. A moderate decrease of β-catenin expression and an increase in Axin1 and Tankyrase1-2 expression were observed in both cell lines treated with XAV939. A decrease of phospho-Erk1/2 was observed in SW13 cells treated with XL147 and a slight decrease of P70 and Akt was perceived in H295R cells treated with XL147.

Conclusion. The antiproliferative effects of XAV939 and XL147 have been investigated in adrenocortical cell lines. The results obtained are promising, but further studies in primary adrenocortical tumor cells and on ACC xenograft mouse models are in progress to fully explore their role as potential anticancer drugs.

Intra-Tumour heterogeneity of *TERT* Promoter Mutations in Adrenocortical Carcinoma

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TERT is a member of the telomerase enzyme complex, which maintains telomere length. TERT expression is repressed in normal cells with subsequent telomere shortening upon time leading to cell senescence. Evasion of this regulatory mechanism is often found in cancer cells. Recently *TERT* promoter mutation leading to increased TERT expression and aberrant telomere lengthening was found in different types of malignancies including adrenocortical carcinoma. In this study we have analyzed *TERT* promoter mutation status in a large cohort of adrenocortical tumors (n=219), consisting of 95 aldosterone producing adenomas, 55 adrenocortical adenomas and 69 adrenocortical carcinoma. The previously described mutation C228T (-124bp) was found in six tumors and restricted to adrenocortical carcinoma. Multi-regional sampling was performed on 16 tumors resulting in an additional 95 samples. The multi-regional sampling revealed a heterogeneous state of the mutation in two tumors. Copy number variation analysis revealed copy number gain at the *TERT* locus in 53/69carcinoma tumors, but none in the 150 analyzed benign adrenocortical tumors. In conclusion, *TERT* promoter mutations and gene amplifications are frequent in adrenocortical carcinoma but rare (or absent) in benign adrenocortical tumors. A heterogeneous state of *TERT* promoter mutations in adrenocortical carcinoma suggests that this event is more likely a facultative event than obligatory in tumorigenesis, with a possible alternate function to facilitate carcinoma evolution and/or propagation.

Adrenocortical carcinoma cell-derived miR-483-5p and miR-139-5p promote angiogenesis

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Introduction: The interaction between tumor cells and their microenvironment is a crucial aspect of tumor development. Cancer cell-derived microRNAs are secreted in vesicles called exosomes, which allow communication between cells via the transfer of their cargo. We have shown previously that miR-483-5p and miR-139-5p are upregulated in adrenocortical carcinoma (ACC) as well as in the serum of ACC patients. Our aim was to evaluate whether ACC-derived miR-483-5p and miR-139-5p could be transferred to endothelial cells and modify their phenotype.

Methods: MiR-483-5p and miR-139-5p levels were measured in conditioned medium (CM) from NCI H295R cells transfected with miR-483-5p or miR-139-5p inhibitors. Human umbilical vein endothelial cells (HUVEC) were incubated in NCI H295R-derived CM to evaluate their migration and organization capacities. Exosomes from NCI H295R cells were isolated and visualized by electron microscopy. The uptake of GFP-CD63-labeled exosomes by HUVEC was monitored using fluorescence microscopy.

Results: Silencing miR-483-5p and miR-139-5p in NCI H295R cells decreased their extracellular release and impaired HUVEC migration and tube formation in CM. Electron microscopy visualization of NCI H295R-derived exosomes revealed a characteristic cup-shaped morphology, with a diameter of approximately 100 nm. Quantitative PCR analysis indicated that miR-483-5p and miR-139-5p were present in exosomes. NCI H295R cells-derived GFP-labeled exosomes were efficiently internalized by HUVEC.

Conclusion: Our results suggest that NCI H295R cells could transfer miR-483-5p and miR-139-5p to endothelial cells via exosomes and modulate their angiogenic capacities. Characterization of the mechanisms responsible for the reduced angiogenic response of HUVEC to miR-483-5p and miR-139-5p suppression is under investigation.

PRKACA mutations in aldosterone producing adenomas

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Aldosterone producing adenomas (APA) are the most frequent cause of primary aldosteronism (PA). Somatic mutations of *KCNJ5, ATP1A1, CACNA1D, ATP2B3* and *CTNNB1* are involved in APA formation. In contrast, mutations of *PRKACA* coding for the catalytic subunit of protein kinase A have been identified in cortisol producing adenomas.

We performed exome or bidirectional Sanger sequencing of tumor-tissue from 109 patients with APA and evaluated mutations in candidate genes and *PRKACA*. In addition to candidate gene mutation, exome sequencing revealed *PRKACA* somatic mutations in two APAs (1,8%). One APA carried a L206R mutation, previously described only in cortisol-producing adenoma while in the second case a newly identified H88Y mutation was found. Both affected patients were females with hypokalemic hypertension, aldosterone excess and lateralization during adrenal venous sampling. We functionally characterized the enzymatic activity of mutated PKA catalytic subunit *in vitro* and immunoexpression of steroidogenic enzymes in affected tissues.

Functional analysis showed that the newly found H88Y mutation was not associated with gain of function of PKA. Interestingly, while CYP11B2 expression was found in the H88Y-mutated APA, no co-expression of CYP11B1 was present. In contrast, in the L206R-mutated APA, CYP11B1 was expressed while CYP11B2 was weak or negative. Accordingly, biochemical Cushing's syndrome was present only in the patient with the L206R mutation. Following adrenalectomy, both patients improved with a reduced number of antihypertensive medications and normalized potassium levels. Overall, *PRKACA* mutations are rare findings in APAs. As cortisol co-secretion occurs in a sub-group of APAs, other molecular mechanisms are likely to exist.

ZNRF3 a new tumor suppressor gene in adrenocortical tumors

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Adrenocortical carcinoma (ACC) is a rare and highly aggressive tumor with very poor prognosis. Up to now, somatic inactivating mutations of the tumor suppressor gene *TP53* and activating mutations of the protooncogene βcatenin were the most frequent mutations identified in ACC. By a combination of genomic approaches, we have recently analyzed a cohort of 122 ACC, recruited from the European Network ENSAT. This work confirmed recurrent alterations in *CTNNB1* and *TP53* and revealed new genes not previously reported to be altered in ACC. Strikingly, *ZNRF3* (zinc and ring finger 3) was the most frequently altered gene (21%). In a majority of cases, homozygous deletions of *ZNRF3* were observed. *ZNRF3* had never been associated with other tumor types. This original finding suggests that *ZNRF3* could be a novel suppressor gene involved in ACC. Our objective is to demonstrate that *ZNRF3* acts as a tumor suppressor gene in adrenocortical cells and to identify molecular pathways downstream of *ZNRF3*. In adrenocortical cell line H295R, *ZNRF3* silencing by RNAi confers protection against apoptosis induced by staurosporin while *ZNRF3* overexpression increases apoptosis and decreases proliferation. Co-immunoprecipitation experiments were carried out to identify protein partners of *ZNRF3* by mass spectrometry. The choice of the partner(s) of *ZNRF3* identified was promoted by the function of the candidates. Further experiments are required to confirm these interactions and to identify signaling pathways underlying. The results of this research will help to progress toward our understanding of adrenocortical tumorigenesis involving *ZNRF3* alterations.

DNA methylation is an independent prognostic marker of survival in adrenocortical cancer (ACC)

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Previous ENSAT studies have demonstrated the prognosis value of tumor stage and Ki67 in ACC. However after stratification on tumor stage and Ki67, the prognosis remains heterogeneous, suggesting a need for additional markers. Recently pan-genomic analysis of tumor DNA identified CpG islands hypermethylation as pejorative.

Aim: To confirm the prognostic value of methylation on an independent cohort using a commercially available kit and to confront methylation to tumor stage and Ki67.

Methods: DNA methylation was measured by methylation-specific multiplex-ligation-dependent probe amplification (MS-MLPA) in the CpG islands of 27 genes using the ME002-B1 kit (MRC-Holland). MS-MLPA marker was set up in a training cohort of 50 ACC, then validated in an independent cohort of 203 ACC from 21 ENSAT centers. The validation cohort included 64% females, median age 50 years, 80% localized tumors (ENSAT stages I-III). Univariable and multivariable survival analyses were performed with Cox models.

Results: The best methylation marker was obtained as the mean methylation of 4 genes (PAX5,GSTP1,PYCARD,PAX6).
In this cohort, methylation was a strong predictor of disease-free survival (DFS, $p<0.0001$) and overall survival (OS, $p<0.0001$). Combining methylation, Ki67 and ENSAT stage in a multivariable prediction of DFS, methylation ($p=0.0005$) and ENSAT stage ($p<0.0001$) but not Ki67 ($p=0.19$) remained significant. For OS, methylation ($p=0.0006$), ENSAT stage ($p<0.0001$) and Ki67 ($p=0.024$) were independent predictors.

Conclusion: Tumor DNA methylation emerges as a predictor of recurrence and death in ACC patients. MS-MLPA is readily compatible with clinical routine, and should therefore complete the clinical and pathological prognostic markers for precision medicine.

Long Term Follow-Up in Patients Operated on a Phaeochromocytoma or a Paraganglioma: compilation of the ENSAT database

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Following resection of the primary tumour, patients with PPGL are at risk of tumour persistence and of new tumoral events. there are no robust prognostic indices of tumour recurrence in patients with PPGL to guide clinical practice .

Data from 6 centers were compiled, including 1153 patients with PPGL among whom 701 had: (i) resection of the primary tumor, (ii) no evidence of persistent disease at postoperative assessment, and (iii) documented follow-up of 6 months or more.

The ENS@T cohort gave access to individual data and enabled univariate and multivariate analyses of candidate prognostic markers. The records from 701 patients who had no evidence of persistent disease at postoperative assessment and who had a follow-up of 6 months or more were analyzed. Fifty-four percent were women, 80% had at least one phaeochromocytoma, and 34% had a genetic or syndromic disease. Median age at surgery was 46 years [interquartile range (IQR) 33, 57]. Median tumour size was 44 mm [IQR 30, 60], tumour size exceeding 50 mm in 44% of cases. Median follow-up was 54 months [IQR 25, 101]. The risk of new events in the whole population was 10% [95% CI 8, 14] over the first 5 years of follow-up (45% metastatic, 42% new tumour, 13% local recurrence). The incidence of new events did not decline after 5 year of follow-up, but estimates are imprecise after 10 years of follow-up.

These data show that 10% of the patients will have a recurrence or a new tumor during follow up.

[123/131I](R)-1-[1-(4-Iodophenyl)ethyl]-1H-imidazole-5-carboxylic acid azetidinylamide (IMAZA) a novel radiotracer for diagnosis and treatment of adrenocortical tumours - first clinical experience

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[$^{123/131}$I]Iodometomidate (IMTO), a specific CYP11B2- and CYP11B1-tracer, has been proven to be useful for molecular imaging in adrenal incidentalomas and radiotherapy in ACC. IMTO is, however, rapidly inactivated which impairs target-tissue to background activity ratios and therapeutic efficacy, >80 new IMTO derivatives have been designed und evaluated. Here we report the first clinical experience with the best derivative, [$^{123/131}$I]IMAZA, in patients with ACC.

28 patients with known advanced ACC were investigated by SPECT/CT and planar imaging up to 24 h after injection of 185 MBq [^{123}I]IMAZA. Blood levels of tracer and metabolites were determined by radio-HPLC. Dosimetry with 40 MBq [^{131}I]IMAZA was performed over 4 days followed by endoradiotherapy on compassionate use basis in 3 patients.

[^{123}I]IMAZA specifically accumulated in adrenocortical tissue. Comparable to IMTO, IMAZA showed heterogenous uptake in the tumour lesions. We observed rapid clearance of unbound tracer and an up to 5-fold higher tumour uptake compared to IMTO. Analysis of blood samples by radio-HPLC demonstrated significant higher metabolic stability of the tracer. Three patients received high doses of 26-28 GBq [^{131}I]IMAZA leading to tumour doses up to 265 Gy. Treatment was well tolerated. Comparison of FDG-PET and low-dose CTs before and 9 weeks after treatment revealed PD in two patients. After two treatment cycles, a third patient showed a significant metabolic response especially of small lesions in the lungs and liver and a decrease in size of this lesions was observed in CT.

[$^{123/131}$I]IMAZA is a promising radiotracer for molecular adrenal imaging and radiotherapy of adrenocortical carcinoma. The highly specific uptake in the target tissue leads to superior imaging quality and therapeutic potential compared to IMTO.

Abstracts

Poster Session

from all fields of Adrenal Tumors

Long term follow-up of patients with pheochromocytomas and paragangliomas from a regional center in Greece

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Background: Pheochromocytomas (PPCs) and paragangliomas (PGLs) are rare neuroendocrine tumors of the sympathetic and parasympathetic tissue. Although malignancy is defined by the presence of distant metastases, size, Ki-67, PASS and genetic alterations are considered as potential prognostic markers.

Methods: Fifty-three patients (46 PPCs and 7 PGLs) were referred to our tertiary center over the last 15 years. Radiologic and hormonal evaluations were performed at baseline and during the follow-up and were evaluated retrospectively.

Results: Eleven out of 53 (20.7%) patients had metastases either at the time of the diagnosis or during the follow up. No difference in the risk of metastases was found between PPCs and PGLs in this cohort. Patients with metastatic disease were diagnosed at younger age compared to those without metastases (p=0.001). Hormonal secretion did not differ significantly between two groups. Anatomical imaging (CT and MRI) detected the primary tumor in 100% and 94% of the cases respectively whereas MIBG confirmed the diagnosis in 77% and octreoscan in only 66% of PPCs/PGLs. Size and PASS score did not differ between patients with metastatic and patients with no metastatic PPCs/PGLs. However Ki-67% was significantly higher in patients with metastatic disease (p= 0.037). Mortality was significanlty higher in patients with metastatic PPCs/PGLs compared to those without metastases (p=0.04, HR=6.6, 95%CI:1.1-41).

Conclusions: Ki-67% was found to be the most important prognostic marker for metastatic disease in our series. Conventional imaging presented the greater sensitivity for the detection and follow up of PPCs/PGLs. Mortality rate remains significantly higher in metastatic PPCs/PGLs.

RAR signaling contributes to adrenal morphology and functional zonation

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Primary aldosteronism is the most common form of secondary hypertension. Recurrent somatic mutations in *KCNJ5, CACNA1D*, *ATP1A1* and *ATP2B3* have been identified in aldosterone producing adenoma (APA). In parallel, we have identified retinoic acid receptor (RAR) signaling as a central molecular network involved in APA formation independently of the mutation status. The aim of our study was to evaluate the role of RAR signaling in the development of APA.

Whereas treatment of H295R cells with 10^{-6}M of all trans retinoic acid (ATRA) did not affect cell viability, 10^{-5}M of ATRA dramatically decreased cell number in a time-dependent manner. This effect was due to decreased cell proliferation and increased cell apoptosis. However, 9-cis RA did not modify tumor growth in a mouse xenograft model. RARa invalidation by shRNA transduction in H295R cells did not affect their proliferative properties, but induced a major change in cellular phenotype with cells forming spheroid structures able to grow in suspension. Investigation of the adrenal phenotype of *rara* knock-out mice demonstrated that in young and old *rara*$^{-/-}$ mice the characteristic cellular arrangement of the adrenal cortex was replaced by an enlarged zona glomerulosa and a disorganized zona fasciculata, this effect being more pronounced in old mice.

Our results suggest that RAR signaling contributes to normal adrenal morphology and functional zonation. Disruption of RAR signaling could trigger abnormal proliferation of cells in the adrenal cortex, creating a propitious environment for the emergence of specific mutations affecting ionic channels and ATPases leading to increased aldosterone production.

HIGH EXPRESSION OF C-X-C CHEMOKINE RECEPTOR TYPE 4 IN THE ZONA GLOMERULOSA AND IN ALDOSTERONE PRODUCING ADENOMA

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Introduction: Chemokines are small secreted molecules that promote cell survival, proliferation and migration. We recently observed high CXCR4 mRNA expression in normal adrenals (NA) and adrenocortical carcinomas. A PET tracer for selective molecular imaging of CXCR4-expression has recently been established.

Objective: To further investigate CXCR4 expression in NAs and in adrenocortical adenomas (ACA) and to estimate its potential as a target for molecular imaging in primary hyperaldosteronism.

Methods: CXCR4-expression was evaluated by quantitative PCR and immunohistochemistry in specimens of 2 NAs, 117 aldosterone producing adenomas (APA), 25 non-functioning adenomas (NFA), 52 cortisol producing adenomas and in the adjacent adrenal cortex of 194 ACAs. In addition, the expression of its ligand CXCL12, CYP11B2 and 11-beta-hydroxylase was analyzed. Furthermore, we performed binding studies of [68Ga]Pentixafor to frozen tumor tissue of APAs, NFAs and NAs.

Results: In NAs, strongest CXCR4 staining was found in the outer adrenal cortex covering the CYP11B2 positive zona glomerulosa while its ligand CXCL12 was expressed in the inner cortical zone. Both qRT-PCR and immunohistochemistry indicated high CXCR4 expression in most APAs which was significantly higher compared to NFAs (p=<0,0001). [68Ga]Pentixafor exhibited strongest binding to kryosections of APAs, whereas binding to NFAs was significantly lower.

Conclusion: The expression pattern of CXCR4 and CXCL12 in NAs suggests that it may play a role in ultrastructural organization of the adrenal cortex. Because of the high expression in APAs compared to NFAs and the strong binding of [68Ga]Pentixafor to APAs, CXCR4 may be a suitable target for molecular imaging in PA.

PATCHED AS A NEW THERAPEUTIC TARGET FOR ADRENOCORTICAL CANCER

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Keywords: adrenocortical cancer, Patched, multidrug transporter, drug efflux, doxorubicin

Adrenocortical cancer has very poor prognosis with an estimated survival rate of 35% in 5 years and until today pharmacological therapy is based on mitotane in combination with doxorubicin, cisplatin and etoposide. Unfortunately, this treatment has very low response rate and high toxicity on various organ systems.

We have showed that members of Hedgehog pathway and its receptor Patched are overexpressed in human adrenocortical H295R cells. This was also proved by a team of clinicians on cohort of 99 patients. We have showed that Patched is a multidrug transporter onvolved in the resistance of cancer cells to chemotherapy.

Therefore, our laboratory is interested in developing a new treatment for adrenocortical cancer by blocking drug efflux activity of Patched. During the past few months we have done the screening of compounds from the chemical library in combination with doxorubicin on human adrenocortical H295R cells and yeast expressing human Patched. We observed that some compounds inhibit doxorubicin efflux activity of Patched and increase the effect of doxorubicin on viability, proliferation, clonogenicity and apoptosis of H295R cells.

Ongoing project is testing these compounds in vivo.

Epigenetic mutation of the succinate dehydrogenase C promoter in paraganglioma - case report

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Succinate dehydrogenase (SDH) gene mutations are a well-known cause of tumor development in pheochromocytomas/ paragangliomas (PPGL). Epigenetic inactivation by cytosine methylation of the SDHC gene is a more recently discovered phenomenon, which so far has only been described in paraganglioma patients with Carney triad syndrome and gastrointestinal stromal tumors (GISTs). A 33-year old patient presented with two abdominal paragangliomas (PGL) and an adrenocortical adenoma. Both PGLs showed high succinate:fumarate ratios indicative of SDHx-mutations; however, no mutations in any of the known PPGL susceptibility genes were found in leucocyte or tumor DNA. We identified methylation of the SDHC promoter region in both PGLs, which coincided with decreased SDHC expression at mRNA and protein levels and a hypermethylated epigenomic signature (CIMP-phenotype). Low level SDHC promoter methylation was also observed in the adenoma but not in normal adrenal tissue or blood, suggesting postzygotic somatic mosaicism for SDHC promoter methylation in the patient.

Our findings suggest that SDHC promoter methylation may also occur in sporadic PPGLs, emphasizing the importance of considering epigenetic changes and functional readouts in the genetic evaluation of patients not only with GISTs and Carney triad but also with PPGL.

Investigating the possible role of metformin as anticancer agent in H295R cells.

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Despite its rarity, adrenocortical carcinoma presents a very aggressive behavior and a poor prognosis when metastatic at diagnosis. Since radical surgery, associated with mitotane adjuvant therapy, is currently the only available treatment, more specific and effective drugs are urgently required. The use of metformin, a well-established and effective agent for the management of type 2 diabetes mellitus, has been associated to beneficial effects on cancer prevention and treatment, leading to increasing interest in its potential use as an anticancer agent.

Here we evaluate the potential in vitro anti-cancer effect of metformin on the adrenocortical cancer cell line H295R.Increasing doses of metformin affect cell viability and proliferation in a dose- and time-dependent manner, as demonstrated by MTS, cell counting and [^{3}H]thymidine incorporation assay. This anti-proliferative effect seems to be mediated by metformin-induced arrest of the cell cycle as well as by stimulated apoptosis, as investigated by cytofluorimetric assays. Western blot analysis of cell lysates after 6- and 24-hour metformin treatment was used to identify the molecular pathways involved in mediating the drug effect and revealed a dose-dependent inhibition of ERK1/2 phosphorylation and a delayed increase in phosphorylated AMPK, which associates with a decrease in mTOR phosphorylation and IGF-1R expression.

Our data indicate that metformin is effective in inhibiting proliferation in H295R cancer cells, showing an effect which depends on the drug concentration and treatment duration. Further studies are necessary to understand the mechanism of action of this drug and to evaluate its potential role in the treatment of adrenocortical carcinoma.

Bone morphogenetic protein signaling as novel therapeutic target in pheochromocytoma

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Rats affected by the MENX syndrome develop bilateral pheochromocytoma (PCC) with complete penetrance. Gene expression profiling of rat PCC identified *Bmp7* (bone morphogenetic protein 7) as highly expressed in the tumors. By analyzing PCC patients, 72% of cases showed elevated expression of BMP7 in tumor cells, as well.

BMP7 plays pro- or anti-oncogenic roles in cancer in a cell type-dependent manner. To address the role of *Bmp7* in PCC, its level in PCC cell lines was modulated and functional assays were conducted. We observed that upregulation of *Bmp7* enhanced proliferation, migration and invasion of PC12 cells, while downregulation of *Bmp7* impaired these properties in MPC and MTT cells and reduced the viability of rat primary PCC cells.

The small molecule inhibitor DMH1 inhibits selectively BMP type I receptors. To verify whether blocking BMP receptor signaling might be a potential strategy for a targeted therapy of PCC, MTT cells were treated with DMH1 and cell proliferation and migration were significantly suppressed in a dose- and time-dependent manner. Concomitantly, a dose-dependent downregulation of molecular readouts of active BMP signaling in PCC cells was observed. Furthermore, the treatment of MENX rat primary PCC cell cultures with DMH1 was associated with a dose-dependent decrease in cell viability.

Our data demonstrate a pro-oncogenic role for BMP7 in PCC and propose BMP signaling as a novel therapeutic target. Indeed, DMH1 elicits anti-proliferative and anti-migratory responses in PCC cells with active BMP signaling *in vitro* and should in the future be evaluated in PCC models *in vivo*.

Targeting Tyrosine-Kinase in Adrenocortical Carcinoma cell lines

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The main therapeutic option for Adrenocortical Carcinoma(ACC) is surgery. However medical treatment is tried, with Mitotane and/or chemotherapy, with variable results. Understanding the molecular mechanisms that regulate ACC could be useful to identify new therapeutic options. Our study aim to explore the role of different tyrosine-kinase in ACC biology. For this purpose we tested Sunitinib, a multi target receptor tyrosine-kinase inhibitor, Erlotinib a selective EGFR inhibitor, and SCH772984, an ERK1/2 inhibitor, on 2 human adrenocortical carcinoma cell lines (SW13 and NCI-H295). We found that EGF increased proliferation and reduced apoptosis only in SW13 while it did not affect NCI-H295. Sunitinib reduced cell viability in both cell lines, being counteracted by EGF in SW13. We analysed in both cell lines the expression of EGFR family members, which are more expressed in SW13 as compared to NCI-H295. Moreover, we investigated the intracellular signal transduction pathway of EGF in ACC cells. Our results show that in SW13 Sunitinib inhibited EGFR phosphorylation, and counteracted EGF-induced phosphorylation of ERK1/2. In addition we tested Erlotinib in the 2 cell lines. We found that Erlotinib was capable of reducing cell viability and activating caspase 3/7 in SW13, having no effects on NCI-H295. Moreover, Erlotinib inhibited EGFR phosphorylation, and decreased ERK1/2 activation. On the other hand SCH772984 was able to significantly reduce NCI-H295 cell viability, and to activate caspase 3/7. In SW13, SCH772984 blocked the effects of EGF. Our data suggest that EGF/ERK pathway could represent new targets in drug design for ACC treatment.

Long-term outcome after surgery for incidentally-diagnosed subclinical cortisol-secreting adenomas

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Background: Management of subclinical cortisol-secreting adenomas (SCSA) is controversial and available evidence is lacking to assess the superiority of a surgical versus a nonsurgical approach. The aim of this work was to report postoperative and long-term outcome after surgery for incidentally-diagnosed SCSA and compare it with patients having undergone surgery for a cortisol-secreting adenoma (CSA)

Methods: From 1994 to 2011, 107 consecutive patients underwent laparoscopic unilateral adrenalectomy for either SCSA (n=39) or CSA (n=68). Preoperatively, all patients underwent standardized clinical, hormonal, and imaging assessment. Patients were followed for at least two years with serial assessment of body weight, blood pressure, and HbA1c.

Results: Surgical resection of SCSA and CSA did not significantly differ regarding operative time, conversion rate, overall surgical and medical morbidity, and length of stay. For SCSA, comparison between preoperative status and two-year assessment showed a median weight loss of 6% ($p<0.001$), a decrease in median HbA1c of 15% ($p<0.001$), and an improvement or normalization of blood pressure in 50% of patients. Same significant metabolic beneficial effects of surgery with greater improvement were observed in CSA patients.

Conclusion: LA for SCAS is associated with low morbidity, no mortality, and significant improvement of various aspects of metabolic syndrome. Until additional evidence from prospective randomized controlled studies is obtained, LA should be considered as a valid option in the care of patients with SCSA.

Adrenocortical carcinoma with mixed response to sequential therapy – rational to investigate clonal heterogeneity?

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A 48 years old lady presented with Cushing's syndrome and was diagnosed with a left sided adrenal tumour 5cm in size. Staging revealed metastases to the liver. She was operated with adrenalectomy, liver resection and radiofrequency ablation. Pathology report confirmed adrenocortical carcinoma with a Ki67 index of 30%. Postoperativly there were no macroscopic signs of residual tumour. She was started on adjuvant treatment with (1st line) streptozocine + mitotane. New metastases to the liver and bone were noted and the therapy was changed to (2nd line) etoposide, doxorubicin and cisplatin + mitotane. This resulted in stable disease but the therapy had to be discontinued due to side effects. The patient was started on (3rd line) Paclitaxel and was stable for 12 months. Upon progression she started (4th line) treatment with sunitinib. At baseline there were bilateral lung metastases and well as multiple lesions in bone and liver. Evaluation after 3 months revealed a partial response of the metastases in lung and bone but progressive disease within the liver. It was decided to perform local intervention to the liver with radioembolization using Y90-SIR-Spheres and to maintain sunitinib treatment. Subsequent evaluation showed a partial response in the liver and the patient was stable for a total of 18 months from start of sunitinib. The patient diseased 51 months after diagnosis.

Urinary free cortisol measured by LC-MS/MS to rule out Cushing's Syndrome in patients with adrenal incidentaloma

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Introduction and aim: All patients with adrenal incidentaloma (AI) should be investigated for hypercortisolism, the Cushing's syndrome (CS). As recommended by the Endocrine Society, the screening tests for CS are 1-mg dexamethasone suppression test (1-mg DST), late-night salivary cortisol (LNSC), or urinary free cortisol (UFC) measurement. We examined the diagnostic performance of the first-line screening tests for CS in a large series of subjects.
Materials and Methods: We retrospectively collected 237 patients, 191 with AI and 46 with confirmed CS as control group. UFC was measured by liquid chromatography tandem-mass spectrometry (LC-MS/MS), LNSC by radio-immunometric method and serum cortisol by chemiluminescence.
Results: In the whole population, cortisol levels <138 nmol/L after 1-mg DST revealed high specificity (SP, 93%), while the 50 nmol/L cutoff revealed the best sensitivity (SE, 100%) and the worst SP (62%); SE and SP for LNSC were respectively 86% and 92%, and for UFC were 98% and 91%. Overall, UFC revealed the best combination of the highest positive (10.8) with the lowest negative (0.02) likelihood ratio among first-line tests. Similar results were obtained considering the group of patients that performed all 3 first-line screening tests (99 AI and 38 CS), confirming low SP (60%) of 50 nmol/L cutoff after 1-mg DST. UFC was at least as good as each other test alone, paired (DST+LNSC, DST+UFC and LNSC+UFC), or combined (DST+LNSC+UFC) in the ROC-contrast analysis.
Conclusions: Measuring UFC by LC-MS/MS achieves the best accuracy to detect CS among patients presenting with AI.

The role of chronically elevated levels of luteinizing hormone in the cause-effect relationship between the size of adrenal incidentaloma and insulin resistance

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Patients with adrenal incidentalomas (AI), nonfunctioning (NAI) and with subclinical hypercortisolism (SH) have been associated with a high prevalence of insulin resistance (IR). Studies have demonstrated that an increase in IR is related to the size of adrenal tumorous mass (ATS) with dilemma weather hyperinsulinemia plays a role in the growth of the AI. It has also been proposed that luteinizing hormone (LH) could enhance the adrenal steroid production and adrenal tumorigenesis as well.

The aim was to asses the relationship between IR, ATS and LH in nondiabetic menopausal women with AI, NAI and SH.

77 women with AI were drawn from a series of patients who underwent hormonal evaluation in our Department, mean age 60.75±7.4, mean BMI 27.60±4.47kg/m^2, mean FSH 95.83±44.05mIU/l, mean LH 30.66±14.5mIU/L, mean menopause duration at the moment of AI discovery 11.76±7.9years. To evaluate IR we used HOMA index.

49 patients had NAI and 28 had AI with SH. There was no significant difference in age, BMI and LH between NAI and SH. Multivariate regression analysis using HOMA as dependent variable and ATS, LH, cortisol after 1mg dexamethason suppression test, BMI, age and duration of menopause showed that ATS ($p=0.016$, $\beta=.340$) and LH ($p=0.047$, $\beta=.241$) were the predictors of IR. This significance got stronger in the group with SH, ATS ($p=0.001$, $\beta=.600$), LH ($p=0.032$, $\beta=.369$).

The results of our study show that not only ATS but also chronically elevated LH might play a significant role in IR in patients with AI.

Nicotinamide nucleotide transhydrogenase (NNT) as a novel molecular target in adrenocortical carcinoma - impact of NNT knockdown on adrenocortical cell proliferation, redox balance and steroidogenesis

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Nicotinamide nucleotide transhydrogenase (NNT) is a NADPH-generating mitochondrial proton pump with a central role in mitochondrial antioxidant pathways. Recent studies revealed inactivating NNT mutations in patients with familial glucocorticoid deficiency, indicating a selective susceptibility of the adrenal cortex to NNT deficiency and oxidative stress. Here we explored the potential value of NNT as a therapeutic target in adrenocortical cancer. We delineated the distinct effects of NNT loss on cellular proliferation and steroidogenesis, employing two *in vitro* knockdown model: transient, siRNA-mediated knockdown and stable, shRNA-mediated knockdown of NNT in NCI-H295R cells. Transient NNT knockdown impaired cellular redox balance, resulting in a lower ratio of reduced to oxidised glutathione. Assessment of cellular growth kinetics revealed a decrease in proliferation with NNT knockdown, and massive cell death with exposure low doses of oxidising agents. After long-term culture, H295R cells with stable NNT knockdown appeared to develop compensatory mechanisms, restoring their redox balance and proliferative potential. This adaptation was associated with alterations in glycolytic and oxygen consumption rates. Steroid profiling by liquid chromatography-tandem mass spectrometry revealed a distinct profile induced by transient NNT knockdown, comprising lower 17OH-progesterone but, surprisingly, higher androstenedione and cortisol synthesis, with a pronounced increase in 11β-hydroxylase activity. A similar steroidogenic pattern was observed with stable knockdown. Our study suggests a potential role of NNT inhibition as a novel therapeutic approach in advanced adrenocortical carcinoma. Steroid profiling reveals a surprising increase in glucocorticoid synthesis with NNT loss, challenging the previous association of impaired redox balance with adrenal insufficiency.

Advancing Care of Primary Aldosteronism in Japan Study (JPAS): A new systematic multicenter Cohort study on the diagnosis and treatment

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Primary aldosteronism (PA) is one of the common causes of hypertension. Although clinical guidelines have been published, details of the individual steps varied from each other. Aim: To establish evidence for the clinical guideline of PA. Methods: We organized Japan PA study (JPAS) group with 24 referral hospitals by extending our previous WAVES-J with 9 institutes. Patients diagnosed as PA by a positive result in at least one confirmatory testing and underwent AVS between Jan 2006 and Dec 2014 was enrolled. Three major clinical issues included 1) prediction of subtype diagnosis by non-invasive methods, 2) standard protocol and decision criteria of AVS, and 3) comparisons of surgical and medical treatments. The Cohort study was approved by the ethics committee of Kyoto Medical Center and conducted according to the Guideline for Clinical Study in Japan. Currents state: Preliminary survey demonstrated more than 1500 cases could be included in the database. 14 research topics related to the major clinical questions were selected by the Steering committee. All the clinical data will be collected through the WEB after validation. After completion of the WEB and data registration, all research analysis will be done. Conclusions: We have launched the first nation-wide Cohort for PA as the principle infrastructure. Evidence from the study will be essential for improving the quality of clinical guideline. WEB in 2 languages will enable a global collaboration of PA and other adrenal tumors.

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Biochemical and functional characterization of a murine pheochromocytoma cellular model: Role of THE microenvironment

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Solid tumors are very complex tissues comprising not only cancer cells, but also non-malignant stromal cells such as endothelial cells, fibroblasts, immune cells and extracellular matrix, forming the so called tumor microenvironment. In the last few years, it has become more and more evident that the tumor microenvironment plays a pivotal role in modulating cancer progression and metastasis, thus becoming a potential therapeutic target.

In this work, we used two different cell lines: the first was isolated from mouse pheochromocytoma (MPC); the second was derived from the MPC cells and therefore considered metastatic, termed MTT (mouse tumor tissue-derived cells) with a more aggressive phenotype.

At first, we evaluated the possible metabolic and proliferative differences between the two cell lines with the purpose to define their specific profile. When compared to MPC cells, MTT cells showed an altered metabolism characterized by an increase of lactate uptake, while glucose uptake and oxygen consumption remained similar between the two cell lines.

Additionally, MTT cells showed a significant increase in cell proliferation. Interestingly, when co-cultured with mouse primary fibroblasts, both the two cell lines showed a significant increase in cell proliferation versus their mono-cultured counterparts. This effect was even more strikingly evident in co-cultured MTT cells, which showed a 500% increase in cell proliferation vs their mono-cultured counterpart.

Our data demonstrate, that microenvironment, here represented by fibroblasts, strongly affects tumor cell growth capacity and this effect is even more evident in the metastatic cell model compared to the primary tumor cell model.

Identification of candidate genes in a mouse model with hyperaldosteronism

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In an attempt to define novel genetic mechanisms involved in the pathophysiology of primary aldosteronism a mutagenesis screen after treatment with the alkylating agent N-ethyl-N-nitrosourea was established for the parameter aldosterone. One of the identified mouse lines with hyperaldosteronism was characterized phenotypically and genetically. Upon exome sequencing point mutations could be identified in nine candidate genes (*Sspo*, *5730446D14Rik*, *Dguok*, *Clstn3*, *Rrad*, *Atm*, *Tipin*, *Mapk6* and *Matr3*). Animals were grouped to wild type and mutated according to their genotype for each of these candidate genes and a correlation of their genotype to their aldosterone, urea, and creatinine values as well as steroidogenic enzyme expression levels was performed in each group. Although aldosterone, urea and creatinine levels did not differ significantly among wild types and mutated animals in any of the nine 9 genes, the expression levels of steroidogenic enzymes showed significant variation among wild type and mutated mice. More specifically, a significant *Star*, *Cyp11b1* and *Cyp11b2* overexpression could be documented in adrenals from animals carrying mutations in five different candidate genes, whereas *Hsd3b1* overexpression was observed in seven and *Cyp11a1* overexpression in three different candidate genes. It remains so far unclear which of these mutations could be causative for the described phenotype so that further experiments (i.e. renin and corticosterone measurement) need to be performed to narrow down the list to a single causative genetic variant.

Evaluation of the inhibitor of apoptosis protein livin/BIRC7 expression in adrenocortical tumors.

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Livin/ML-IAP/BIRC7 is a member of the inhibitors of apoptosis proteins family, which are involved in tumorigenesis through the inhibition of caspases. Aim was to investigate the expression of livin/BIRC7 and its pathway in adrenocortical tumors and its relationship with histopathological and clinical parameters. Specifically, *BIRC7, caspase-3* and *Smac/DIABLO* mRNA levels were evaluated by qRT-PCR in 84 fresh-frozen tissues (34 carcinomas=ACCs, 25 adenomas=ACAs, 25 normal adrenal glands=NAG), including 19 paired samples of tumor and surrounding adrenal tissue. Livin protein expression was assessed by immunohistochemistry in 286 paraffin-embedded tissues (195 ACCs, 67 ACAs, 24 NAG).

BIRC7 mRNA expression was higher in ACCs (0.06±0.12) than in both ACAs and NAG (0.01±0.01 and 0.01±0.02, respectively,*P<0.005*), being consistently higher in tumors than in surrounding adrenal tissues. C*aspase-3* levels were slightly higher in ACAs (0.024±0.012) than in ACCs (0.017±0.011*)* and NAG (0.018±0.011, *P=0.056*), being inversely correlated with tumor size (*P=0.005,HR=0.36*). No differences in *Smac/DIABLO* mRNA levels were found. In immunohistochemistry, livin cytoplasmatic expression was higher in ACCs than in ACAs and NAG (H-score:2.0±0.6 *vs* 1.75±0.6 *vs* 1.76±0.6 respectively, *P=0.08*). Otherwise, nuclear expression was higher in ACAs than in ACCs and NAG (H-score:1.3±0.7 *vs* 0.1±0.6 *vs* 0.8±0.7 respectively, *P<0.05*). No significant correlation was observed between protein levels and tumor size, Weiss score, ki67, metastasis and survival.

Conclusion: livin/BIRC7 is specifically over-expressed in ACCs, suggesting that it may be involved in adrenocortical tumorigenesis and represent a potential therapeutic target. Cytoplasmatic or nuclear distribution of livin could denote different functions of this protein that need to be further investigated.

THE METHYLATION PATTERN OF *IGF2* REGULATORY REGIONS AS A NOVEL BIOMARKER TO DISTINGUISH ADRENOCORTICAL CARCINOMAS FROM ADENOMAS

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To distinguish adrenocortical carcinomas (ACCs) from adrenocortical adenomas (ACAs) is challenging. High *IGF2* expression is associated with malignancy, but is not fully discriminating. We investigated whether methylation of *IGF2* regulatory regions could serve as a valuable biomarker to distinguish ACCs from ACAs. By pyrosequencing we analyzed methylation of the differentially methylated region-0 and -2 (DMR0, DMR2), the imprinting control region (ICR, consisting of CTCF3 and CTCF6) and the *H19* promoter. Expression of *IGF2* and *H19*, assessed by real-time quantitative PCR, and methylation percentages, were studied in three ACC cell lines (vehicle and demethylating drug 5'-Aza-2'-deoxycytidine (AZA) treated cells), 24 ACCs, 14 ACAs, and 11 normal adrenals. Methylation patterns in neoplasms were transformed into standard deviation scores based on methylation in normal adrenals, of which the diagnostic accuracy was assessed using Receiver Operating Characteristic.

In ACC cell lines, methylation of regulatory regions and *IGF2* mRNA expression decreased after AZA treatment, while *H19* mRNA expression increased. In adrenocortical neoplasms, methylation in the *H19* promoter, and the ICR, was higher in ACCs compared to ACAs ($p=0.001$, $p=0.008$, respectively). Methylation in the *H19* promoter, CTCF3, and DMR0, were positively correlated with *IGF2* expression in ACCs ($p<0.05$). Methylation in the most discriminating regions distinguished ACCs from ACAs with a sensitivity of 100%, specificity of 93% and an area under the curve of 0.997±0.005.

Conclusion: methylation patterns of *IGF2* regulatory regions appear to discriminate ACCs from ACAs with high accuracy, and may therefore be of additional diagnostic value in the assessment of malignancy of adrenocortical tumors.

EFFECTS OF TEMOZOLOMIDE ON HUMAN ADRENOCORTICAL CANCER CELLS AND THE ROLE OF THE O6-METHYLGUANINE-DNA METHYLTRANSFERASE GENE

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Patients with adrenocortical carcinoma (ACC), who are treated with mitotane, often deal with toxicity and tumor progression. We evaluated the effects of temozolomide (TMZ) on ACC cells and assessed the role of the DNA repair gene O6-Methylguanine-DNA methyltransferase (*MGMT*). Three ACC cell lines, and ACC primary cultures were studied. IC_{50} values of TMZ on cell growth were 17 µM for H295R and HAC-15, and 41 µM for SW13. In all cell lines, TMZ induced apoptosis ($p<0.001$), and had a cytotoxic and cytostatic effect by reducing the surviving fraction of ACC colonies ($p<0.001$) and the mean colony size ($p<0.05$). One of three primary cultures strongly responded to TMZ by cell number reduction and an induction of apoptosis, both already at 5 µM TMZ. At 25 µM TMZ treatment, H295R cells accumulated in the S and G2/M phase, and HAC-15 cells in the G2/M phase. After 7 days 25 µM TMZ treatment, growth of 3D spheroids was inhibited by 91.5% in H295R and 45.0% in HAC-15. *MGMT* promoter methylation was low in ACC cell lines (2.7%±1.0), normal adrenals (2.5%±1.8, *n*=6), adrenocortical adenomas (ACAs; 2.1%±0.84, *n*=14), and ACCs (5.3%±9.3, *n*=21). Mean relative *MGMT* mRNA expression was 0.13±0.10 in ACC cell lines, 0.20±0.23 in normal adrenals, 0.26±0.16 in ACAs, and 0.11±0.10 in ACCs. In ACCs, *MGMT* mRNA expression and methylation were inversely correlated ($\rho=-0.754$, $p<0.001$). Conclusion: despite *MGMT* expression, TMZ has antitumor effects on ACC cells at clinically relevant concentrations of TMZ (~25 µM). Clinical studies are warranted to assess efficacy *in vivo*.

Adrenocortical Carcinoma: Experience of a tertiary referral center in Greece

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BACKGROUND: Adrenocortical carcinoma (ACC) in adults is a rare tumor with an overall poor prognosis unless it is diagnosed early. SUBJECTS AND METHODS: Thirty patients (13 males) diagnosed with ACC since 2002 (median age of diagnosis 52.7 years) were retrospectively analysed considering tumor characteristics, stage, histological features (Weiss criteria), secretory status and clinical course. Thirteen patients presented with symptoms related to hormone hypersecretion and 2 due to local mass effect. Cortisol was the most frequently hypersecreted hormone. RESULTS: Six patients had metastatic disease at diagnosis. All, except one with unresectable metastatic disease, were submitted to an adrenalectomy. Histological diagnosis was based on a >=3 score according to the Weiss criteria. Overall median follow up was 37 months (2-139). Thirteen patients developed disease progression (PD) or relapse after a median progression free survival (PFS) of 7 months (2-58) irrespective of stage and treatment. Nine of the 13 patients with PD died after a median overall survival (OS) of 22 months (6-65). Capsular invasion was found to be a negative prognostic factor of relapse after surgery (OR 95%=13.33(1.3-134). Secreting tumours and those with a Ki-67 >7% were also associated with a worse OS [HR 95%=6.7(1.8-25) and 4.4(1.3-16) respectively]. CONCLUSIONS: Adrenocortical carcinoma, although rare, is a highly aggressive malignancy with poor overall survival, especially in secreting tumours and ki67 >7%. Our study also showed that histological invasion of the adrenal capsule correlated with disease progression after adrenalectomy.

Tumor tissue aldosterone content measurement for identification of aldosterone producing adenomas

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Unilateral primary aldosteronism is a prevalent and curable form of hypertension. Aldosterone producing adenomas (APAs) represent a common form of this disease. These tumours can present as solitary adenomas or display surrounding multinodular hyperplasia, raising the question of what nodules are functional. The recent development of an antibody directed against CYP11B2 have aided in the diagnosis of aldosterone production. In this study we wanted to investigate a new form of diagnosing functionality by direct measurement of aldosterone in tumour tissue. We included forty-seven APAs, an additional three nodules from multinodular unilateral PA, one breast tumour and one cortisol producing adenoma in the analysis. The cohort had previously been screened for mutations in *KCNJ5*, *ATP1A1*, *ATP2B3*, *CACNA1D* and *CTNNB1*. Immunohistochemistry for aldosterone synthase was performed. Aldosterone was measured in lysed tumour tissue using a chemiluminescent immunoassay used in routine clinical practice. All tumors with known mutations contained aldosterone at higher levels than the negative controls. No difference in aldosterone content was observed between APAs with G151R (n=13) and L168R *KCNJ5* mutations (n=11). *ATP1A1*/*ATP2B3* mutated tumours contained a significantly higher amount of aldosterone compared to *KCNJ5* mutated tumours, 4.3 vs 1.3 pmol/mg (p=0.0041). In the multinodular adrenals, a higher level of aldosterone was observed in the mutated nodules. CYP11B2 expression analysis showed low expression in nodules with low aldosterone content. Importantly, of the tumours classified as non-mutated, 6/13 (46%) did not contain aldosterone, suggesting a non-functional nodule. This method may represent an easy and inexpensive way of diagnosing aldosterone production.

Activating Mutations in *CTNNB1* in aldosterone producing adenomas

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Aldosterone producing adenomas (APAs) represent a common form of hypertension. The WNT signalling pathway is activated in APAs. A common cause of aberrant activation of the WNT pathway is somatic mutations in the *CTNNB1* gene. The frequency of these mutations in APAs is still unknown. We resequenced exon 3 of *CTNNB1* in a cohort of 198 APAs, including 114 (57.6%) APAs with mutations in *KCNJ5*, *ATP1A1*, *ATP2B3* and *CACNA1D*, and 84 (42.4%) tumors without mutations in known genes. We observed somatic *CTNNB1* mutations in 10 APAs (5.1% of the total cohort and 11.9% of the tumors without mutation). *CTNNB1* mutations occurred mutually exclusive to other known mutations. All *CTNNB1* mutated tumours displayed cytoplasmic/nuclear expression of β-catenin, or increased levels of activated β-catenin. Expression of the downstream target AXIN2 was increased in APAs with *CTNNB1* mutations. CYP11B1 and CYP11B2 expression analysis revealed two phenotypically distinct groups, one group displaying diffuse expression of CYP11B2 with low CYP11B1 expression, and the other group displaying diffuse CYP11B1 expression with heterogeneous expression of CYP11B2. Aldosterone measurement on tissue lysates from three tumours with *CTNNB1* mutations showed levels similar to *KCNJ5* mutated APAs. Tumours with *CTNNB1* mutations were of similar size as *KCNJ5* mutated APAs, but larger than non-mutated tumours, 6/10 were removed from female patients, no difference in S-aldosterone levels was observed. Mutations in *CTNNB1* occur in roughly 5% of APAs. These tumours are large and display a variable pattern of CYP11B1/CYP11B2 expression.

The Paradifference Foundation

Amanda Gustavsson

The Paradifference foundation, Svenljunga, Sweden

The Gustavsson family founded the Paradifference Foundation in February 2014. Our aim is to financially support research in the field of paraganglioma and pheochromocytoma, with special regard to malignant paragangliomas caused by the SDHB mutation. We hope to aid in the development of more efficient treatment, and essentially finding a cure for the disease. The foundation is registered in Sweden as a non-profit foundation. We have so far raised and commited over $ 10 million to projects in the field.

Cortisol producing adrenocortical carcinoma in a patient with Gardner's syndrome.

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Gardner's syndrome is an autosomal dominant syndrome caused by mutations in the APC gene. Adrenal masses are rare manifestations in Gardner's syndrome, and functional adrenal tumours are exceedingly rare. In this report we describe a patient with Gardner's syndrome that succumbed to a cortisol secreting adrenocortical carcinoma.

Case description: The patient was diagnosed with Gardner's syndrome by clinical and genetical workup. At age 47 she was diagnosed with a tumour lesion on her left adrenal with a concomitant enlarged right adrenal gland. Laboratory results were unremarkable and U-cortisol was within normal range. A benign adrenocortical tumour was removed on her left side. Retrospective molecular analysis of the tumour tissue revealed a 3bp in-frame deletion in *PRKACA* (p.del293Lys), and a *TERT* promoter mutation (C< T -124). Six years later she developed cushings syndrome and CT revealed enlarged lymph nodes and a 3 cm mass at the location of her previous surgery. Small needle biopsy of the mediastinal lesion revealed an ACC, and she was started on lysodren, cisplatin and zanosar but passed away 3 months later.

Discussion: We describe a patient with Gardner's syndrome with a cortisol-secreting carcinoma. Molecular analysis revealed both a *TERT* mutation and an in-frame *PRKACA* deletion. *TERT* mutations occur frequently in ACCs. If molecular analysis had been undertaken at the first presentation, a higher suspicion of malignant disease could have been suspected. We suggest that molecular analysis of adrenal tumour tissue in high-risk patients such as those with FAP/Gardener's should be undertaken to determine clinical follow up.

Dexamethasone test follow-up after two years provide limited information about changes in cortisol secretion in most patients with incidentally detected adrenal adenomas

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Background: Cortisol at 1 mg dexamethasone suppression ($Cortisol_{DST}$) is a key component evaluating cortisol secretion in patients with adrenal incidentalomas (AIs). We wanted to evaluate $Cortisol_{DST}$ follow-up after two years for diagnosing biochemical progress in individual patients with unilateral AIs.

Methods: We retrospectively studied 114 patients with AIs examined with $Cortisol_{DST}$ at baseline and at follow-up (2.2±0.05 years). The patients were divided according to baseline $cortisol_{DST}$ into quintiles. $Cortisol_{DST}$ was log transformed and within subject standard deviation (SD_{WS}) estimated with linear mixed models. We defined statistically significant progression of cortisol secretion as an increase larger than 2 SD_{WS}. The Regression to mean effect (RTMe) was calculated for the lowest and highest quintile.

Results: Mean $cortisol_{DST}$ was in the quintiles 20±0.9, 30±0.3, 37±0.6, 49±1.5 and 92±5.2 nmol/l and the changes from baseline to follow-up 41±5, 21±5, 8±6, 9±7 and 0±9%, respectively. The calculated SD_{WS} was 25% and 2 SD_{WS} 57%. The criterion for biochemical progress was fulfilled in 13% of the patients. The RTMe was 14% in both the lowest and highest quintile. Compensating for this, the changes from baseline were approximately 24% in the lowest and 16% in the highest quintile. This reclassified biochemical progress in 2 patients.

Conclusion: $Cortisol_{DST}$ retesting after two years detects statistically significant progress in a minor group of patients but in most patients no conclusion can be made. $Cortisol_{DST}$ seems to increase at all baseline $Cortisol_{DST}$ levels after adjusting for RTMe. The large variation in $Cortisol_{DST}$ and the RTMe should be considered evaluating follow-up results.

Development of the first mobile application for assistance in decision making in clinical genetic testing of pheochromocytoma and paraganglioma

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Background: Paraganglioma and pheochromocytoma are rare tumours arising from peripheral ganglia and the adrenal medullae respectively. To date 14 susceptibility genes for pheochromocytoma and paraganglioma (PPGL) have been discovered and over half of all pheochromocytomas can be attributed to constitutional genetic alterations. Mutation status has prognostic value, with certain mutations being associated with increased risk of malignancy. The presence of a germline mutation warrants more frequent follow-up, as well as genetic testing and surveillance of the offspring. Currently, genetic testing is recommended for all patients with PPGL. Sequencing of all susceptibility genes is costly and time-consuming. The advent of next-generation sequencing technologies holds promise to mitigate these problems, but the effects of this can not be seen in clinical practice in the near future.

Methods: Using the published literature and our own large cohort of PPGL, we have developed a scheme for prioritization in screening of susceptibility genes, based on age, tumour location, secretory phenotype, presence of other symptoms, as well as availability of tumour tissue. Our algorithm has been implemented in a mobile application for use by clinicians.

Results: The first version of the first mobile application for assistance in decision making in the clinical genetic testing for pheochromocytoma and paraganglioma susceptibility genes will be released for download during the ENSAT Scientific Meeting.

RET GENE MUTATIONS IN PHEOCHROMOCYTOMA: DEALING WITH UNEXPECTED FINDINGS IN THE NEXT GENERATION SEQUENCING ERA

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Genetic screening in Pheochromocytoma/Paraganglioma (PHEO/PGL) is widely adopted in clinical practice. Progresses in speed, quality and cost of high-throughput sequencing technology allow sequencing of an increasing number of causal genes, with complete exome covering. In this new context, RET gene sequencing, thus far usually limited to to exons 10,11,13,14,15 and 16, can easily include all exons.

At our Center we routinely use a NGS technique to perform genetic screening in PHEO/PGL patients. Our clinical panel includes the complete coding region of nine genes.

In a partial series of 44 PHEO/PGL patients we identified two subjects with a c.C166A (pL56M) heterozygous mutation in exon 2 of RET gene. Both patients, 24 and 62 years old, respectively, presented an isolated adrenal pheocromocytoma.

This finding prompted us to re-evaluate all the patients previously submitted to genetic screening for RET mutations who did not show any mutation in "conventional" RET exons.. Focused analysis of L56M mutation was completed in 182 subjects: (i) 65 patients with Pheo/Pgl, (ii) 117 patients with medullary thyroid cancer (MTC), (iii) 20 patients with C-Cell hyperplasia (CCH). The L56M mutation was identified in a third patient, a 55 years old man affected by adrenal pheocromocytoma. No patient with MTC or CCH presented this mutation. So far, the L56M variant has been described in patients with Hirschsprung disease, but never reported in PHEO/PGL or MTC patients.

Our finding suggest a potential causative role of RET L56M variant in the pathogenesis of a MEN2 variant with preferential expression the pheochromocytoma phenotype.

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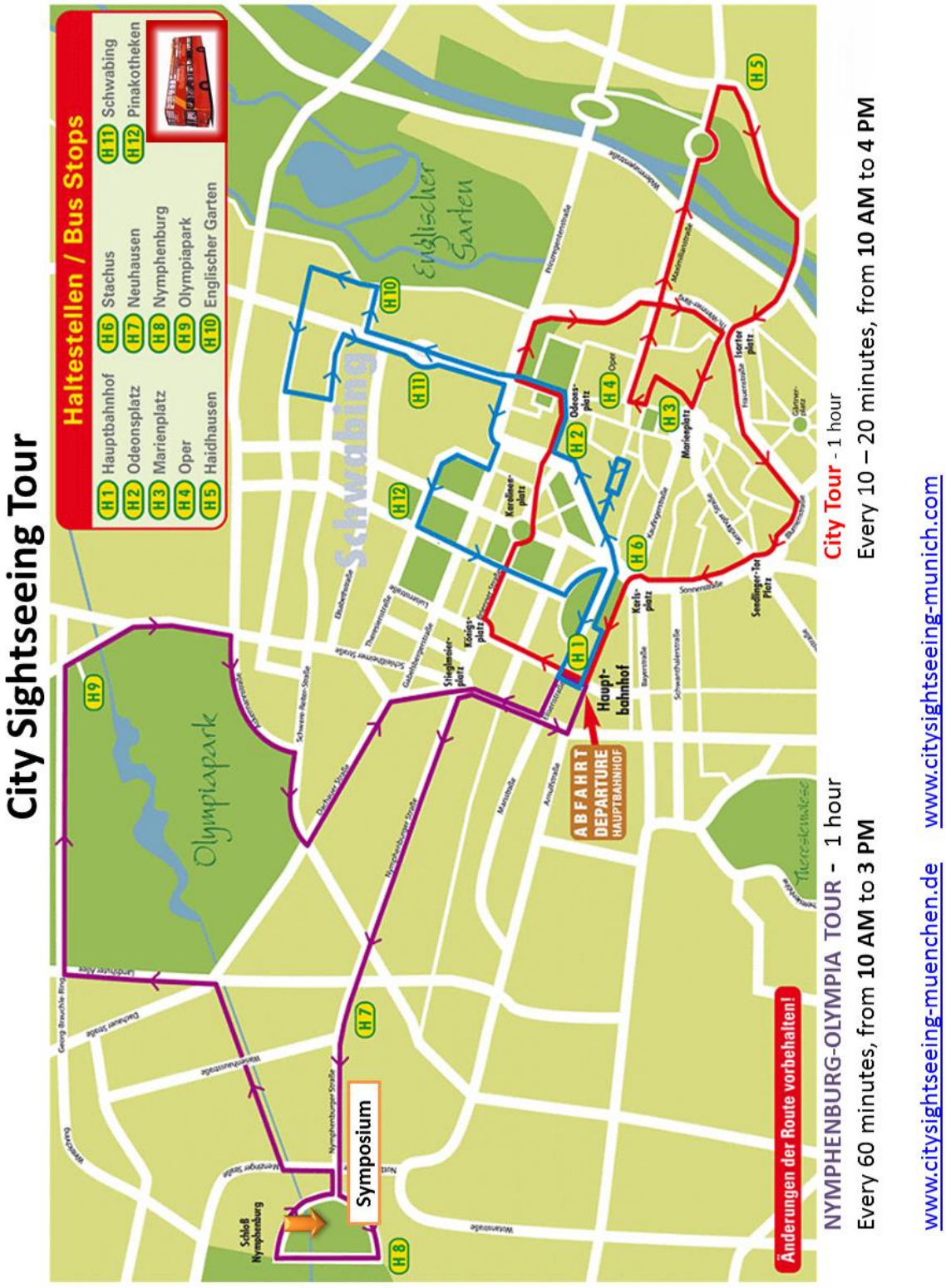

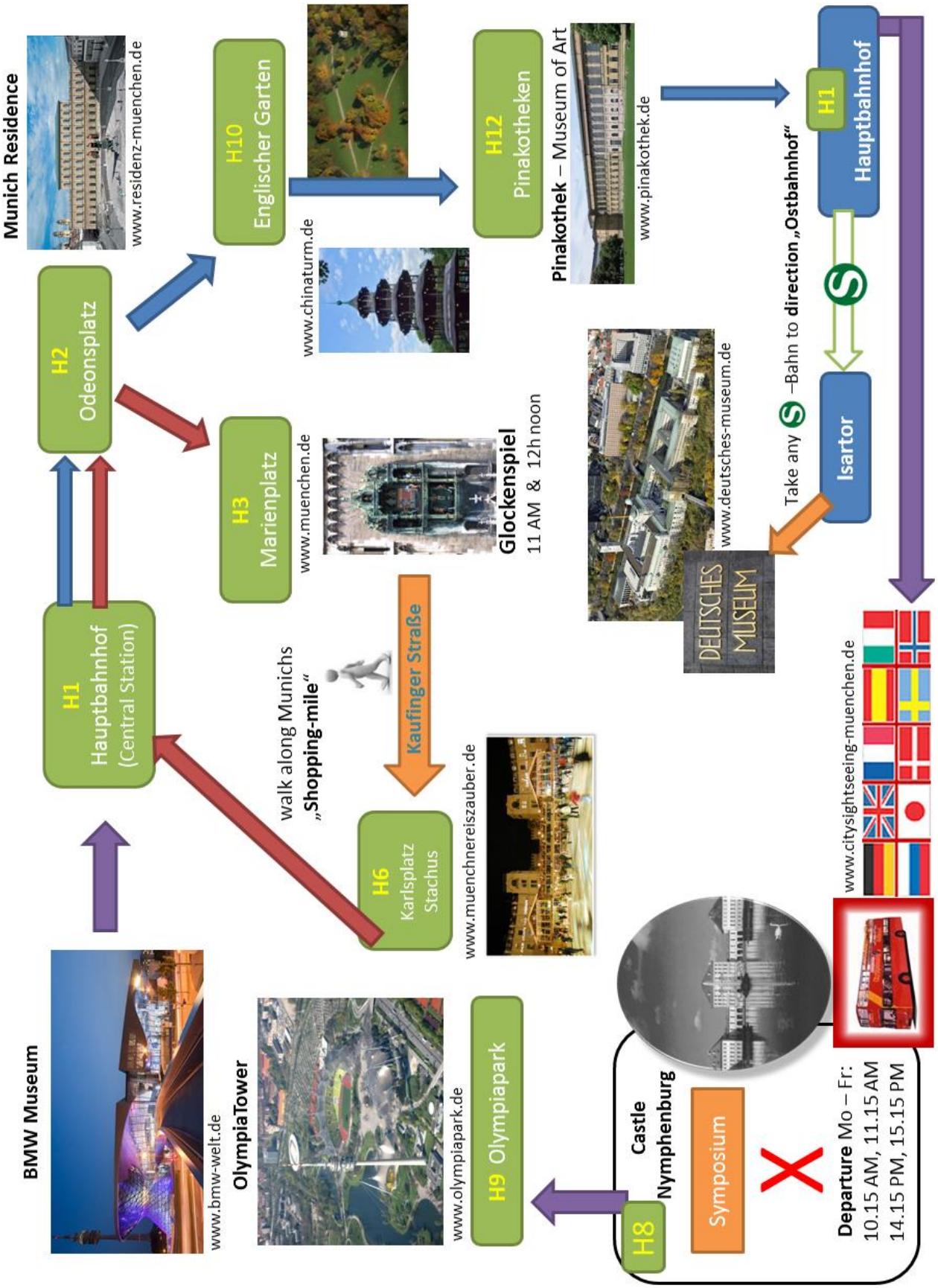
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