***Scientific report from 11th Scientific Meeting of ENS@T*, November 23rd - 24th, 2012.**
Local organizers:

Mercedes Robledo, Hereditary Endocrine Cancer Group. CNIO, Madrid, Spain.
Javier Aller, Endocrinology Department, Clínica Puerta de Hierro, Madrid, Spain.

1- Summary

ENS@T (*European Network for the Study of Adrenal Tumours*) is composed of four working groups that cover clinical and basic scientific aspects of adrenocortical cancer (ACC), pheochromocytoma/paraganglioma (PCC/PGL), aldosterone producing adenomas (APA) and non-aldosterone producing adenomas (NAPACA), respectively. The Meeting was focused on five topics of interest for this field, selected by the members of the Scientific Organising Committee (composed by Felix Beuschlein, Pierre-Francois Plouin, Harm Haak, Graeme Eisenhofer, Jacques Lenders, Jérôme Bertherat, Anne-Paule Gimenez-Roqueplo, Maria Christina Zennaro, Henri Timmers, Massimo Terzolo, Massimo Mannelli, Martin Fassnacht, and Mercedes Robledo):

1- Comprehension of molecular mechanisms in the improvement of differential diagnosis and risk stratification of adrenal cancer.
2- Identification of novel biomarkers and screening for molecular mechanisms as the basis to improve treatment response.
3- Functional imaging approaches for adrenal tumours.
4- Evidence based therapeutic strategies to improve clinical outcome of patients with adrenal cancer.
5- Overview of clinical trials already in place and novel therapeutic strategies.

This two days event counted with the participation of 104 attendees (50 females and 54 males) from 14 countries (Australia, Bulgaria, Croatia, France, Germany, Hungary, Israel, Italy, Poland, Spain, Sweden, The Netherlands, United Kingdom, and United States). A total of 47 abstracts were received. Twenty of them were selected by the Scientific Organising Committee to be presented as oral communications, which were distributed in two general sessions, related to on APA/NAPACA/ACC, and PCC/PGL respectively. There were 27 additional abstracts that were selected to be presented as poster to be discussed during the coffee-breaks.

The programme included four parallel sessions to present and discuss new projects on APA, NAPACA, ACC, and PCC/PGL. This initiative was essential to establish new collaborations between the ENS@T groups. The results of discussions about the topics reviewed on the parallel sessions were presented on a General Assembly to increase the network of potential collaborators interested in each specific project.

2- Description of the scientific content of and discussions at the event (up to four pages).

The quality and topics of the abstracts allowed us to reach the aims of the event. Sixteen abstracts were related to molecular mechanisms involved in tumorigenesis of adrenal tumours, three focused on epidemiological data, ten on biomarkers, two on functional imaging, three on therapeutic strategies to improve clinical outcome, and 13 on clinical trials already in place or potential novel therapeutic targets.

Several studies demonstrated **the robustness of to genomic profiles** in order to identify pathways specifically related to genetic conditions, and diagnostic biomarkers. In this regards,

- a group of genes were linked to causal *KCNJ5* mutations in the formation of aldosterone producing adenoma (APA), and related to cellular growth and differentiation, nuclear receptor transcription regulation and lipid metabolism (Sheerazed Boulkroun et al., oral communication).
- A genome-wide association study was able to demonstrate a correlation between high aldosterone to renin ratio and a locus at 5q32. *SLC26A2*, a sulfate transporter gene hosted in this locus, was found to be highly expressed in mouse adrenal glands, and investigated into this gene's potential role in the etiology of primary aldosteronism (Tarik Bozoglu et al., oral communication).
- Using SNP array analysis several copy number alterations (CNA) in both adrenocortical adenomas (ACA) and carcinomas (ACC) were observed. In particular, only cortisol-secreting tumors (13% of 15 ACA and 29% of 14 ACC) presented an allelic loss of *SGK1* (serum glucocorticoid kinase 1), a glucocorticoid-responsive kinase gene involved in multiple cellular functions and in Wnt/beta-catenin signaling pathway regulation (Cristina Ronchi et al., oral communication).
- Genome-wide methylation analysis revealed the existence of hypermethylated ACCs, with a poorer prognosis. Hypermethylation in these tumors is important for silencing specific tumor suppressor genes (Guillaume Assié et al., oral communication).
- A mapping of chromosome aberrations by single nucleotide polymorphism (SNP) array, microarray-based expression profiling and immunohistochemistry (IHC), demonstrated the implication of NF1 somatic alterations in a significant proportion of PCCs and PGLs (Judith Favier et al., oral communication).
- Using the Infinium HumanMethylation27 BeadChip, methylation signatures specific to and common among various genetic classes of PCC/PGL has been identified. As occurs in ACC, there is a subgroup of PCC/PGLs that exhibits a methylator phenotype, associated with poor prognosis.

It is worthy to mention several studies which aims were the identification of **biomarkers** and to assess their utility on differential diagnosis, risk stratification of adrenal cancer, and treatment response predictors.

- Recent studies have demonstrated that serum miRNAs represent potential non-invasive cancer biomarkers. A French group has explored in detail the utility of microRNAs as biomarkers in adrenal tumours, concluding that the circulating miR-195 displayed the higher diagnostic accuracy in discriminating ACA from ACC patients (Olivier Chabre et al., oral communication).
- An analysis of plasma free, urine deconjugated and urine free normetanephrine, metanephrine and methoxytyramine (the latter test with urine catecholamines) by UPLC-MS/MS, has demonstrated that combined measurements of plasma free normetanephrine, metanephrine and methoxytyramine offer the test with the highest efficacy for diagnosis of PPGLs. Among urine tests, measurements of the free metabolites appear to offer advantages over the deconjugated metabolites (Graeme Eisenhofer et al., oral communication).
- Among histological parameters with potential prognostic value, Ki67 was identified as the single most important prognostic marker for disease recurrence in ACC patients following R0 resection (Felix Beuschlein et al., oral communication).
- A study from a Group of Rotterdam revealed negative SDHA immnunostaining on a group of GISTs, indicating the potential presence of *SDHA* germline mutations as causal genetic factor (Lindsey Oudijk et al., oral communication).
- A study provided evidences that aldosterone excess has a direct negative effect on beta cell function in patients with Primary aldosteronism. Accordingly, following adrenalectomy, early insulin secretion improves significantly in these patients (Evelyn Fischer et al., poster).
- It was discussed a study based on circulating tumor cells assessment of ACC as biomarkers for early diagnosis and drug efficacy monitoring. The authors described that CTC were isolated in all ACC but not in ACA samples, suggesting that they may represent valid diagnostic markers, and that CTC levels might eventually be useful also for the patient's follow-up (Elisa Corsini et al, poster).
- An Italian group showed a genetic profile predictive of mitotane levels that may be potentially useful to select the start-up mitotane regimen (low-dose vs. high¬dose) (Vittoria Basile et al., poster).

Regarding **therapeutic strategies to improve clinical outcome** of patients with adrenal cancer, **clinical trials** already in place and **novel therapeutic strategies**, some of the most interesting studies are summarized below:

- Genomic approaches have revealed that SF-1 has an important role in regulating proliferation of adrenocortical cells, as well as other biological

processes as angiogenesis, adhesion to the extracellular matrix, cytoskeleton dynamics, transcriptional and post-transcriptional regulation of gene expression and apoptosis in the adrenal cortex. These finding will open new avenues for therapeutic intervention in adrenal diseases (Mabrouka Doghman & Enzo Lalli, oral communication).

- A study on morphological and functional effects of mitotane in ACC cells has revealed that mitochondrial disruption represents one of the main factors contributing to the cytotoxic effect of MTT (Giada Poli et al., oral communication).
- Mitotane therapy in ACC induces CYP3A4 and inhibits 5α-reductase explaining the need for personalized glucocorticoid and androgen replacement (Vasileios Chortis et al., oral communication).
- A study using animal models has demonstrated that PDP (paclitaxel, doxorubicin, cisplatin), LEDP (etoposide, liposomal doxorubicin, liposomal cisplatin) and specially LPDP (nab-paclitaxel, liposomal doxorubicin, liposomal cisplatin) represent in combination with M (mitotane) promising novel treatment options compared with classical EDP (etoposide, doxorubicin, cisplatin) plus M scheme (Constanze Hantel, et al., poster).
- Sunitinib is associated with tumor size reduction, decreased 18-F-FDG PET/CT uptake, disease stabilization, and hypertension improvement in some patients with progressive metastatic PCC/PGL (Camilo Jiménez, oral communication).
- Everolimus may be an effective treatment in ACC and may be potentiated by the association with SOM230 (Massimo Terzolo et al., poster).
- A deep characterization of the role of G-protein-coupled estrogen receptor (GPER) suggests that i) GPER can be considered a new target to control ACC cell proliferation; ii) activation of GPER can modulate ACC growth with similar molecular mechanisms observed in other estrogen-dependent tumors; and iii) the use of GPER agonists and/or SERM able to activate GPER and to inhibit ESR1 (such as Tamoxifen) could be a very effective new therapy for controlling ACC growth (Rosa Sirianni et al., poster).
- It was reviewed a study on 177Lu-DOTA-Octreotate therapy of PCC and PGL. The authors showed data demonstrating that 177Lu-DOTA-Octreotate therapy is associated with favorable outcome. In this regards, high tumour proliferation and ENETS grading are potential negative predictive factors for response to therapy (Joakim Crona et al., poster).

3- Assessment of the results and impact of the event on the future directions of the field.

This event will have a direct impact on the future directions of the field across the scientific collaborations that were established during the parallel session as well as the General Assembly. There were presented numerous projects focused on the different topics reviewed during the Meeting, and there were discussions on the pros and cons of the objectives and the experimental approaches required. Below appears a list of the projects presented and the corresponding principal investigator. This list represents a road for the future:

I) Update on current projects

1. Current status ENSAT ACC Registry (Martin Fassnacht, Munich, Germany).
2. ENSAT CANCER omics and next generation sequencing projects (Jerome Bertherat, Paris, France).
3. EURINE-ACT (Evaluation of URINE Steroid Metabolomics in the diagnosis of AdrenoCortical Tumours) - progress report and invitation to participate (Wiebke Arlt, Birmingham, UK).
4. The ENSAT Adrenal Tumor Tissue Micro Array project (Thomas Papathomas, Rotterdam, The Netherlands).

II) Proposals of new collaborative projects

1. Circulating miR-483-5P and miR-195 as biomarkers for recurring adrenocortical carcinoma (Nadia Cherradi, Grenoble, France).
2. Pregnancy in patients with ACC (Marie-Laure Raffin-Sanson, Boulogne, France).
3. Pre-clinical targeting of PI3K/Akt/mTOR and RAF/MEK/ERK signaling pathways in ACC: impact on steroidogenesis, cell proliferation and apoptosis (Dorota Dworakowska, Gdansk, Poland).
4. ARMITO (Androgen Replacement in MITOtane-treated men) - invitation to participate (Wiebke Arlt, Birmingham, UK).
5. Molecular markers in FIRMACT and ENSAT ACC datebase: role as prognostic factors or predictors of response (Eric Baudin, Villejuif, France).
6. Evaluation of metabolome and steroidobolome signatures for prediction of diagnosis of PA (versus essential hypertension), prediction of subtype differentiation (APA vs IAH vs FHA type 1-3) and prediction of underlying somatic or germ-line mutations of PA susceptibility genes in patients primary aldosteronism of various subtypes (Martin Reincke, LMU Munich, Germany).
7. Evaluation of the role of DNA methylation in the development of Aldosterone Producing Adenomas (Pierre Val. UMR CNRS 6293, INSERM U1103 GReD. University of Clermont Ferrand, France).

III) Clinical trials

1. Short update on ADIUVO (Massimo Terzolo, Turino, Italy).
2. New trials (currently submitted to a EU FP7 call, coordinated by Massimo Terzolo).

3. ADIUVO-II trial (mitotane or mitotane plus cisplatin in high risk patients (Massimo Terzolo, Turin).
4. SuMiTrial (Sunitinib vs. Mitotane in advanced ACC in mitotane-naive patients) (Matthias Kroiss, Martin Fassnacht, Würzburg, Munich, Germany).
5. SimpleFACT trial (Cisplatin + Mitotane vs. EDP-Mitotane in advanced ACC (Eric Baudin, Villejuif, France).

Annexes 1: List of participants

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11th Scientific Meeting of ENS@T

November 23rd-24th 2012

CNIO, Madrid, Spain.

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european network for the study of adrenal tumors

We appreciate our sponsors for supporting this Meeting

Dear Colleague,

Please receive our warmest welcome to Madrid. We hope that you will enjoy your stay during the ***11th Scientific Meeting of ENS@T*, November 23rd - 24th, 2012.**

In order to facilitate your stay, we have compiled this bag for you that contains the conference booklet including the programme, abstracts and the list of participants. Please use the special lunch vouchers provided.

We would like to take this opportunity to draw your attention to a few additional points:

Coffee-breaks will be served in the hall of the building.

Lunches are available in the main dining room of the building.

Please use your **badge** throughout the conference.

We would like to let you know that the CNIO building is a **wireless** hotspot and does not require a password.

Please note that the CNIO building and surroundings are a **smoke free area**.

Please remember that if you wish to make any local calls from your **mobile phone**, you will need to dial the prefix **(00 34)** before the number you require. Please ensure they are completely **switched off in the auditorium** or interference with microphones will cause disturbance.

With the best wishes on behalf of the Scientific Organising Committee.

Sincerely,

Mercedes Robledo and Javier Aller

(Local Organizing Committee)

11th Scientific Meeting of ENS@T

Venue: Auditorium CNIO. Melchor Fernández Almagro, 3. 28029 Madrid; www. cnio.es
Date: November 23rd-24th, 2012

Friday, November 23rd

12.45 – 14.00 Registration and lunch (at the cafeteria of CNIO)
14.00 – 14.15 Welcome Address (M. Robledo)

14.15 – 15.30 Session I. ENS@T Projects on APA/NAPACA/ACC
Chairs: Felix Beuschlein and Enzo Lalli

14.15 - Tanja Dekkers. *Plasma metanephrine for assessing the selectivity of adrenal venous sampling*.
14.30 - Martin Reincke. *Female patients with primary aldosteronism are diagnosed earlier and have a better cardiovascular outcome after treatment*.
14.45 - Sheerazed Boulkroun. *Molecular correlates of KCNJ5 mutation status and relationship with adrenal cortex remodeling*.
15.00 - Tarik Bozoglu. *Investigation of a Solute Carrier's (SLC26A2) Role in Aldosterone Production*.
15.15 - Cristina Ronchi. *Low SGK1 expression in adrenocortical tumors is associated with ACTH independent glucocorticoid secretion and poor prognosis.*
15.30 - Nadia Cherradi. *Circulating miR-195 and miR-483-5P are promising non-invasive biomarkers of aggressive adrenocortical carcinoma.*

15.45 – 16.30 Coffee break and poster viewing.

16.30 – 18.15 Session II. ENS@T Projects on APA/NAPACA/ACC
Chairs: Wiebke Arlt and Massimo Mannelli

16.15 - Guillaume Assié. *Genome wide methylation analysis: a new step in the molecular classification of adrenocortical tumors by integrated genomics revealing a subgroup of aggressive cancers with a CpG island methylator phenotype.*
16.30 - Enzo Lalli. *Beyond steroidogenesis: the impact of genomic studies for the identification of novel SF-1 target genes.*
16.45 - Felix Beuschlein. *Prognostic value of histological markers in localized adrenocortical carcinoma after complete resection.*
17.00 – Rosella Libé *Prognostic factors of synchronous advanced unresectable stage III and IV ENS@T adrenocortical carcinomas (ACC).*
17.15 - Giada Poli. *Morphological and functional effects of mitotane on mitochondria in human adrenocortical cancer cells.*
17.30 - Vasileios Chortis. *Mitotane therapy in adrenocortical cancer induces CYP3A4 and inhibits 5α-reductase explaining the need for personalized glucocorticoid and androgen replacement.*
17.45 - Constanze Hantel. *Investigation of novel chemotherapeutic combinations for the treatment of adrenocortical carcinoma.*

18.30 Bus to the Hotel
19.00 – 20.15 ESF/ENS@T Steering Committee Meeting
21.00 Dinner and "Award ceremony"

11th Scientific Meeting of ENS@T

Saturday, November 24th

8.15 Bus to the CNIO

8.45 – 10.30 Session III. ENS@T Projects on PCC/PGL

Chair: Anne-Paule Gimenez-Roqueplo and Ronald de Krijger

8.45 – Giuseppe Opocher. *The natural history of endemic paraganglioma syndrome type 1: growth rate and predictors of tumour growth*.

9.00 - Graeme Eisenhofer. *UPLC-MS/MS analysis of plasma versus urine catecholamine O-methylated metabolites for diagnosis of phaeochromocytoma.*

9.15 - Jyotsna Upendra Rao. *Correlation between energy metabolism and catecholamine content in pheochromocytoma and paraganglioma.*

9.30 - Lindsey Oudijk. *SDHA Mutations in Adult and Pediatric Wild-Type Gastrointestinal Stromal Tumors.*

9.45 - Judith Favier. *Somatic NF1 inactivation is a frequent event in sporadic pheochromocytoma.*

10.00 - Aguirre A. de Cubas. *DNA Methylation profiling of pheochromocytoma and paragangioma. Adding more pieces of the puzzle.*

10.15 - Camilo Jiménez. *Current and future systemic therapies against metastatic pheochromocytoma and sympathetic paraganglioma.*

10.30 – 11.00 Coffee break and poster viewing

11.00 – 13.00 Parallel sessions on APA/NAPACA/ACC/PCC projects

Sessions on APA. Chair: Maria Christina Zennaro
Sessions on NAPACA. Chair: Massimo Terzolo
Sessions on ACC. Chair: Martin Fassnacht.
Sessions on PCC/PGL. Chair: Anne-Paule Gimenez-Roqueplo

13.00 – 14.00 General assembly and presentation of future ENS@T projects

14.00 – 15.00 Lunch

15.00 – 16.30 PRESSOR business meeting

ORAL PRESENTATIONS

Friday, November 23rd

14.15 – 15.30 Session I. ENS@T Projects on APA/NAPACA/ACC
Chairs: Felix Beuschlein and Enzo Lalli

14.15 - Tanja Dekkers. *Plasma metanephrine for assessing the selectivity of adrenal venous sampling*.

Tanja Dekkers[1], Jaap Deinum[1], Dirk Blondin[2], Mirko Peitzsch[3], Leo Schultzekool[1], Oliver Vonend[2], Holger Willenberg[2], Ad. R. Hermus[1], Lars C. Rump[2], G. Antoch[2], Fred C.G.J. Sweep[1], Jacques WM Lenders[1] and Graeme Eisenhofer[3].

1- Radboud University Nijmegen, Nijmegen, the Netherlands; 2- University Medical Center Düsseldorf, Düsseldorf, Germany; 3- University Hospital Carl Gustav Carus, Dresden, Germany.

Context: Adrenal vein sampling (AVS) provides the gold standard for distinguishing unilateral from bilateral causes of primary aldosteronism. More reliable parameters than cortisol are needed for assessing correct positioning of catheters during AVS. Plasma metanephrine represents one such alternative.
Objective: To determine the utility of adrenal vein measurements of metanephrine concentrations to establish correct positioning of catheters during AVS.
Design and methods: We included 86 AVS procedures from two medical centers: 52 ACTH-stimulated and 34 non-stimulated sequential procedures. Plasma concentrations of cortisol, metanephrine, normetanephrine, adrenaline and noradrenaline were measured in adrenal venous (AV) and peripheral venous (PV) samples. The success of AVS, according to a cortisol AV:PV selectivity index (SI) of 3, was compared with that for metanephrine using a SI for the latter determined by ROC curve analysis.
Results: Among AVS procedures assessed as selective by cortisol, the median AV:PV plasma metanephrine ratio was 6-fold higher ($P<0.0001$) than that for cortisol (94.0 versus 15.5). There were significant positive relationships between AV:PV ratios for cortisol and metanephrine during ACTH-stimulated samplings ($r=0.819$, $P<0.0001$), but not during non-stimulated samplings ($r=0.082$). ROC curve analysis indicated an AVS SI for plasma metanephrine of 10. There was 96% concordance in rates of success of AVS determined by cortisol and metanephrine derived SIs during ACTH-stimulated AVS. In contrast, without stimulation the concordance was only 53%; success rates of AVS determined by metanephrine were much higher than those determined by cortisol (91% versus 58%).
Conclusions: Metanephrine provides an alternative analyte to cortisol for sensitive assessment of AVS selectivity that appears to be particularly useful in sampling performed without ACTH stimulation.

14.30 - Martin Reincke. *Female patients with primary aldosteronism are diagnosed earlier and have a better cardiovascular outcome after treatment*.

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Introduction. Primary aldosteronism (PA) is the most frequent curable form of hypertension. Although early diagnosis is important because of associated cardiovascular and renal morbidity, PA often goes undiagnosed for many years.

Our aim was to study the influence of gender on diagnosis and outcome in a cohort of PA patients.

Methods. A total of 73 consecutive patients prospectively studied since 2008 in the Munich center of the German Conn's Registry were analyzed at diagnosis and 1 year after specific treatment (adrenalectomy or mineralocorticoid antagonist treatment).

Results. Median age at diagnosis was 53(43;60) years. Female patients (n= 26, 36%) were diagnosed earlier than males [43.1(37.3;49.5) vs. 58.2(46.7;63.3) y, p<0.001]. The time from the initial recognition of hypertension to the diagnosis of PA was 8(5;22) y, with 7(1;12) in females vs. 10(5;24) y in males (p=0.028). Although different by age, other baseline parameters, such as blood pressure [(157(146;181)/94(89;100) vs. 160(149;180)/94(87;105) mmHg, p=0.557/0.944], minimal serum potassium concentration [2.8(2.4;3.0) vs. 2.9(2.7;3.2) mmol/L, p=0.334] or cardiovascular and metabolic comorbidities did not differ between sexes. At 1 year follow-up, 98% of all operated patients had biochemical remission. Median ambulant blood pressure was reduced from 160 (148;180)/94(87;105) to 139(128;147)/86(78;93) mmHg in all patients (p<0.001), with no differences between genders. Male patients were taking more antihypertensive drugs compared to female patients [3(2;4) vs. 1(0;2), p<0.001]. Females were significantly less likely to have renal insufficiency (12 vs. 38%, p=0.016), left ventricular hypertrophy (14 vs. 42%, p=0.021) or coronary artery disease (0% vs. 16%, p=0.029).

Conclusion. Women with PA are diagnosed 15 years younger than men and have a shorter history of arterial hypertension. Specific treatment improves cardiovascular outcome in both sexes but this effect is much more pronounced in females.

14.45 - Sheerazed Boulkroun. *Molecular correlates of KCNJ5 mutation status and relationship with adrenal cortex remodeling*.

Sheerazed Boulkroun[1,2], Jose Felipe Golib Dzib[3], Benoit Samson-Couterie[1,2], Amanda J. Rickard[1,2], Tchao Meatchi[1,2,4], Laurence Amar[1,2,4], Arndt Benecke[3,5], and Maria-Christina Zennaro[1,2,4]

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Potassium channels play a major role in controlling the membrane voltage of adrenal zona glomerulosa cells. Recently, recurrent somatic mutations of the potassium channel Kir3.4 have been implicated in the formation of aldosterone producing adenoma (APA) in up to 34% of unselected APA. While the causal link between *KCNJ5* mutations, membrane depolarization and aldosterone production has been formally established, the precise mechanism by which these mutations promote cell proliferation and APA formation remains unclear. The aim of our study was to correlate KCNJ5 mutation status to transcriptome profiles, morphological and functional characteristics of the adrenal cortex adjacent to APA. Immunohistochemistry showed Kir3.4 expression in APA as well as in the zona glomerulosa of the adjacent cortex, which was not correlated to *KCNJ5* mutation status. There was no correlation between *KCNJ5* mutation status and morphological measures of adrenal cortex remodeling, including nodulation (red sirius labeling, p=0.9457), vascularization (CD34 immunostaining, p=0.5544) and expression of Cyp11B2 by in situ hybridization (p=1.000). APA cell composition (zona fasciculata-like cells vs zona-glomerulosa like cells) was not significantly different between groups (HES, p=0.1643). Transcriptome analyses identified 893 genes which were significantly differentially regulated (post hoc $p<0.001$) in APA. GO/PANTHER enrichment analyses revealed biological processes and molecular functions related to cellular proliferation and differentiation, calcium signaling, and cell-cell signaling. We selected genes that were differentially expressed (post hoc $p<0.05$) between KCNJ5-associated and KCNJ5-independent APA and identified 29 genes, which are central to the *APA* gene network and are regulated by the *KCNJ5* mutation status. 24 (83%) of these genes constitute two structured subnetworks related to cellular growth and differentiation, nuclear receptor transcription regulation and lipid metabolism.

15.00 - Tarik Bozoglu. *Investigation of a Solute Carrier's (SLC26A2) Role in Aldosterone Production*.

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A correlation between high aldosterone to renin ratio and a locus at 5q32 was indicated by a genome-wide association study among participants of a population based survey. SLC26A2, a sulfate transporter gene hosted in this locus, was found to be highly expressed in mouse adrenal glands. In order to conduct an investigation into this gene's potential role in the etiology of primary aldosteronism, the human adrenocortical cell line NCI H295R was utilized. Endogenous SLC26A2 expression was found to be increased by potassium but mildly decreased by angiotensin II in both H295R cells and primary adrenocortical cells. Lentiviral transduction of H295R cells with SLC26A2 specific shRNA resulted in four-fold knockdown and a concomitant increase in aldosterone production along with aldosterone synthase (CYP11B2) expression. This increase was sustained in presence of aldosterone biosynthesis stimulators potassium, angiotensin II or forskolin. Further molecular characterization revealed upregulation of genes with key functions in steroid biosynthesis, CYP11A1 and HSD3B2, and in CAM kinase pathway, CAMK1, NR4A1 and NR4A2. Aptly, inhibition of CAM kinase activity in knockdown cells by the compound KN-93 nullified the increase in aldosterone production. However, so far no ample differences in intracellular calcium content between SLC26A2 wild type and knockdown cells could be demonstrated. Elucidation of other potentially involved pathways by microarray studies points towards activation of MAPK dependent pathways. To assess the gene function in an in vivo context, gene expression from adrenal glands from SLC26A2 knock-out mice was analyzed. These results suggest a tendency towards higher expression of CYP11B2 and HSD3B6, enzymes with key functions in aldosterone production, as opposed to lower expression tendency of STAR, CYP11A1 and HSD3B1, genes with unspecified expression patterns, was suggested. Further investigations into the endocrine phenotypes of these mice are ongoing.

15.15 - Cristina Ronchi. *Low SGK1 expression in adrenocortical tumors is associated with ACTH independent glucocorticoid secretion and poor prognosis.*

C.L. Ronchi[1], S. Sbiera[1], E. Leich[2], F. Tissier[3], S. Steinhauer[1], T. Deutschbein[1], M. Fassnacht[1], B. Allolio[1]

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Background: Using SNP array analysis we observed several copy number alterations (CNA) in both adrenocortical adenomas (ACA) and carcinomas (ACC). In particular, only cortisol-secreting tumors (13% of 15 ACA and 29% of 14 ACC) presented an allelic loss of the SGK1 (serum glucocorticoid kinase 1), a glucocorticoid-responsive kinase involved in multiple cellular functions and in Wnt/beta-catenin signaling pathway regulation.

Aim: To analyze SGK1 expression levels in adrenocortical tumors and to characterize its role in ACTH-independent cortisol secretion, tumor progression, and prognosis.

Materials and methods: 227 adrenocortical tumors (40 ACA and 187 ACC) and 25 normal adrenals were included. Among them, 62 frozen tumor samples were analysed for mRNA expression of SGK1, SGK3, and CTNNB1 (coding for betacatenin) and 203 tumors on tissue microarrays or full standard slides were investigated by immunohistochemistry for SGK1, nuclear beta-catenin and phosphorylated AKT (pAKT). The relationship between SGK1 expression and clinical parameters was then evaluated.

Results: SGK1 mRNA levels were lower in cortisol-secreting than in non cortisolsecreting tumors ($P<0.005$), while no significant difference was observed between ACA and ACC. Only non cortisol-secreting neoplasias showed a significant correlation between SGK1 and CTNNB1 levels ($P<0.001$, $r=0.57$). Low SGK1 protein levels, but not nuclear beta-catenin and pAKT, were associated with poor overall survival in ACC patients ($P<0.005$, HR=2.0, 95%CI=1.24-3.24), independently of tumor stage and cortisol secretion.

Conclusion: CN microdeletion at SGK1 locus and low SGK1 expression are frequent in cortisol-secreting adrenocortical tumors, suggesting a potential role for SGK1 in autonomous glucocorticoid secretion and in intra-adrenal glucocorticoid feedback. Low SGK1 levels are associated with poor survival in ACC, possibly through complex interactions with other factors, representing a new prognostic factor.

15.30 - Nadia Cherradi. *Circulating miR-195 and miR-483-5P are promising non-invasive biomarkers of aggressive adrenocortical carcinoma.*

Olivier Chabre[1,2], Rossella Libé[3], Guillaume Assie[3], Xavier Bertagna[3], Jérôme Bertherat[3], Jean-Jacques Feige[1] and Nadia Cherradi[1].

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Adrenocortical carcinoma (ACC) is an aggressive tumor showing frequent metastatic spread and poor survival. Genome-wide studies of ACC have clearly contributed to our understanding of the disease. However, major challenges remain for both diagnostic and prognostic assessments. Recent studies have demonstrated that serum miRNAs represent potential non-invasive cancer biomarkers. We and others have previously reported that miR-195 and miR-335 were markedly downregulated in ACC as compared to adrenocortical adenoma (ACA) tumor samples. We further brought evidence that miR-139-5P was upregulated in aggressive as compared to non-aggressive ACCs. The aim of this study was to assess whether the miRNAs that we have previously found deregulated in ACC tumor tissues were detectable in the serum of ACC patients and whether they could have diagnostic/prognostic value. Serum miRNA levels were determined using real-time quantitative PCR (Taqman miRNA assays) on a cohort of 14 ACAs, 15 aggressive (ACC A) and 8 non-agressive ACCs (ACC B, minimum follow-up of two years) and 19 healthy controls. We found that circulating miR-195 and miR-335 were remarkably decreased whereas miR-139-5P was significantly increased in ACC patients compared with ACA patients. Among the circulating miRNAs tested, miR-195 displayed the higher diagnostic accuracy in discriminating ACA from ACC patients (AUC: 0.95; Sensitivity: 90.9; Specificity: 100.0, $p<0.0001$). miR-483-5P, a miRNA which has been previously reported to be overexpressed in ACCs, was not detected in the serum of either ACA or ACC B patients. By contrast, circulating miR-483-5P was detectable in ACC A samples and was even higher in metastatic patients, thus providing a highly specific marker of aggressive ACCs. In addition, low levels of circulating miR-195 and high levels of circulating miR-483-5P were associated with poor prognosis, indicating important tumor suppressive/oncogenic functions of these miRNAs in ACC biology.

16.30 – 18.15 Session II. ENS@T Projects on APA/NAPACA/ACC
Chairs: Wiebke Arlt and Massimo Mannelli

16.15 - Guillaume Assié. *Genome wide methylation analysis: a new step in the molecular classification of adrenocortical tumors by integrated genomics revealing a subgroup of aggressive cancers with a CpG island methylator phenotype.*

Guillaume Assié[1,2,4,5], Olivia Barreau[1,2,3,4], Hortense Wilmot-Roussel[1,2,4], Bruno Ragazzon[1,2], Camille Baudry[1,2,4,5], Karine Perlemoine[4], Fernande René-Corail[4], Xavier Bertagna[1,2,4,5,6,7], Bertrand Dousset[1,2,4,8], Nadim Hamzaoui[9], Frédérique Tissier[1,2,4,10], Aurélien de Reynies[3], Jérôme Bertherat[1,2,4,5,6]

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DNA methylation is a mechanism for gene expression silencing in cancer. Limited information is available for adrenocortical carcinomas (ACCs). The aim is to characterize the methylation in ACCs, to assess its clinical significance, and its impact on gene expression. Patients and methods: Methylation patterns of CpGs islands in promoter regions of 51 ACCs and 81 adenomas were studied by the Infinium HumanMethylation27 Beadchip (Illumina). Methylation of 33 genes was studied by MS-MLPA in 15 ACCs. Gene expression data were available for 87 tumors from a previous study (HGU133Plus2.0 Affymetrix). Clinical information, including patient features and survival, were available for all tumors. Results: Methylation was higher in ACCs than in adenomas (p=3.1e-9). Unsupervised clustering of DNA methylation profiles identified two groups of ACCs, one with an elevated methylation level, evoking a "CpG island methylator phenotype" (CIMP).The subgroup of hypermethylated ACCs was further divided in two subgroups, with different levels of methylation ("CIMP-High" and "CIMP-Low"). This classification could be confirmed by MS-MLPA. Hypermethylation was associated with a poor survival (cox model p= 0.02). The transcriptome/methylation correlation showed 1741 genes (out of 12 250) negatively correlated; among the top genes were H19 and other tumor suppressors (PLAGL-1, G0S2, NDRG2). Previously identified subgroups of ACCs (based on transcriptome and TP53 / CTNNB1 mutations) showed specific methylation patterns, with high methylation in 2 of the aggressive subgroups of ACCs, including the TP53 mutated ACCs. Conclusions: This genome-wide methylation analysis reveals the existence of hypermethylated ACCs, with a poorer prognosis. Hypermethylation in these tumors is important for silencing specific tumor suppressor genes. These results further support our molecular classification of ACCs. We are currently analyzing methylation in a European cohort (ENSAT-CANCER) by MS-MLPA.

16.30 - Enzo Lalli. *Beyond steroidogenesis: the impact of genomic studies for the identification of novel SF-1 target genes.*

Mabrouka Doghman & Enzo Lalli.

Institut de Pharmacologie Moléculaire et Cellulaire CNRS UMR 7275 and University of Nice -Sophia Antipolis.

Steroidogenic Factor-1 (SF-1; NR5A1) is a transcription factor able to bind as a monomer to nuclear receptor half-sites that was identified by its capacity to coordinately regulate the expression of steroidogenic P-450 enzymes. Based on these data, SF-1 has a recognized role as a global regulator of steroidogenesis in the adrenal cortex and gonads. Further evidence for the essential role of SF-1 as a master steroidogenic gene came from experiments showing that its forced expression in embryonic and mesenchymal stem cells is sufficient to activate steroidogenic genes and to initiate steroid expression. Multilayered regulation of SF-1 activity is achieved by association with positive and negative cofactors, posttranslational modifications, phospholipid ligand availability, tissue-specific/epigenetic gene expression regulation and gene dosage. Recent studies using genomic approaches have revealed that SF-1 also has an important role in regulating proliferation of adrenocortical cells and have revealed its role in the control of a variety of biological processes as diverse as angiogenesis, adhesion to the extracellular matrix, cytoskeleton dynamics, transcriptional and post-transcriptional regulation of gene expression and apoptosis in the adrenal cortex, considerably increasing the portfolio of SF-1 transcriptional targets. The identification of the full set of SF-1 target genes will be of great importance to open new avenues for therapeutic intervention in adrenal diseases.

16.45 - Felix Beuschlein. *Prognostic value of histological markers in localized adrenocortical carcinoma after complete resection.*

F. Beuschlein, J. Obracay, W. Saeger, M. Kroiss, M. Quinkler, U. Lichtenauer, T. Deutschbein, C. L. Ronchi, H. S. Willenberg, N. Reisch, M. Reincke, R. Libe, E. Baudin, J. Bertherat, H. Haak, P. Loli, M. Terzolo, B. Allolio, HH. Müller, M. Fassnacht.

University Clinic Munich, University Clinic Wuerzburg, Marienhospital Hamburg, Charite Berlin, University Clinic Duesseldorf, Cochin Institute Paris, Insitute Gustave Roussy, Paris, Máxima Medisch Centrum, Eindhoven, Ospedale Niguarda Cà Granda, Milano, Medicina Interna I, Orbassano, Institute for Medical Biometry, Munich.

Purpose: Recurrence of adrenocortical cancer (ACC) even after complete resection is a common clinical problem. The aim of this study was to identify histological parameters with potential prognostic value.

Patients and Methods: From the German ACC registry 318 patients with ENSAT stage I-III were identified with R0 resection and available histological annotations. Histological markers used for Weiss scoring as well as Ki67 indices were correlated with recurrence free (RFS) and overall survival (OS). As an independent validation cohort, patients (n=253) from five ENSAT centers (from Italy, France and the Netherlands) were utilized.

Results: Within the German cohort univariate analysis identified age at diagnosis (log rank p=0.01), tumor size (p<0.01), lymph node positivity (p=0.002) among others as predictors of recurrence. For histological markers, only Ki67 index provided prognostic information for RFS (HR 1.042 per each % increase, p<0.0001; Ki67 ≥5%, HR 2.616, p=0.0002; Ki67 ≥10%, HR 2.743 p<0.0001; Ki67 ≥15%, HR 2.810 p<0.0001; Ki67 ≥20%, HR 3.526 p<0.0001; Ki67 ≥25%, HR 3.050 p<0.0001) and was superior to markers evaluated for calculation of the Weiss score. Similar results were obtained for OS (HR 1.051 per % increase, p<0.0001). Following multivariate analysis including age, tumor size, mitotane treatment, or other histological parameter, Ki67 index remained informative. Validation of these findings on the European cohort is under way and will be presented.

Conclusion: In conclusion, Ki67 was identified as the single most important prognostic marker for disease recurrence in ACC patients following R0 resection.

17.00 – Rosella Libé. *Prognostic factors of synchronous advanced unresectable stage III and IV ENS@T adrenocortical carcinomas (ACC).*

Rossella Libé, Isabelle Borget, Cristina Ronchi, Massimo Terzolo, Michaela Haaf, Federica Laino, Thomas Kherkhof, Elisa Corsini, Antoine Tabarin, Harm Haak, Olivier Chabre, Massimo Mannelli, Françoise Borson-Chazot, Christelle de la Fouchardière, Patricia Niccoli, Delphine Drui, Brigitte Delemer, Bernard Goichot, Philippe Caron, Alfred Penfornis, Felix Beuchlein, Jérôme Bertherat, Alfredo Berruti, Martin Fassnacht, Eric Baudin for the ENS@T group.

Introduction. The prognosis of stages III-IV ACC patients is dismal. The 5-yr survival rate ranges between 0-13% in three multicentric studies. Several reports suggest a greater heterogeneity of advanced ACC prognosis that initially thought.
Aim. The primary objective of our study was to analyse the prognostic factors of overall survival (OS) of advanced unresectable stage III-IV ACC patients collected in the ACC-ENS@T registry.
Methodology: The primary end-point of the study was overall survival (OS). Secondary objectives were: to define 1,2, 5 yr-OS, to determine a prognostic score, to analyse the role of early ACC management and delays in treatment. All relevant clinical, pathological relevant parameters that characterized ACC and therapeutic management were captured Patients. Three hundreds and thirty-four adult patients were enrolled (100 stage III and 234 stage IV ACC) seen between 2000 and 2009 in one of the ENSAT centre. Inclusion criteria were: age> 18 years, unresectable ACC (R1, R2, Rx), available follow-up.
Results. The median follow-up was 60 months. Median OS was 20 months. The 1-, 2- and 5-yrs survival rates were 67%, 42% and 19%.Two hundreds fifty patients (74%) died of their disease
At multivariate analysis, preliminary results showed that age > 50 years (HR:1.4 , CI: 1.1-1.9, p=0.005), stage IV (HR: 8.3, CI: 2.4-28.7, p=0.0007), adipose tissue infiltration (HR: 1.8, CI: 0.8-2.5, p=0.0022) , Ki-67: 5-20% (HR: 2.7, CI: 1.1-6.5, P=0.02) significantly increase the risk of death, whereas adrenalectomy (HR:0.3, CI: 0.2-0.5,p<0.0001), early polychemotherapy (HR: 0.7, CI: 0.4-1, p=0.05) and only one organ involved (HR: 0.3, CI: 0.1-0.9, p<0.04) were associated with significant reduction of death.
Conclusion. Based on the largest prognostic study ever done in advanced ACC patients a prognostic score will be presented that will drive future therapeutic development in advanced stage III-IV ACC.

17.15 - Giada Poli. *Morphological and functional effects of mitotane on mitochondria in human adrenocortical cancer cells.*

Poli Giada[1], Guasti Daniele[2], Rapizzi Elena[1], Fucci Rossella[1], Bandini Alessandra[3], Cini Nicoletta[3], Bani Daniele[2], Mannelli Massimo[1], Luconi Michaela[1]

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Mitotane (dichlorodiphenyltrichloroethane -DDD) is at present the drug of first line approach for the treatment of advanced adrenocortical carcinoma (ACC). Despite clear evidence that the drug can reduce the clinical signs of steroid excess in the secreting forms of ACC, the mechanism underlying the possible toxic effect of mitotane on tumor cells still remains obscure. This study investigated the intracellular events underlying the toxic effect of mitotane by studying alteration in the morphology and functions of mitochondria in the human adrenal cortical cancer cell line H295R. Increasing concentrations of mitotane resulted in a rapid intracellular accumulation of the drug and of its lipophylic metabolite dichlorodiphenyldichloroethene (DDE). Cytotoxic effect was already evident starting from low concentrations of mitotane (10 μM) at 48 hours reaching a maximum after 7 days of treatment with higher doses (inhibition: 92.0 ± 1.12 % at 7 d, $P< 0.0001$, 30 μM MTT). Electron microscopy analysis of cell mitochondria displayed a mitotane-induced dose-and time-dependent alteration in the morphology of the organelle, characterized by a marked swelling and a decrease in the number of respiratory internal cristae, accompanied by a significant depolarization of the mitochondrial membrane potential finally leading to the disruption of the organelle. A drastic reduction of oxygen consumption (inhibition: 74.1 ± 6.63 %, $P<0.001$, with 30 μM MTT and 82.1± 10.38 %, $P<0.001$, with 50 μM MTT) was observed due to mitochondrial membrane damage rather than to specific alteration in the respiratory chain enzymes. These findings contribute to better understand the intracellular mechanism of action of mitotane in adrenocortical cancer cells, showing that mitochondrial disruption represents one of the main factors contributing to the cytotoxic effect of MTT.

17.30 - Vasileios Chortis. *Mitotane therapy in adrenocortical cancer induces CYP3A4 and inhibits 5α-reductase explaining the need for personalized glucocorticoid and androgen replacement.*

Vasileios Chortis, Angela E. Taylor, Petra Schneider, Jeremy W. Tomlinson, Beverly A. Hughes, Donna M. O'Neil, Rossella Libé, Bruno Allolio, Xavier Bertagna, Jérôme Bertherat, Felix Beuschlein, Martin Fassnacht, Niki Karavitaki, Massimo Mannelli, Franco Mantero, Giuseppe Opocher, Emilio Porfiri, Marcus Quinkler, Mark Sherlock, Massimo Terzolo, Peter Nightingale, Cedric H.L. Shackleton, Paul M. Stewart, Stefanie Hahner, Wiebke Arlt.

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Mitotane (o,p'DDD) is the first-line treatment for metastatic adrenocortical carcinoma (ACC) and is also regularly used in the adjuvant setting after presumed complete removal of the primary tumor. Mitotane is considered an adrenolytic substance, but there is limited information on distinct effects on steroidogenesis. However, adrenal insufficiency and male hypogonadism are widely recognized side effects associated with mitotane treatment. Here we aimed to define the impact of mitotane treatment on in vivo steroidogenesis in patients with ACC. We employed gas chromatography/mass spectrometry for steroid profiling of 24-h urine samples (n=127) collected from patients with ACC before and during mitotane therapy in the adjuvant setting (n=23) or for metastatic ACC (n=104). We found a sharp increase in the excretion of 6β-hydroxycortisol (6βOHF) over cortisol ($P<0.001$), indicative of a strong induction of the major drug-metabolizing enzyme CYP3A4. The contribution of 6βOHF to total glucocorticoids increased from 2% (median, IQR 1-4%) to 56% (39-71%) during mitotane treatment. Furthermore, we documented strong inhibition of 5α-reductase activity, indicated by a significant decrease in 5α-reduced steroids, including 5α-tetrahydrocortisol, 5α-tetrahydrocorticosterone and androsterone (all $P<0.001$). The degree of inhibition was similar to that

in patients with inactivating 5α-reductase type 2 mutations (n=23) and patients receiving finasteride (n=5). The degree of CYP3A4 induction, but not 5α-reductase inhibition, correlated significantly with plasma mitotane levels. In summary, CYP3A4 induction by mitotane results in rapid inactivation of more than 50% of administered hydrocortisone, explaining the need for doubling hydrocortisone in mitotane-treated patients. Strong inhibition of 5α-reductase activity in line with the clinical observation of relative inefficiency of testosterone replacement in mitotane-treated men, calling for replacement by 5α-reduced androgens.

17.45 - Constanze Hantel. *Investigation of novel chemotherapeutic combinations for the treatment of adrenocortical carcinoma.*

Constanze Hantel, Sara Jung, Martin Reincke, Felix Beuschlein.

Endocrine Research Unit, Medical Hospital and Poliklinik IV, Ludwig-Maximilians-University, Munich, Germany.

Medical treatment of adrenocortical carcinoma (ACC) is limited to common cytotoxic agents, which are usually given in combination with mitotane (M). However, all systemic treatments achieve only partial responses while complete remissions are rarely observed. Recently, we detected an extraordinary uptake phenomenon of liposomes by adrenocortical tumor cells which indicated that encapsulated drugs could represent an interesting novel treatment option for ACC. In a first step we investigated in combination with M the effects of two different chemotherapies on human NCIh295 cells in vitro: 1. EDP (etoposide, doxorubicin, cisplatin) and 2. a novel paclitaxel containing scheme PDP (paclitaxel, doxorubicin, cisplatin) indicating superiority of PDP over EDP regarding reduction of cell viability (% of 100% basal; EDP: 69.7±0.9%, PDP: 40.7±1.7%; p= 0.001), induction of apoptosis (EDP: 473.3±11.7%, PDP: 629±16%; p= 0.001) and inhibition of proliferation (EDP: 105.3±9.4%, PDP: 14.5±4.4%; p= 0.0009). In a second step, we performed short-term therapeutic experiments with M and EDP, PDP as well as the liposome based variants LEDP (etoposide, liposomal doxorubicin, liposomal cisplatin) or LPDP (nab-paclitaxel, liposomal doxorubicin, liposomal cisplatin) on NCIh295 xenografts. Reflecting the clinical situation EDP treatment resulted in leucopenia and did not induce a significant loss of tumor cells (% of basal; 83.9±10.1) while PDP (83.6±1.9%, p=0.003) LEDP (72.5±1.9%, p=0.003) and LPDP (61.2±3.5%, p=0.0002) resulted overall in a significant reduction in the number of tumor cells compared with controls (100%). LEDP (150.3±8.3%, p=0.004) and LPDP (146.7±13.7%, p=0.02) induced apoptosis and only minor grades of leucopenia [leukocytes G/l; controls: 6.1±1.3] after treatment with LPDP (3.2±0.2) compared with EDP (1.9±0.2; p=0.003). In summary PDP, LEDP and specially LPDP could represent in combination with M promising novel treatment options compared with classical EDP plus M scheme.

8.45 – 10.30 Session III. ENS@T Projects on PCC/PGL

Chairs: Anne-Paule Gimenez-Roqueplo and and Ronald de Krijger

8.45 – Giuseppe Opocher. *The natural history of endemic paraganglioma syndrome type 1: growth rate and predictors of tumour growth*.

Giuseppe Opocher[1,2], Serena Dematté[3], Daniela Di Sarra[3], Giulia Casagranda[3], Elisa Casagranda[2], Paola Sartorato[1] and Francesca Schiavi[1]

1- Familial cancer clinic and oncoendocrinology, Veneto Institute of Oncology, Padova, Italy; 2- Department of Medicine -DIMED, University of Padova, Italy; 3- Hospital of Trento, Italy.

Paraganglioma syndrome type 1 (PGL1, OMIM # 168000) is an autosomal dominant disease characterized by head and neck paragangliomas (HNPGL), thoracic or retroperitoneal paragangliomas and pheochromocytoma. Mutations of *SDHD* confer a high susceptibility to disease development. PGL1 is inherited with a parent-of-origin effect with only carriers of the paternal allele at risk of developing the tumors. Following an anecdotal report of a suspected high incidence of head and neck paraganglioma in Trentino, we identified and characterized the largest founder effect to-date for the SDHD c.341A>G p.Y114C mutation causing endemic PGL1 syndrome, its origin and spread to neighboring areas. In this large and genetically homogenous population, we investigated the natural history of paraganglioma to acquire relevant data for an evidence based strategy in the challenging clinical management of this rare tumor. We evaluated by TC/RMN 38 individuals for a total of 71 paraganglioma. The mean follow up period ranged from 9 to 257 months, with a mean of 56 and a median of 35 . Seventeen of 68 paragangliomas (25%) showed a volume increase > to 20 % and the median annual volume variation was 9,7 mm3 (mean 641± 3062 mm3); 26 paragangliomas, with an annual volume increase larger than 64 mm3 were considered growing. We registered 3 de novo paraganglioma. Basal volume, but not diameter, age at diagnosis, gender or altitude, were significant predictors of tumor growth. During the follow up period, neurological symptoms were not observed. In conclusion, in the endemic area, paragangliomas grows very slowly and their growth may be predicted by basal volume. Low growth rate of this essentially benign tumor may argue against an aggressive treatment and favor a wait and see strategy.

9.00 - Graeme Eisenhofer. *UPLC-MS/MS analysis of plasma versus urine catecholamine O-methylated metabolites for diagnosis of phaeochromocytoma.*

Graeme Eisenhofer[1], Mirko Peitzsch[1], Daniela Pelzel[1], Roland Därr[1], Anthony Stell[2], Felix Beuschlein[3], Martin Fassnacht[4], Aleksander Prejbisz[5] and Andrzej Januszewicz[5]

1- University Hospital Carl Gustav Carus, Dresden, Germany; 2- University of Melbourne, Melbourne, Victoria, Australia; 3- Medical Clinic Innenstadt, LMU, Munich, Germany; 4- Medical University Hospital, Wurzburg, Germany; and 5- Institute of Cardiology, Warsaw, Poland.

Background: Measurements of plasma metanephrines (normetanephrine and metanephrine) provide a sensitive test for diagnosis of pheochromocytomas and paragangliomas (PPGLs), but do not allow detection of dopamine-producing tumours. There also remains controversy over use of the plasma compared to the urine test.

Objective: To determine the utility of UPLC-MS/MS measurements of plasma metanephrines compared to urine free or deconjugated metanephrines for diagnosis of PPGLs and to establish any further utility of measurements of methoxytyramine.

Methods: Preliminary analysis of data from the Prospective Monoamine-producing Tumour study, including 54 patients with diagnosed PPGLs and 323 patients in whom PPGLs were not confirmed. Plasma free, urine deconjugated and urine free normetanephrine, metanephrine and methoxytyramine (the latter test with urine catecholamines) were determined by UPLC-MS/MS in samples from all patients. Receiver-operating characteristic (ROC) curve analysis was used to assess diagnostic efficacy.

Results: Areas under ROC curves (AUROCC) suggested diagnostic advantages of measurements of plasma over urine free or deconjugated normetanephrine and metanephrine (AUROCC = 0.974, 0.961 & 0.956), all of which were superior to measurements of urine norepinephrine and epinephrine (AUROCC = 0.887). Additional measurements of methoxytyramine or dopamine respectively increased AUROCC to 0.999, 0.988, 0.968 and 0.922. Associated values for diagnostic sensitivity and specificity respectively increased from 95% and 97% to 100% and 98% for the plasma metabolites, whereas for the urine free metabolites sensitivity increased from 89% to 96% at the expense of a small drop in specificity (98% to 96%). Little change in diagnostic sensitivity and specificity from 90% and 97% to 90% and 98% for urine deconjugated metabolites and from 87% and 85% to 87% and 83% for urine catecholamines did not indicate any advantages of measurements of methoxytyramine or dopamine for these two tests.

Conclusions: Among all 4 tests examined, combined measurements of plasma free normetanephrine, metanephrine and methoxytyramine offer the test with the highest efficacy for diagnosis of PPGLs. Among urine tests, measurements of the free metabolites appear to offer advantages over the deconjugated metabolites.

9.15 - Jyotsna Upendra Rao. *Correlation between energy metabolism and catecholamine content in pheochromocytoma and paraganglioma.*

JU Rao[1], UF Engelke[1], RJ Rodenburg[1], RA Wevers[1], K Pacak[2], G Eisenhofer[3], B Kusters[1], AG Goudswaard[1], JW Lenders[1], AR Hermus[1], AR Mensenkamp[1], DH Kunst[1], FC Sweep[1], HJ Timmers[1]

1- Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; 2- National Institutes of Health, Bethesda, USA; 3- University Hospital Carl Gustav Carus, Dresden, Germany.

Background: Genotype specific differences have been observed in energy and catecholamine metabolism in pheochromocytoma and paraganglioma (PGL). *RET* and *NF1* related PGLs produce both epinephrine and norepinephrine and have low rate constants for catecholamine secretion, while *SDHx* and *VHL*–related tumors have lower catecholamine content, mainly produce norepinephrine and have high rate constants for catecholamine secretion. The sequestration of catecholamines into chromaffin granules through vesicular monoamine transporters is dependent on the H+ gradient generated by ATP dependent vesicular membrane proton pump. Chromaffin granules contain high ATP concentrations which contribute to the stability and maintenance of catecholamine stores. Thus, we investigated relationships between genotype-specific differences in mitochondrial function and catecholamine content in PGL tumor tissues.

Design and Methods: 94 primary PGL tissues collected from patients with the following genotypes were investigated: *SDHB* (n=13), *SDHD* (n=9), *SDHAF2* (n=1), *VHL* (n=9), *NF1* (n=8), *MAX* (n=4), MEN-2 (n=13) and sporadic (n=37). ATP/ADP/AMP and catecholamine levels in PGLs were measured using 500MHz 1H-NMR spectrometer in deproteinized tumor tissue. The tumor tissue homogenates were assayed for RCCs (complex I-IV) on Konelab20XT autoanalyzer.

Results: Low complex II activity in tissues with *SDHx* mutations was significantly associated with low tumor tissue ATP/ADP/AMP levels, succinate accumulation and norepinephrine dominant tumor catecholamine content. The respiratory chain enzyme activities and ATP/ADP/AMP levels showed a significant positive correlation with epinephrine, norepinephrine ($p<0.05$) and total catecholamine content ($p<0.01$). Passing-Bablok regression statistics demonstrated a significant linear relationship between complex I, II and III activities and total catecholamine content.

Conclusion: Positive correlation and linear relationship was found to be present between respiratory chain function and tumor tissue ATP/ADP/AMP content and catecholamine metabolism. This suggests that differences in energy metabolism contribute to the lower tumor tissue catecholamine contents in cluster 1 than in cluster 2 tumors.

9.30 - Lindsey Oudijk. *SDHA Mutations in Adult and Pediatric Wild-Type Gastrointestinal Stromal Tumors.*

Lindsey Oudijk[1], José Gaal[1], Esther Korpershoek[1], Francien H. van Nederveen[1], Lorna Kelly[2]; Gaia Schiavon[3], Jaap Verweij[3], Ron H.J. Mathijssen[3], Michael A. den Bakker[1], Rogier A. Oldenburg[4], Rosa L.E. van Loon[4], Maureen O'Sullivan[2], Ronald R. de Krijger[1], Winand N.M. Dinjens[1]

1- Department of Pathology, Erasmus MC, University Medical Center Rotterdam, The Netherlands; 2- Department of Pathology, Our Lady's Children's Hospital, Crumlin, Dublin 12, Ireland; 3- Department of Medical Oncology, Erasmus MC, University Medical Center Rotterdam, The Netherlands; 4- Department of Clinical Genetics, Erasmus MC, University Medical Center Rotterdam, The Netherlands.

Most gastrointestinal stromal tumors (GISTs) harbor oncogenic mutations in KIT or platelet-derived growth factor receptor-α (PDGFRA). However, a small subset of GISTs lacks such mutations and is termed “wild-type GISTs”. Germline mutation in any of the subunits of succinate dehydrogenase predisposes individuals to hereditary paragangliomas and pheochromocytomas. However, germline mutations of the genes encoding SDH subunits A, B, C or D (SDHA, SDHB, SDHC or SDHD; collectively SDHx) are also identified in GISTs. SDHA and SDHB immunohistochemistry are reliable techniques to identify pheochromocytomas and paragangliomas with mutations in SDHA, SDHB, SDHC and SDHD. In this study we investigated if SDHA immunohistochemistry could also identify SDHA-mutated GISTs. Twenty-four adult wild-type GISTs and nine pediatric/adolescent wild-type GISTs were analyzed with SDHB and where this was negative, then with SDHA immunohistochemistry. If SDHA immunohistochemistry was negative, sequencing analysis of the entire SDHA coding sequence was performed. All nine pediatric/adolescent GISTs and seven adult wild-type GISTs were negative for SDHB immunohistochemistry. One pediatric GIST and three SDHB immunonegative adult wild-type GISTs were negative for SDHA immunohistochemistry. In all four SDHA-negative GISTs a germline SDHA c.91C>T transition was found leading to a nonsense p.Arg31X mutation. Our results demonstrate that SDHA immunohistochemistry on GISTs can identify the presence of an SDHA germline mutation. Identifying GISTs with deficient SDH activity warrants additional genetic testing, evaluation and follow-up for inherited disorders and paragangliomas.

9.45 - Judith Favier. *Somatic NF1 inactivation is a frequent event in sporadic pheochromocytoma.*

Favier J, Burnichon N, Buffet A, Parfait B, Letouzé E, Laurendeau I, Loriot C, Pasmant E, Abermil N, Valeyrie-Allanore L, Bertherat J, Amar L, Vidaud D, Gimenez-Roqueplo AP.

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Germline mutations in the *RET, SDHA, SDHAF2, SDHB, SDHC, SDHD, MAX, TMEM127, NF1* or *VHL* genes are identified in about 30% of patients with pheochromocytoma or paraganglioma and somatic mutations in *RET*, *VHL* or *MAX* genes are reported in 17% of sporadic tumors. In the present study, using mutation screening of the *NF1* gene, mapping of chromosome aberrations by single nucleotide polymorphism (SNP) array, microarray-based expression profiling and immunohistochemistry (IHC), we addressed the implication of NF1 somatic alterations in pheochromocytomas and paragangliomas. We studied 53 sporadic tumors, selected because of their classification with RET/NF1/TMEM127-related tumors by genome wide expression studies, as well as a second set of 11 independent tumors selected on their low individual levels of NF1 expression evaluated by microarray. Direct sequencing of the NF1 gene in tumor DNA identified the presence of an inactivating NF1 somatic mutation in 41% (25/61) of analyzed sporadic tumors, associated with loss of the wild-type allele in 84% (21/25) of cases. Gene expression signature of NF1-related tumors highlighted the downregulation of NF1 and the major overexpression of SOX9. Among the second set of 11 tumors, two sporadic tumors carried somatic mutations in *NF1* as well as in another susceptibility gene. These new findings suggest that NF1 loss of function is a frequent event in the tumorigenesis of sporadic pheochromocytoma and strengthen the new concept of molecular-based targeted therapy for pheochromocytoma or paraganglioma.

10.00 - Aguirre A. de Cubas. *DNA Methylation profiling of pheochromocytoma and paragangioma. Adding more pieces of the puzzle.*

Aguirre A de Cubas[1], Iñaki Comino-Méndez[1], Esther Korpershoek[2], Ronald de Krijger[2], Nan Qin[3], Graeme Eisenhofer[3], Felix Beuschlein[4], Henry Timmers[5], Giuseppe Opocher[6], Massimo Mannelli[7], Alberto Cascón[1] and Mercedes Robledo[1].

1-Hereditary Endocrine Cancer Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain; 2- Department of Pathology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands; 3- University Hospital Dresden, Dresden, Germany; 4- Endocrine Research Unit, Medizinische Klinik Campus Innenstadt, Klinikum der LMU, Munich, Germany; 5- Department of Endocrinology, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands; 6- Department of Medicine, University of Padova, Padova, Italy; 7- Department of Clinical Pathophysiology, University of Florence. Florence, Italy.

Pheochromocytoma and paragangiloma (PCC/PGL) are genetically heterogeneous tumors and can present highly variable clinical behavior. It is now common knowledge that up to 40% of PCC/PGLs are hereditary and associated with mutations in any one of more than ten known susceptibility genes. While this has improved disease management and facilitated genetic counseling, still relatively little is known about the molecular mechanisms involved in its pathogenesis and disease progression. It is therefore essential to continue to identify tumor-specific molecular alterations so that effective diagnostic screening and curative therapies may be developed. In this regard, several high-throughput profiling studies on PCC/PGL have added to our understanding of the molecular biology behind these tumors, but to date, no study has assessed the alterations to the epigenetics of PCC/PGL, including DNA methylation. Here, we test the hypothesis that PCC/PGL can be distinguished on the basis of global DNA methylation patterns. Using the Infinium HumanMethylation27 BeadChip, we preformed methylation profiling across 27578 CpG sites in a large series of fresh-frozen hereditary (*VHL, SDHB, SDHD, RET, NF1*, and *MAX*-related) and sporadic PCC/PGL (n=89) and adrenal medullas (n=5), to identify epigenetic alterations to further understand the molecular biology of this disease and identify potential diagnostic markers. We show that unsupervised analysis of DNA methylation profiles divides PCC/PGLs into subsets according to genetic background. We identified methylation signatures specific to and common among various genetic classes of PCC/PGL, providing evidence that differences in DNA methylation can be used as a platform for biomarker discovery and development.

10.15 - Camilo Jiménez. *Current and future systemic therapies against metastatic pheochromocytoma and sympathetic paraganglioma.*

Camilo Jimenez. Department of Endocrine Neoplasia and Hormonal Disorders. The University of Texas MD Anderson Cancer Center.

Approximately 17% of pheochromocytomas (PH) and sympathetic paragangliomas (PG) are metastatic. Treatment against metastatic disease includes systemic chemotherapy and radiopharmaceutical agents that are mostly nonspecific and indiscriminately target dividing cells. Patients with metastatic tumors have high morbidity and mortality rates from excessive catecholamine secretion, cardiovascular complications, and bulky disease, for which no curative treatment is available. The 5-year overall survival rate for patients with metastatic tumors ranges is approximately 50%. Up to 50% of metastatic cases could be associated with hereditary germline mutations. This knowledge is now leading to the development of new therapies based on the molecular mechanisms involved in the formation of malignant PH/PG. In a recently published retrospective study, sunitinib, a multiple tyrosine kinase receptor inhibitor, was associated with clinical benefits in patients with progressive metastatic PH/PG. Approximately, 47% of patients experienced partial responses or prolonged stabilization of disease, including four with predominant skeletal metastases on 18-FDG-PET/CT scan. Of 14 patients who had hypertension, 6 became normotensive and 2 discontinued antihypertensives. The median overall survival from the time sunitinib was initiated was 26.7 months with a PFS of 4.1 months (95% CI 1.4-11.0). Most of the responsive patients were carriers of SDHB mutations.
Conclusion: sunitinib is associated with tumor size reduction, decreased 18-F-FDG PET/CT uptake, disease stabilization, and hypertension improvement in some patients with progressive metastatic PH/PG. Prospective multi-institutional clinical trials are needed to determine the true benefits of sunitinib or other molecular targeted therapies.

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POSTERS

P.1 *Cytotoxic effects of everolimus in combination with mitotane or SOM230 in adrenocortical cancer cell lines*.

Germano A, Rapa I*, Volante M*, Basile V, Ardito A, Duregon E*, Papotti M*, Terzolo M.

Internal Medicine 1, Department of Clinical and Biological Sciences; Pathology*, Department of Oncology, San Luigi Hospital, University of Turin, Italy.

Background: Mitotane is the most effective agent in the treatment of adrenocortical carcinoma (ACC), although affected by significant toxicity and incomplete efficacy. Therefore, there is an urgent need of novel treatments to improve the therapeutic strategies in ACC patients. In vitro data suggest that the IGF/mTOR pathway is involved in the pathogenesis of ACC and may represent a therapeutic target. SOM230 inhibits adrenal hormone secretion but it remains unknown whether SST analogs are effective in ACC.

Aim: to investigate in vitro the effects of different agents (mitotane, SOM230 and everolimus) on the growth of human ACC cells. Methods: H295R and SW13 ACC cell lines were incubated with mitotane, SOM230 and everolimus, either alone or in combination. Cell viability was determined by WST-1 method, and the interaction between drugs was calculated using the combination index (CI).

Results: Mitotane determined a cytotoxic activity in H295R cells only. Everolimus determined cytotoxic effects in both ACC cell lines tested, whereas SOM230 was not effective in both cell lines. The association of SOM230 and mitotane determined an antagonistic effects (CI= 7.56) in SW-13 cells and a synergistic effect (CI=0.63) in H295R cells. By contrast, SOM230 enhanced everolimus activity in both SW-13 and H295R cells (CI=0.56 and 1E-05 respectively).

Conclusions: Everolimus may be an effective treatment in ACC and may be potentiated by the association with SOM230. The combination of SOM230, or everolimus, and mitotane needs further in vitro studies to be proposed in clinics.

P.2 *Assessment of the HPA axis in patients on adjuvant mitotane treatment following removal of adrenocortical cancer.*

A. Ardito, F. Laino, B. Zaggia, F. Daffara, V. Basile G. Reimondo, C. Sciolla, M.C. Zatelli*, M. Terzolo

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Background: mitotane has a well-known adrenolytic effect but it remains unsettled if the drug may also affect pituitary ACTH release. Aim of the study: to evaluate the HPA axis in patients on adjuvant mitotane treatment after radical resection of adrenocortical cancer (ACC).

Patients and methods: ovine CRH test (100 mcg iv. with blood drawing at -15, 0, 15, 30, 45, 60 min for ACTH and cortisol measurement) was performed in 16 patients on adjuvant mitotane treatment and steroid supplementation with cortone acetate for more than 6 months and no evidence of disease at the time of study. Average duration of therapy was 34 months (12-63), median mitotane plasma level was 13.5 mg/l (1.7-24.3) and median dose of cortisone acetate was 62.5 mg daily (50-100), corresponding to 0.8 mg/kg (0.57 – 1.22).

Results: 7 patients showed a basal ACTH level <111 pg/ml (the median value of the cohort; range 30-554), among these 6 patients (85.7%) received a dose of cortisone acetate >0.8 mg/kg and 4 patients (57%) had mitotane levels <14 mg/l. Nine patients had a basal ACTH value >111; among these 7 (78%) received a dose <0.8 mg/kg cortisone acetate and 5 patients (56%) had mitotane levels <14 mg/l. After CRH, an ACTH % increment >50% was seen in 13 patients (81.2%) with a median value of 185% (20-830). Serum cortisol was undetectable in 6 patients (37.5%), and only 2 patients (12.5%) showed serum cortisol >8 mcg/dl. All patients showed low levels of salivary cortisol and elevated levels of CBG. After CRH, only one patient (6.2%) had a cortisol % increment > 20%.

Conclusion: the present data show that the inhibitory effect of mitotane on the HPA axis is played mainly at the adrenal level blunting cortisol secretion. In patients with ACC on adjuvant mitotane, ACTH levels vary more as a function of replacement therapy than mitotane levels.

P.3 *Investigation of β-catenin, N-cadherin and E-cadherin expression in adrenocortical tumors.*

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Background: Adrenocortical tumors (ACT) are classified as adenomas (ACA) or carcinomas (ACC). β¬catenin constitutive activation is a frequent alteration in benign and malignant ACT. E-cadherin was discovered as a protein associated with β-catenin which plays a crucial role in cadherin-mediated cell adhesion. N-cadherin seems to be involved in the development of malignant ACT, but information regarding expression of N-cadherin or E-cadherin in ACT is very limited.
Aim: to evaluate the expression of N-cadherin, E-cadherin and β-catenin in ACT and in ACC cell line models (H295R and SW13).

Methods: We analyzed differential expression of β-catenin, N-cadherin and E-cadherin by immunohistochemistry and by quantitative Real time-PCR in 71 sporadic ACT. This study included 8 normal adrenal cortex samples (NA), 24 ACC, 18 aldosteronomas (APA), 23 cortisol producing adenomas (CPA) and 6 non-secreting incidentalomas (NSA).

Results: Real-time PCR: Compared with NA, β-catenin was over-expressed in 50% of ACC (12/24) and 51% of ACA (24/47); N-cadherin was down-regulated in 75% of ACC (18/24) and in 60% of ACA (28/47). IHC: 47% of ACC (7/15) and 33% of ACA (11/33) presented increased cytoplasmic and/or nuclear β-catenin accumulation; furthermore 100% of ACC (15/15) presented down-expression of N-cadherin and 18 of 33 ACA (55%) were down-regulated. We did not find expression of E-cadherin in any ACT. Interestingly, Spearman analysis showed correlation between β-catenin and N-cadherin expression (ACC vs ACA).

Conclusion: Our preliminary data suggest that β-catenin overexpression together with the aberrant expression of N-cadherin may participate to progression of ACT. Identification of these and other differentially expressed genes may enhance our understanding of the molecular biology of ACT development, and may contribute in creating new diagnostic and prognostic tools.

P.4 *DKK3 is a component of the genetic circuitry regulating aldosterone biosynthesis in the adrenal cortex.*

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Primary aldosteronism (autonomous aldosterone production from the adrenal cortex) causes the most common form of secondary arterial hypertension, which is also the most common curable form of hypertension. Recent studies have highlighted an important role of mutations in genes encoding potassium channels in the pathogenesis of primary aldosteronism, both in human disease and in animal models. Here we have exploited the unique features of the hyperaldosteronemic phenotype of Kcnk3 null mice, which is dependent on sexual hormones, to identify genes whose expression is modulated in the adrenal gland according to the dynamic hyperaldosteronemic phenotype of those animals. Genetic inactivation of one of the genes identified by our strategy, dickkopf-3 (Dkk3), whose expression is increased by calcium influx into adrenocortical cells, in the Kcnk3 null background results in the extension of the low-renin, potassium-rich diet insensitive hyperaldosteronemic phenotype to the male sex. Compound Kcnk3/Dkk3 animals display increased expression of Cyp11b2, the rate-limiting enzyme for aldosterone biosyntheis in the adrenal zona glomerulosa. Our data show that Dkk3 can act as a modifier gene in a mouse model for altered potassium channel function and suggest its potential involvement in human primary aldosteronism syndromes.

P.5 *First phase insulin secretion is impaired by aldosterone excess in primary Aldosteronism.*

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Context: Primary aldosteronism (PA) represents the most frequent cause of secondary arterial hypertension. Conflicting data have been published regarding the effect of aldosterone excess on glucose and lipid metabolism.

Objective: Our aim was to analyze insulin sensitivity and beta cell function in a cohort of PA patients. Prospective follow-up investigations were performed in a subgroup of patients before and after adrenalectomy to assess the metabolic outcome.

Design: Oral glucose tolerance test, combined intravenous glucose tolerance test (ivGTT) - hyperinsulinaemic-euglycaemic glucose clamp test and arginine test were carried out after a 12-hour fasting period. Patients: 22 consecutive PA patients with both unilateral (n=14) and bilateral (n=8) disease were recruited through the Munich center of the German Conn's Registry. A cohort patients with essential hypertension (EH, n=11) of corresponding age, gender and BMI and a normotensive cohort (n=11) were recruited as controls.

Results: At baseline, first phase insulin reaction in ivGTT was significantly reduced in patients with PA as compared to normal controls (36.0 [24.0;58.7] vs. 90.1 [52.6;143.8] µU/ml, p=0.031) and lower in comparison to EH (53.2 [30.8;73.3] µU/ml, p=0.123). The study was repeated 6 months after adrenalectomy in 9 consecutive patients with unilateral disease. At this time point, blood pressure was normalized in the majority of patients while BMI remained unchanged. The first phase insulin reaction in response to glucose significantly increased at follow-up (from 36.0 [25.5;58.7] to 48.5 [30.4;95.2] µU/ml, p=0.038). In contrast, insulin sensitivity, insulin resistance and response to i.v. arginine did not differ before and after adrenalectomy.

Conclusion: These findings provide evidence that aldosterone excess has a direct negative effect on beta cell function in patients with PA. Accordingly, following adrenalectomy, early insulin secretion improves significantly in these patients.

P.6 *A filtration-and isolation-by-size technique (SCREENCELL®) identifies circulating tumor cells (CTC) as a diagnostic marker of adrenocortical carcinoma: preliminary results of a monocentric study.*

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Objective. Adrenocortical carcinoma (ACC) is a rare malignancy, whose prognosis is mainly dependent on the stage at diagnosis. The identification of disease-associated markers representing solid biomarkers for early diagnosis and drug efficacy monitoring is mandatory to improve survival rate and life quality of patients. CTC are tumor cells originating from primary tumor or metastases. The tumor-induced angiogenesis and invasion allow aggressive tumors to release CTC into blood stream before any detectable metastases are established. Therefore, CTC detection may have enormous potential of assisting malignancy diagnosis, estimating prognosis and monitoring the disease. The presence of CTC in ACC patients have never been investigated so far.

Design & Methods. CTC analysis was performed in 11 ACC and 10 adrenocortical adenoma (ACA) patients. Blood samples obtained before (n=4 patients) and after (n=10 patients) surgery were filtered on Screencell devices (Screencell®), polycarbonate membranes with 8 µm pores which isolate CTC on size-base.

Results. CTC were isolated in all ACC but not in ACA samples. A statistically significant reduction in the number of CTC recovered was evident between samples obtained before and after surgery (mean CTC number/blood ml±SD:before, 3.80±4.4 and after, 0.64±0.62, P=0.007). Immunocytochemistry on CTC compared to the primary tumors revealed positivity for adrenocortical markers (anti-MART-1, alpha-inhibin and synaptophysin) in the 3 patients analyzed. A statistically significant linear relationship was found between the number of CTC recovered after surgery and the diameter (R=0.867, R2=0.752, P=0.001) or the Ki67% (R=0.795, R2=0.632, P=0.007).

Conclusions. CTC were identified for the first time in blood stream of ACC but not of ACA patients, suggesting that they may represent a valid diagnostic marker. Moreover, as the number of ACC recovered is reduced after surgery, CTC levels might eventually be useful also for the patients follow-up.

P.7 *Genomic effects of mitotane in the NCI-H295R adrenocortical cancer cell line.*

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Background: The mechanism of action of the adrenalytic agent mitotane is poorly elucidated. To the best of our knowledge, the pangenomic effect of mitotane has not been studied yet. We have studied mitotane-induced mRNA expression changes in the NCI-H295R adrenocortical cancer cell line.

Materials and methods: Cell viability and hormone assays were used to select the optimal mitotane concentration effectively inhibiting hormone secretion without affecting cell viability. Total RNA isolated from cultures treated for 48 and 72 hours was subjected to Agilent 4x44K microarray platforms. Microarray results were validated by quantitative reverse-transcription polymerase chain reaction (qRT-PCR).

Results: Based on the analysis of cell viability and hormone secretion, 5x10-6 M mitotane concentration has been selected for the study of gene expression. Altogether, 117 significantly differentially expressed genes have been detected at 48h and 72h ($p<0.05$) in mitotane-treated samples relative to controls. 3 significantly underexpressed genes involved in steroid hormone biosynthesis (3¬beta-hydroxysteroid dehydrogenase types 1 and 2, 21-hydroxylase) and 4 significantly overexpressed genes (growth differentiation factor 15, aldehyde dehydrogenase 1L2, Homo sapiens tribbles homolog 3 (Drosophila) and serpin peptidase inhibitor E2) have been validated.

Conclusion: Gene expression changes might be involved in the adrenal action of mitotane and in the inhibition of hormone secretion. The relevance of mitotane¬induced overexpression of the validated four genes is unclear.

P.8 *Expression of Aurora kinases in adrenocortical tumors.*

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Background: Adrenocortical tumors (ACT) are diseases with a usually benign behavior. Among these, adrenocortical carcinomas (ACC) show poor prognosis and metastatic potential. Aurora kinase (AK) family members are serine/threonine kinase involved in the regulation of mitosis.

Aim: to investigate the expression of Aurora kinase A, B, C (AKA, AKB, AKC) in adrenocortical tumors and to evaluate the pan-Aurora kinase inhibitor, MK-0457, in adrenocortical cell lines. Materials and methods: 12 ACT were analyzed: 4 ACC, 3 aldosterone producing adenoma (APA), 3 cortisol producing adenomas (CPA) and 2 non-secreting adenomas (NSA). Also 3 normal adrenal tissues and SW13 and H295R cells were studied. All the samples were evaluated by quantitative RT-PCR for AURKA, AURKB, AURKC. MTT test and 3H thymidine assay were performed in SW13 and H295R cells after treatment with MK-0457.

Results: All tissues and cell lines expressed AKA, AKB and AKC. ACC samples overexpressed AKA and AKB, while CPA showed increased AKA. MK-0457 inhibited SW13 cell viability at 72h with IC50 of 85nM. Furthermore we observed a significant time-dependent reduction in cell proliferation for SW13 cells at 24 and 72h. No appreciable change was perceived in H295R cells.

Conclusions: our preliminary results demonstrated the expression of AKA, AKB and AKC in ACT. Overexpression of AKA in ACC and in CPA may suggest the potential use of AK inhibitor in adrenocortical cell lines. Nevertheless MK-0457 seems to act only in SW13 cells. Further analysis are needed to substantiate these data.

P.9 *Genetic determinants of mitotane pharmacokinetics in adrenocortical cancer patients.*

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Mitotane is the reference agent for treatment of adrenocortical carcinoma (ACC), a rare tumor with a dismal a 5-year survival of less than 50%. Mitotane antitumor efficacy is observed with plasma concentrations >14 mg/l while severe toxicity is associated with concentrations >20 mg/l. Mitotane levels correlate grossly with the assumed dose, but other factors contribute to attainment of blood levels. Mitotane metabolism is mainly mediated by CYP2B1, 3A1, 2B6, and 3A4. Our aim was to assess the potential impact of genetic determinants on mitotane pharmacokinetics and on patient response. We performed a retrospective analysis on 66 ACC patients treated with mitotane. Drug plasma levels were determined by HPLC-UV; pharmacogenetic analysis was performed with RT-PCR on CYP2B6_516G>T, ABCB1_1236C>T, ABCB1_2677G>T and ABCB1_3435C>T. Patients with wild type CYP2B6_516 had significantly lower mitotane levels than those with mutated gene after 3 and 6 months of treatment (p=0.03 and p=0.05, respectively). Afterwards, difference was not longer significant due to dose adjustments based on mitotane levels. In univariate analysis there was no difference in clinical outcome between the 2 groups. Patients with wild type ABCB1_1236 and ABCB1_2677 showed higher mitotane levels at 6 months of therapy (p=0.01 and p=0.04, respectively). ABCB1_3435 had no influence on mitotane levels. In conclusion, we identified a genetic profile predictive of mitotane levels that may be potentially useful to select the start-up mitotane regimen (low-dose vs. high¬dose).

P.10 *Adrenocortical carcinoma: a population based study on incidence and survival in the Netherlands since 1993.*

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Background: Adrenocortical carcinoma (ACC) has a reported annual incidence of 0.5-2.0 cases per million individuals. Updated population-based studies on incidence are lacking and most studies use data from specialized centers or networks and are confounded by selection and referral bias.

The aim of this study was to describe the incidence, prevalence and survival rate of ACC in the Netherlands based on a nationwide survey. Secondary objectives were to evaluate whether the number of patients undergoing surgery or survival rates of ACC changed during the study period.

Methods: Data on all ACC patients diagnosed between 1993 and 2010 were obtained from the Netherlands Cancer Registry (NCR). Data on demographics, stage of disease, primary treatment modality and overall survival were used. Results: Between 1993 and 2010, 359 patients were diagnosed to have ACC. Median age at diagnosis was 56 years (range 1-91). There were 196 females (55%). The 5-year age-standardized incidence rate ranged between 0.78 and 1.75 per one million person-years. On December 31, 2011, 96 patients were alive diagnosed between 1993 and 2010. Median survival for patients with stage I-II, stage III and stage IV was 159 months (95% CI 93-225 months), 26 months (95% CI 4-49 months) and 5 months (95% CI 2-8 months), respectively (P< 0.001). Improvements in survival were not observed: period of diagnosis was not significantly associated with mortality: 2005-2010, HR 0.88 (0.63-1.23) compared to 1993-1998. The percentage of patients not receiving treatment decreased significantly from 24% in 1993-1998 to 12% in 2005-2010 (P=0.047), explained by an increase of surgery in stage III-IV patients (n=45 out of 83 in 1993-1998, n=53 out of 76 in 2005-2010).

Conclusion: We here present epidemiological data on ACC in the Netherlands during 1993-2010. The incidence rate of ACC remained stable. Survival rates did not change during this period despite an increased number of patients undergoing surgery.

P.11 *Activation of G-Protein-Coupled estrogen receptor (GPER) inhibits growth of adrenocortical cancer (ACC) cells in vitro and in vivo suggesting a new target for ACC treatment.*

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Our previous study indicated a central role for Estrogen receptor (ER) alpha (ESR1) in both estrogen dependent and independent (induced by IGF-II/IGF1R pathway) proliferation of ACC cells. In fact, treatment with Selective Modulator of Estrogen receptor Tamoxifene (Tam) significantly suppressed the growth of adrenocortical carcinoma H295R xenograft model. In this study we investigated the role of G-protein-coupled estrogen receptor (GPER), a membrane ER, on ACC cell proliferation. We revealed the GPER mRNA and protein expression in samples of human ACC tissues as well as in H295R cells. Surprisingly, we revealed that activation of GPR30 by the receptor-specific, non-estrogenic ligand, G-1 inhibited the growth of adrenocortical tumor H295R cells in vitro and H295R xenografts in vivo. Moreover, treatment of H295R cells with G-1 induced DNA damage and apoptosis. Silencing GPER with a specific siRNA significantly blocked G-1 effects on H295R cell proliferation and apoptosis confirming a specific involvement of GPER in pathway(s) inhibiting ACC growth. Experiments focused on clarifying the molecular mechanism underlying G-1-induced inhibition of ACC growth suggest the involvement of sustained activation of ERK1/2. In conclusion these results, together with our previous studies, suggest that i) GPER can be considered a new target to control adrenocortical carcinoma cell proliferation; ii) activation of GPER can modulate ACC growth with similar molecular mechanisms observed in other estrogen-dependent tumors; iii) the use of GPER agonists and/or SERM able to activate GPER and to inhibit ESR1 (such as Tamoxifen) could be a very effective new therapy for controlling ACC growth.

P.12 *Abrogation of TLR4 and CD14 Expression and Signaling in Human Adrenocortical Tumors.*

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Context: Adrenocortical carcinoma (ACC) is a rare tumor with poor prognosis. The expression of innate immunity receptor Toll-like receptor 4 (TLR4) was recently reported in various human tumors, and TLR4 was shown to regulate tumor immune escape processes, proliferation, and resistance to chemotherapeutical agents.

Objective: The aim of this study was to investigate TLR4 expression, signaling, and function in the process of tumorigenesis in the human adrenal cortex.

Measurements and Main Results: Real-time PCR analysis of human ACC, adenoma, and ACC cell lines (SW13, NCI-H295R, and HAC15) revealed a significant down-regulation of TLR4, MD2 (myeloid differentiation protein-2), and cluster of differentiation 14 (CD14) mRNA compared with normal human adrenal cortex and adrenocortical cells in primary culture. Furthermore, immunohistochemistry revealed an abrogation of TLR4 and CD14 expression in ACC but not adenomatissues. Western blot analysis of MAPK, AKT, activator protein-1, and nuclear factor-_B signaling revealed that the ACC cell lines are unresponsive to lipopolysaccharide action. Restoration of TLR4 signaling by stable transfection of TLR4-CD14 plasmid into NCI-H295R cells sensitized them to lipopolysaccharide incubation as shown by nuclear factor-□B activation and decreased cell viability and induced apoptosis in these cells.

Conclusion: Our results demonstrate a significant reduction in the expression of TLR4 and CD14 and an inactivation of TLR4 signaling in ACCs. Furthermore, our data show that reintroduction of TLR4 expression in ACCs may provide a novel therapeutic strategy for adrenal cancer.

P.13 *Prevalence of benign and malignant secondary neoplasms in patients with primary aldosteronism.*

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Context: Primary aldosteronism (PA) is the most common cause of secondary hypertension. In vitro, aldosterone excess can cause oxidative stress leading to DNA damage. Single case reports describe a coincidence of PA with renal cell carcinoma and other tumors. However, no data on the prevalence of benign and malignant neoplasms in patients with PA exists.

Methods: In the multicentre MEPHISTO study the prevalence of benign and malignant tumors was investigated in 338 patients with confirmed PA both retro- and prospectively.

Results: Of the 338 patients (199 male and 139 female) 56 (16,6%) had been diagnosed with a tumor at any time of their life, 14 had more than one tumor diagnosis. In total, 75 neoplasms were identified which were in 51% of benign and in 40% of malignant dignity (9% unknown dignity).
58% (n=22) of all benign secondary neoplasms were derived from endocrine tissue (thyroid, parathyroid and pituitary). The remaining benign neoplasms were located in skin (13%), female reproductive organs (5%), lung, brain, prostate, or were characterized as lipoma, hemangioma and tumor of the sebaceous glands (together 24%).
By contrast, only 10% (n=3) of the malignant tumors were of endocrine origin (all thyroid carcinomas). Most of the malignant tumors were skin tumors (21%). Renal cell carcinoma was diagnosed in 5 patients (17%), prostate cancer in 4 patients (13%). Less frequently diagnosed were malignant tumors in gastrointestinal tract (13%), breast cancer and malignant tumors in lung, gastrointestinal tract, larynx, reproductive organs or brain (together 33%).

Conclusion: In this cohort of PA patients a very high prevalence of benign endocrine neoplasms was found. Interestingly, a relatively high prevalence of renal cell carcinoma (17% of malignant neoplasms) was observed. Renal cell carcinoma generally accounts for only 3,3-4,4% of all malignant tumors in Germany[1]. Probable pathophysiological backgrounds are subject of ongoing studies.

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P.14 *[123I]Iodometomidate Imaging in Adrenocortical Carcinoma.*

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Context: Imaging with [123I]iodometomidate ([123I]IMTO) has been shown to diagnose adrenocortical lesions with high sensitivity and specificity.

Objective: Clinical utility of [123I]IMTO SPECT imaging in adrenocortical carcinoma (ACC).
Patients and Interventions: 61 patients with histologically confirmed ACC (ENSAT stage I n=1, stage III n=2, stage IV n=58) received 185 MBq of the radiotracer [123I]IMTO. Sequential planar whole body scans until 24 hours p.i. and SPECT/CT hybrid imaging 4 - 6 h p.i. were performed.

Main Outcome Measures: Sensitivity of [123I]IMTO imaging for detection of ACC lesions. Number of patients with metastatic disease non-invasively characterized as ACC by [123I]IMTO. Number of patients eligible for [131I]IMTO therapy.

Results: Of the 437 lesions detected by conventional imaging, 30% showed strong, 8% moderate and 62% no relevant tracer accumulation. 12 additional lesions were found by [123I]IMTO in 6 patients. 37 of the 61 (61%) patients had at least one [123I]IMTO-positive lesion. Cortisol and aldosterone secretion by ACC was positively correlated to [123I]IMTO uptake; cytotoxic chemotherapy and mitotane treatment did presumably not influence tracer uptake. 23 patients (37.7%) had radiotracer uptake in all lesions ≥ 2 cm and therefore were potential candidates for targeted systemic radiotherapy with [131I]IMTO.

Conclusion: [123I]IMTO detects both primaries and metatastatic lesions of ACC. However, a substantial percentage of lesions fails to show [123I]IMTO uptake. Based on [123I]IMTO imaging more than a third of patients are eligible for radionuclide therapy with [131I]IMTO.

P.15 *Functional characterisation of adrenal lesions using [123I]IMTO-SPECT/CT.*

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Background: Adrenal tumors are highly prevalent and represent a wide range of different pathological entities. Conventional imaging often provides only limited information on the origin of these lesions. Novel specific imaging methods are, therefore, of great clinical interest. We have recently developed [123I]iodometomidate ([123I]IMTO) as a new radiotracer for specific molecular imaging of CYP11B1/2 enzymes.

Objective: Evaluation of the clinical utility of [123I]iodometomidate ([123I]IMTO) imaging for non-invasive characterization of adrenal masses

Methods: 51 patients with an adrenal lesion underwent [123I]IMTO imaging after injection of 185 MBq [123I]IMTO. Sequential planar whole body scans until 24 hours p.i. and SPECT/CT imaging 4 - 6 h p.i. were performed.

Results: Adrenocortical tissue showed high and specific tracer uptake with short investigation time and low radiation exposure. Qualitative analysis of SPECT/CT data resulted in a sensitivity of 89% and a specificity of 85% for differentiating adrenocortical tumors from lesions of non-adrenocortical origin. ROC analysis of semiquantitative data revealed a sensitivity of 83% and a specificity of 86% for identification of adrenocortical lesions at a cut-off value of tumor- to-liver ratio of 1.3.

Conclusions: [123I]IMTO is a highly specific radiotracer for imaging of adrenocortical tissue with short investigation time and low radiation exposure. Due to the general availability of SPECT technology, [123I]IMTO scintigraphy has the potential to become a widely used tool to non-invasively characterize the biology of adrenal lesions.

P.16 *Phase II study of dovitinib in first line metastatic or non resectable primary adrenocortical carcinoma (ACC). SOGUG study 2011-03.*

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Background: Dovitinib is a novel targeted therapy, that has proven to inhibit, among other tyrosin kinases, the fibroblast growth factor receptor (FGFR). Since this pathway has been proposed to play a relevant role in ACC, we aim to test the clinical efficacy of dovitinib in this tumor.

Methods: An open label phase II trial was designed in patients with advanced non-resectable ACC. Taking as a basis the two-stage Gehan model, 15 patients were scheduled to be included in the first stage to demonstrate a treatment efficacy of at least 15%. Sample size calculation was done based on the following parameters, probability of Type I error $\alpha = 0.05$, power of the test $(1 - \beta) = 0.8$. Main inclusion criteria were advanced non-resectable disease and no prior therapy (other than mitotane). Since this is an extremely unfrequent disease 7 institutions, with the support of the SOGUG (Spanish Oncology Genitourinary Group), have participated.

Results: 17 patients (5 male; 12 female) have been included from January 2012 to August 2012. Up to date, main toxicity has been astenia that has reached grade 3 in two cases. Other grade 3 secondary effects have been hypertension (one case) and GGT raise (one case). One patient presented grade 3 diarrhea, rectal bleeding and renal insuficiency, all together, but were considered not related to the drug since the patient had been withdrawn from treatment due to disease progression. Regarding efficay no objective response has been achieved, 5 patients experienced disease stabilitation (one lasting longer than 6 months) and 4 disease progression. 8 patients have not yet been evaluated. One patient died from tumor progression Updated data regarding efficacy and toxicity will be presented at the ENSAT meeting. A translational research, including whole exome analysis, will be performed in order to improve our scarce knowledge of ACC.

EudraCT number: **2011-002873-47**

ClinicalTrials.gov Id: **NCT01514526**

P.17 *A case of high risk adrenocortical adenocarcinoma (ACC).*

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ACC is a rare tumor with poor prognosis. Radiotherapy may reduce local recurrence and mitotane therapy continues to be a cornerstone of ACC treatment.

We report a 50y patient with a 25 cm-high risk ACC treated with radical resection, adyuvant radiotherapy and mitotane treatment, with an excellent response after 22 months of follow up.

The patient had a left adrenal mass of 25 cm infiltrating the upper kidney pole. Presurgery PET-TC did not show distant metastasis. Multiple lung 5-7 mm micronodules were detected on TC.

Radical R0 surgery was performed with splenecthomy and left nephrectomy. Vascular invasion, necrosis and atypical mitosis were found. Surgical margin was affected by the tumour.

Abdominal radiotherapy was performed after surgery and mitotane was started on low dose strategy until radiotherapy was finished and then rapidly increased (see table).

Mitotane	M1	M2	M4	M6	M8	M10	M11	M13	M22
Dose (gr/d)	3	3	6	4.5	5.5	4.5	4	4.5	4.5
Levels (mg/L)	2.4	3.4	23.3	11.7	43.7	23	11	22.6	18

After a 22 months follow up, neither local relapse nor distant metastasis were found. Lung micronodules did no change over time. This is an example of good response to adyuvance therapy in a high risk ACC.

P.18 *The transcriptome of cortisol secreting adenomas : tumors classification, cortisol production genes, and cAMP/Protein Kinase A pathway alterations.*

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Context: Adrenocortical adenomas can be either hormonally silent or responsible for hypercortisolism. The molecular mechanisms are unknown.

Objective: To identify the gene expression profile associated with cortisol secretion in adrenocortical adenomas.

Patients & Methods: The transcriptome of 22 unilateral adrenocortical adenomas (5 non-secreting, 6 subclinical cortisol producing, 11 cortisol producing) was correlated with cortisol secretion. Phosphodiesterase 8B (PDE8B) expression was measured by Western-Blot. Cyclic AMP (cAMP) levels and Protein Kinase A (PKA) activity were studied in 25 adenomas.

Results: Transcriptome unsupervised clustering identifies 2 groups of adenomas, one including only cortisol producing adenomas 8 (/11), while the other is an admixture of the non-secreting, the subclinical cortisol producing and 3 (/11) cortisol producing adenomas (Fisher exact p=0.002). This cluster is driven by genes related to cortisol secretion, and to extracellular matrix.

Several hundreds of genes correlate with cortisol secretion. Among the positively correlated were the steroidogenic enzymes, cholesterol metabolism genes, glutathione S-transferases. Among the negatively correlated genes were translation genes, and GATA-6.

PDE8B, which inactivates the PKA, showed unexpectedly the strongest positive correlation with cortisol secretion, confirmed by Western Blot. Accordingly, the cAMP was low in cortisol producing adenomas (p=0.009). Despite low cAMP, PKA activity remained unchanged suggesting a mechanism of constitutive activation in secreting adenomas.

Conclusion: The transcriptome of cortisol secretion identifies 2 types of adenomas and a list of genes presenting an expression level correlated with cortisol secretion. Up-regulation of PDE8B, a gene demonstrated in knock-out mice to play a major role in glucocorticoid secretion, suggests a counter-regulatory mechanism to counteract an original cAMP/PKA signalling alteration in cortisol secreting adenomas.

P.19 *Anti-tumor effects of peptide analogues targeting neuropeptide hormone receptors in rodent pheochromocytoma cells.*

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Pheochromocytoma (PHEO) is a rare but potentially lethal chromaffin cell tumor. The prognosis for malignant pheochromocytoma is particularly poor and there are currently no effective treatments. Interestingly, peptide hormone receptors are frequently overexpressed on endocrine tumor cells and can be specifically targeted by highly effective anti-tumor peptide analogs. Indeed, we have previously demonstrated an aberrant expression of various neuropeptide hormone receptors on both tumors and tumor cell lines of the adrenal medulla and cortex and evidenced significant anti-tumor effects mediated by peptide ligands targeting these receptors in adrenomedullary PC12 and adrenocortical SW13 tumor cell lines. Here, we further studied the expression of somatostatin receptor 2 (sst2), of growth hormone-releasing hormone (GHRH) receptor or its major splice variant SV1 as well as of luteinizing hormone-releasing hormone (LHRH) receptor on mouse pheochromocytoma cells (MPC) and on more aggressive mouse tumor tissue-derived (MTT) cells. Focusing on these cells, we could further demonstrate significant anti-tumor effects mediated by cytotoxic somatostatin analogs AN-162 and AN-238, by LHRH antagonist Cetrorelix, by cytotoxic LHRH analog AN-152 and by GHRH antagonist MIA-602 on MPC. Furthermore, similar anti-tumor effects were evidenced also for AN-152 and MIA-602 on more aggressive MTT cells. Altogether, our study demonstrates that both benign and malignant pheochromocytoma cells abundantly express various neuropeptide hormone receptors and that targeted peptide analogs binding to these aberrantly expressed receptors could mediate strong anti-tumor effects, as demonstrated by significant reductions in pheochromocytoma cell survival and proliferation as well as increases in tumor cell apoptosis. Based on s.c. and i.v. injections of MPC cells into athymic nude mice, we are establishing two mouse models of pheochromocytoma for testing the most effective peptide analogs in vivo.

P.20 *Identification of chromosome 11 parental origin in a population with endemic PGL1 syndrome.*

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Paraganglioma syndrome type 1 (PGL1) is a rare autosomal dominant disease with maternal imprinting characterized by the development of head-and-neck paragangliomas and pheochromocytoma, associated with germ-line mutations of SDHD gene. We identified a PGL1 founder effect caused by the SDHD c.341A>G p.Tyr114Cys mutation and we have so far collected more than 100 families resident or native from a restricted area around Trento mountain in North Italy. The genetic evaluation of 4025 resident volunteers allowed to identify 59 carriers of the founder mutation, resulting in a prevalence of 1.5% among the general population. The identification of a large numbers of carriers with an unknown inheritance highlights the lack of a tool to discriminate the parental origin of the mutated chromosome. To this aim we isolated the chromosome 11 using the conversion technology and analyzed the methylation pattern of 11p15.5 region. Hybrids were generated by PEG-mediated fusion of lymphoblastoid cell lines with mouse RAG cell line and were cultured in a selective medium. After hybrid clones were grown, we had determined by genotyping which were haploid for chromosome 11. Each of these clones were characterized for the presence of the founder mutation with an allelic discrimination taqman assay and for the methylation pattern of the 11p15.5 region with the MLPA kit ME030-B2. We have obtained hybrid clones using lymphoblastoid cell lines from two carriers with known inheritance, and the analysis of the methylation pattern with the MLPA kit ME030-B2 confirmed the parental origin of the chromosome 11. Using this validated method, we therefore investigated a patient with an unknown inheritance and the analysis of the methylation pattern revealed the maternal origin of the mutation. Preliminary results indicate that this approach may be useful to demonstrate parental origin of the SDHD mutation, allowing paraganglioma risk estimation in individuals with unknown inheritance.

P.21 *Functional assessment of MAX variants of unknown significance using PC12 cells.*

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Background. Since the recent discovery of Myc associated factor X (MAX) as a new susceptibility gene of pheochromocytoma, multiple variants of unknown significance have been found. A functional characterization of these variants in order to provide a proper clinical follow-up and genetic counseling is required.

Aim: To set-up an approach to classify MAX variants of unknown significance using PC12 as a cell line model.

Methods: MAX coding region was cloned in an expression vector, incorporating wild¬type and mutant variants, and co-transfected in PC12 cells with a luciferase vector containing E-Boxes binding sites. In addition we incorporated in silico predicted effects in crystallized MAX protein model. Immunohistochemical MAX staining was performed in order to determine the localization of MAX mutants.

Results: The synonymous variant S138S showed a similar luciferase activity as MAX wt, and the truncating pathogenic variant R33X significantly increased the luciferase activity ($P<0.001$). We transfected 11 different missense variants and we could demonstrate the pathogenic character at least for nine of them. Crystallographic predictions as well as the aberrant localization of some of the MAX variants analyzed, supported its pathogenicity.

Conclusion: We propose a functional model to assess pathogenicity of MAX variants. This model could be incorporated to the genetic counseling offered to these patients.

P.22 *Favourable outcome in patients with pheochromocytomas and paragangliomas treated with 177Lu-DOTA-Octreotate.*

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Background: Prognosis for patients with metastatic pheochromocytoma (PCC) and paraganglioma (PGL) remains poor and there are few treatment options available. Treatment with radiolabeled somatostatin analogues have showed positive results in gastroenteropancreatic neuroendocrine tumours as well as in PCC and PGL.

Methods: All consecutive patients with progressive PCC and PGL treated with 177Lu-DOTA-Octreotate (n=13) at Uppsala University Hospital from 2006 to 2012 were analysed. A median of four (range 3-6) treatments with 7,4 GBq activity were administrated every 8th week. Response was evaluated using post treatment 177Lu-DOTA-Octreotate scans, computed tomography (CT) / magnetic resonance imaging (MRI) and tumour biomarkers (catecholamines and chromogranin A).

Results: The overall response rate in 12 evaluable patients was 25%; three patients had (25%) partial responses (PR) while none had complete response (CR). Eight patients (67%) had stable disease (SD) and one had (8%) progressive disease (PD). 10 patients (83%) achieved PR by at least one modality without progressive disease. Median progression free survival (PFS) was 17 months. Overall response was observed in 3/9 patients with Ki67 corresponding to ENETS grade 1 and 2 but not in ENETS grade 3. ENETS grading showed significant correlation to PFS (log rank, p=0.03). No haematological toxicity grade 3-4 was reported, however one patient had hypertension grade 3-4.

Conclusion: 177Lu-DOTA-Octreotate therapy of PCC and PGL is associated with favourable outcome. High tumour proliferation and ENETS grading are potential negative predictive factors for response to therapy.

P.23 *SDHD immunohistochemistry: A new tool to confirm SDHx mutations in pheochromocytoma/paraganglioma.*

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Inherited pheochromocytomas and paragangliomas can be caused by germline mutations in genes encoding one of the 4 succinate dehydrogenase subunits: SDHA, SDHB, SDHC and SDHD (SDHx). Germline mutations in SDHB gene are associated with malignancy and poor prognosis. In 2009, SDHB immunohistochemical procedure was established to detect patients harbouring SDHx mutations: SDHx mutated samples presented a negative immunostaining while it was positive in tumours carrying mutations in the other predisposing genes and in sporadic tumours. In 2011, we have shown that SDHA immunochemistry (IHC) was specifically negative in SDHA-mutated tumours and allowed detecting patients with germline SDHA mutations. In this study, we addressed if the same procedure could be applicable to detect patients with germline SDHD mutations, by using a new commercially available anti-SDHD antibody. We performed a retrospective study on 172 tumours, which included 137 pheochromocytomas and 27 paragangliomas in which we investigated SDHD protein expression. Unexpectedly, 26 of 27 SDHx-mutated tumours showed positive SDHD immunostaining. For the 137 tumours without SDHx mutation, we observed 128 negative staining and 9 samples with a positive SDHD IHC. This technique thus allows detecting SDHx tumours with a sensibility of 96,3% and a specificity of 94,12%. In conclusion, our results demonstrate that we are able to confirm SDHx mutations with a positive staining using SDHD IHC. One hypothesis to explain these results is the conformation of active or inactive succinate dehydrogenase complex. It is postulated that in the presence of an SDHx mutation, the disassembly of the complex exposes the epitope recognized by the antibody, which is apparently hidden when the complex is active, in non-SDHx tumours. Such IHC could be a useful tool to complement SDHB IHC to identify SDHx-related patients.

P.24 *Catecholamine Phenotypes and Hypoxia-Related Gene Expression in Hereditary Phaeochromocytomas: Insights from MAX.*

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Background: Phaeochromocytomas and paragangliomas are highly heterogeneous tumours with variable catecholamine biochemical phenotypes and diverse hereditary backgrounds due to mutations of numerous genes (*RET, VHL, SDHA/B/C/D, SDHAF2, NF1* and *TMEM127*). Gene expression profiling studies indicate two dominant expression clusters: cluster 1 (*VHL/SDH*) and cluster 2 (*RET/NF1/TMEM127*). Mutations of MYC associated factor X (*MAX*), the 10th identified tumour susceptibility gene, lead to tumours that belong to cluster 2, but which show an intermediate catecholamine phenotype of unusually low adrenaline production.

Hypothesis: We propose that HIF1α disrupts MAX/MYC, acting with Sp1 as a cis-activating transcription factor, and stimulates PNMT gene expression. HIF2α stabilizes MAX/MYC complexes, in turn promoting MYC DNA binding at both the E box and Initiator element (Inr). We hypothesize that HIF1α and HIF2α may have opposing effects on PNMT regulation in a MAX/MYC dependent fashion, thereby explaining variations in clinical catecholamine phenotypes of phaeochromocytomas.

Methods: We analyzed gene expression of HIF1α, HIF2α, phenylethanolamine N¬methyltransferase (PNMT) and V-myc myelocytomatosis viral oncogene (c-Myc) in 61 patient tumours (32 cluster 1, 23 cluster 2 and 6 MAX tumours) by real-time RT-PCR. PNMT activity was measured by an in-house LC-MS/MS based stable isotope method. Nucleofector technology was used for cell transfection of MAX, HIF1α and HIF2α in Mouse phaeochromocytomas cells (MPC) and PC12 cells.

Results: HIF2α gene expression in cluster 1 tumours was 2-to 3-fold higher than in cluster 2 tumours, whereas HIF1α and c-Myc showed no differences. *MAX* tumours showed an intermediate catecholamine phenotype, intermediate gene expression of HIF2α and significantly lower gene expression of HIF1α and c-Myc than in cluster 1 and 2 tumours, which did not differ. After silencing

MAX in MPC cells and HIF1α in PC12 cells, PNMT gene expression was decreased. After silencing HIF2α in PC12 cells, PNMT gene expression was increased.

Discussion: These data support a model in which HIF1α and HIF2α alter MYC/MAX binding, acting in apposing directions to regulate PNMT expression. HIF1α enhances PNMT gene expression while HIF2α inhibits expression. This mechanism may explain distinct catecholamine phenotypes in cluster 1 and cluster 2 phaeochromocytomas, the intermediate phenotype in *MAX* tumours and may also contribute to the general regulation of PNMT.

P.25 *Synergistic effect of simultaneous inhibition of PI3K/mTORC1/2-and MEK/ERK¬signalling pathways in mouse phaeochromocytoma cells.*

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Malignant phaeochromocytomas and paragangliomas are generally resistant to most conventional therapies. In previous studies, using two mouse phaeochromocytoma cell lines (MPC and MTT cells), we have shown that the dual PI3K/mTORC1/2-inhibitor NVP-BEZ235 (Novartis) decreases cell proliferation, but that addition of lovastatin will add to this effect. Our data suggested that this additive response might be due to inhibition of the MEK/ERK-pathway. We have now used the specific MEK-inhibitor MEK162 (Novartis) alone and in combination with NVP-BEZ235, and found that these agents show potent effects singly, a synergistic effect at therapeutically relevant low doses (50nM NVP-BEZ235/1.25µM MEK162, combination index (CI): 0.32; 100nM NVP-BEZ235/2.5µM MEK162, CI:0.30), and very strong synergism at high doses (1.6µM NVP-BEZ235/40µM MEK235, CI: 0.08). We have now also recalculated our results on NVP-BEZ235 and lovastatin, and show that this combination is also synergistic rather than being simply additive (synergistic effect at 50nM NVP-BEZ235/10µM Lovastatin, CI: 0.43; strong synergistic effect at 100nM NVP-BEZ235/10µM Lovastatin, CI: 0.18; very strong synergistic effect at 500nM NVP-BEZ235/10µM Lovastatin, CI: 0.03). We conclude that combined inhibition of PI3K/mTORC1/2 and MEK is a very potent antagonist to cell survival in a phaeochromocytoma cell line, and both animal in vivo and human clinical studies are a matter of priority.

P.26 *Succinate dehydrogenase subunit B mutations deeply affect cell metabolism.*

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Cancer cells undergo deep changes in their metabolism. The aim of this project was to investigate whether diverse SDHB mutations account for different metabolic alterations. The neuroblastoma cell line (SK-N-AS) was stably transfected with the wild-type human SDHB, or different SDHB mutated constructs carrying the most significant mutations found in our patients affected by PGLs. We evaluated SDH activity of the different cell clones, and found a significant decrease in all the SDHB-mutated clones compared to SDHB wild-type sequence clone. Since it is well known that the respiration carried out by the mitochondrial respiratory chain is coupled with a phosphorylation ending in ATP production and oxygen reduction, we assessed mitochondrial oxygen consumption. As further confirmation of the decreased SDH activity, we found that also oxygen reduction was significantly lower in all the SDHB-mutated cells. To ascertain whether the different SDHB mutations might induced alterations on glycolysis, we measured the glucose uptake. Surprisingly and unexpectedly, we observed that glucose uptake was slightly, but significantly decreased in all the mutated clones. To address the possibility that the inversion of the Warburg phenotype could be dependent on the metabolic preference of lactate versus glucose, we measured the lactate levels in the culturing medium, which was significantly lower in the media of all the mutated clones, suggesting an increase in the utilization of this metabolite. Moreover, we also found that ATP production following cell stimulation was significantly higher in SDHB-mutated clones. Overall, these data demonstrated that SDHB mutations deeply affect cellular metabolism.

P.27 *Chromaffin Progenitor Cells in the Murine Adrenal Medulla. Influence of Repeated Stress.*

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The adrenal gland has a high plasticity; it therefore has been suggested that a subpopulation of proliferation and differentiation competent progenitor cells continues to exist in the adult adrenal medulla. In our previous studies, we characterized these progenitor cells by the expression of neural crest and neural progenitor markers, establishing the method to in vitro enrich these cells from bovine and human adrenals in sphere cultures (1;2). These cells are able to differentiate to the chromaffin and the neural lineage.
In this study nestin-GFP mice are used as a tool to identify progenitor cells in the adrenal medulla. These nestin-GFP positive cells found in adrenal medulla were cultured in specific conditions that stimulated the formation of spheres – chromospheres. In these chromosphere cultures, GFP+ cells in vitro proliferated. To study in situ the involvement of chromaffin progenitor cells in the gland's adaptation to stress, nestin-GFP mice were submitted to repeated immobilization stress. In contrast to control mice, restrained mice lost bodyweight while the adrenals increased in size. Within the adrenals of stressed mice, the number of GFP+ progenitor cells as well as of BrdU+ proliferating cells differed significantly from control animals.
In conclusion the transgenic nestin-GFP mice proved to be an ideal model to visualize and isolate and in vitro study these cells. Stress experiments indicate the involvement of these progenitor cells in the gland's adaptation to increased physiological needs with increased catecholamine synthesis. We will apply our findings to identifying common molecular pathways in progenitor cells and tumour cells, since several lines of evidence indicate the involvement of persistent sympathoadrenal progenitors in the adrenal medulla in the development of pheochromocytomas. Characterization of these cells will form the basis for identifying genetic changes leading to tumourigenic development.

(1) Chung KF et al. Stem Cells 2009; 27:2602-2813; (2) Vukicevic V, Rubin de Celis MF et al., J Mol Neurosci 2012; 48:420-426.

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Notes