Scientific report for the 13th Scientific meeting of ENS@T (European Network for the Study of Adrenal Tumours)

Summary

The 13th Scientific meeting of ENS@T (European Network for the Study of Adrenal Tumours) was held on November 21-22 in Nice (France) with the support of the European Science Foundation, French National Agency for Research, Nice City Council, University of Nice – Sophia Antipolis, Canceropôle PACA, French Society of Endocrinology, La Ligue contre le Cancer (which offered support for six travel grants for young scientists) and HRA Pharma. The conference was attended by 160 participants and represented the most important opportunity of the year for European clinicians and basic scientists involved in the study of adrenal tumours to meet together and to discuss about the results of projects supported by ENS@T and to start new collaborative projects.

Scientific content of and discussions at the event

ENS@T is a European network created to foster research on all kinds of adrenal tumours (benign and malignant). Those tumours are rare and therefore progress with regard to their diagnosis and treatment can only be achieved by combining the efforts of researchers and clinicians from several countries. To overcome these difficulties and to achieve significant progress benefiting the affected patients, scientists from many European countries have created a Network on Adrenal Tumours at a European-wide level. ENS@T aims to improve the understanding of the genetics, tumourigenesis and hormonal hypersecretion in patients with adrenal tumours and associated familial syndromes. It aims to improve the prediction of recurrence and the management of malignant adrenal tumours, which are particularly rare. The study of adrenal tumours is likely to reveal new molecular mechanisms of tumour growth and also provide insight into the role of hormones as the cause of hypertension.

ENS@T was founded in 2002 by the merging of three already existing National Adrenal Networks (Comete in France, GANIMED in Germany, and NISGAT in Italy) and teams from the United Kingdom, all dedicated to the study of adrenal tumours. In 2009, ENS@T became a membership-based society with statutes and bye-laws and a large number of European clinicians and scientists have joined in the efforts of the Network by becoming members of ENS@T. A specific Research Networking Programme (RNP) funded by the ESF (ESF-ENS@T) is dedicated to support ENS@T activities.

Each year the ENS@T holds its annual meeting in a different European venue. The 13th annual meeting of ENS@T took place on November 21-22, 2014 at the Radisson Blu hotel in Nice (France). This conference has represented the most important opportunity of the year for European clinicians and basic scientists involved in the study of adrenal tumours to meet together and to discuss about the results of projects supported by ENS@T and to start new collaborative projects. The meeting gathered 160 participants coming from all over Europe and, in small number, also from Canada, United States, Brazil and Australia.

ENS@T is organized into 4 working groups: ACC (adrenocortical cancer); APA (aldosterone-producing adenomas); NAPACA (non-aldosterone-producing adenomas); PCC/PGL (pheochromocytoma/paraganglioma), each one of which is

directed by a head elected among the members of each working group. A Steering committee elected among all members of ENS@T coordinates the activities of the working groups. The Steering committee and heads of working groups are also responsible for the selection of the abstracts submitted to the annual ENS@T meeting and repartition into oral and poster communications.

According to the organization of ENS@T into working groups, the 13th Scientific meeting started in the morning of its first day (November 21) with oral communications in the field of adtrenocortical cancers (ACC). Dr. Guillaume Assié (Cochin Institute, Paris, France) presented his recent results on the integrated genomic chracterization of ACC and the search of new molecular prognostic markers, while Dr. Emilia Pinto (St. Jude Children's Hospital, Memphis TN – USA) presented her data on genomic profiling of children adrenocortical tumors. Further talks concerned the reproducibility of the Ki67 index, an important prognostic parameter in ACC (Dr. Emilio Pucci, Erasmus University, Rotterdam, The Netherlands), the efficacy of mitotane-retinoic acid combination in a preclinical model of ACC (Dr. Peter Igaz, Semmelweis University, Budapest, Hungary), the action of transcription factor SF-1 in modulating the invasive capacities of adrenocortical cancer cells (Dr. Carmen Ruggiero, IPMC, Sophia Antipolis, France), the search for inhibitors of Patched, a membrane protein implicated in multidrug resistance in ACC cells (Dr. Isabelle Mus-Veteau, IPMC, Sophia Antipolis, France) and the role of the Notch pathway in adrenocortical tumours (Dr. Cristina Ronchi, University of Würzburg, Germany). The following session involved a presentation of the results of a European consensus conference on the surgical treatment of adrenocortical cancer (Dr. Sébastien Gaujoux, Institut Cochin, Paris, France and Dr. Radu Mihai, Oxford University, UK). The ACC working group meeting then ensued, which saw the presentation of several proposals for collaborative studies in this domain. After the lunch break, a crowded poster session followed. 58 poster communications were presented and a jury chose the 4 best posters (one in each category), that received an award during the conference dinner. A tutorial was also organized by Dr. Anthony Stell (University of Melbourne, Australia) to teach participants to access and use the ENS@T patients database. In the afternoon, communications within the APA and NAPACA working groups were presented. In the APA session, Dr. Paolo Mulatero (University of Turin, Italy) presented a study on aldosterone suppression on contralateral adrenal during adrenal vein sampling. Dr. Fabio Fernandes-Rosa

(HEGP-PARCC, Paris, France) showed data of a recent study on the effect of somatic mutations in aldosterone-producing adenoma, while Dr. Anastasios Mangelis (Technical University of Dresden, Germany) described the results of a computational data-driven model of steroidogenesis in human adrenocortical tumour cells. After the APA working group meeting, which also saw the presentation of several collaborative projects in the domain, the NAPACA session ensued with two different presentations about the *ARMC5* gene, whose mutations are a newly discovered cause of bilateral macronodular adrenal hyperplasia (BMAH). In the first presentation, Dr. Rossella Libé (Cochin Institute, Paris, France) showed the results of a study aimed to investigate the prevalence of ARMC5 mutations in a large cohort of BMAH patients, while in the second presentation Dr. Ludivine Drougat (Cochin Institute, Paris, France) described the functional effect of *ARMC5* mutants in human adrenocortical cells. The first day of the meeting ended with the meeting of the NAPACA working group, where several proposals for collaborative projects within ENS@T were presented.

The second day of the meeting (November 22) was dedicated to pheochromocytoma/paraganglioma (PCC/PGL), chromaffin tumors of the adrenal medulla and paraganglia that produce catecholamines. Several communications were dedicated to the genetic and genomic aspects of those tumours. Dr. Luis Castro-Vega (HEGP-PARCC, Paris, France) presented a study on the integrated genomic characterization of PCC/PGL, while Dr. Alexandre Buffet (HEGP-PARCC, Paris, France) showed how germline mutations in the fumarate hydratase gene, which is involved in the Krebs cycle, confer predisposition to malignant PCC/PGL. Dr. Samuel Backman (University of Uppsala, Sweden) presented data on promoter methylation as a tool to identify malignant PCC/PGL and Dr. Joakim Crona from the same group talked about genomic heterogeneity in PCC/PGL. A new xenograft mouse model of malignant PCC/PGL was presented by Dr. Charlotte Lepoutre-Lussey (HEGP-PARCC, Paris, France), while Dr. Lucie Evenepoel (Erasmus University, Rotterdam, The Netherlands) talked about her promising results for identification of molecular markers for malignant behaviour of PCC/PGL tumours. To finish up this session, a new biochemical method for molecular diagnosis of PCC/PGL was presented by Dr. Mirko Peitzsch (Technical University of Dresden, Germany) and Dr. Thomas Papathomas presented results of a multicenter study of SDHA/SDHB immunohistochemistry of PCC/PGL. After the PCC/PGL working group

meeting, the conference was closed by the ENS@T General assembly, during which the locations for next meetings during the following years were chosen and approved by the participants.

In addition to the European Science Foundation, support for the 13th Scientific meeting of ENS@T was also obtained from the French National Agency for Research, Nice City Council, University of Nice – Sophia Antipolis, Canceropôle PACA, French Society of Endocrinology, La Ligue contre le Cancer (which offered support for six travel grants for young scientists) and HRA Pharma.

Assessment of the results and impact of the event on the future directions of the field

ENS@T is a vibrant, dynamic scientific community dedicated to the advancement of basic and clinical research in the field of adrenal tumours. The contents of the 13th Scientific meeting of ENS@T were unanimously appreciated by all participants, who praised the great quality of all oral and poster presentations at the meeting.

A special emphasis during the meeting was given to the presentation and discussion of new collaborative projects among ENS@T members during the ACC, APA, NAPACA and PCC/PGL working groups meetings. Those projects will represent an important part of the ENS@T scientific activities during the years to come. A number of projects submitted to the EEC Horizon 2020 call was also presented during the meeting, which will contribute to shape the profile of European reserch in the field of adrenal tumours during the following years.

ANNEXES

1) Programme of the meeting

2) List of participants

13th Scientific Meeting of ENS@T
November 21-22, 2014
Radisson Blu Hotel, Nice (France)

EUROPEAN SCIENCE FOUNDATION
SETTING SCIENCE AGENDAS FOR EUROPE

ens@t
european network for the study of adrenal tumors

Dear Colleague,

Please receive our warmest welcome to Nice. We hope that you will enjoy your stay during the 13th Scientific Meeting of ENS@T.

We thank our sponsors for supporting this meeting

Program

November 21

8.15 – 9.00 Registration

8.30 – 9.00 ESF-ENS@T Steering committee meeting (**Fregate room**)

9.00 – 9.10 Welcome address (*E. Lalli, F. Beuschlein*) (**Baie des Anges room**)

9.10 – 11.00 ACC communications (I) (**Baie des Anges room**)
Chairs: W. Arlt, J. Bertehrat

9.10 "Genome-wide identification of genes involved in adrenocortical tumors: towards sequencing-based molecular predictor?" ***G. Assié*** *Cochin Institute - Paris (France)*

9.25 "Genomic landscape of pediatric adrenocortical tumors" ***E.M. Pinto*** *St. Jude Children's Research Hospital - Memphis TN (USA)*

9.40 "An international Ki67 reproducibility study in adrenocortical cancer: a multicenter interobserver variation analysis using virtual microscopy" ***E. Pucci*** *Erasmus MC - Rotterdam (The Netherlands)*

9.55 "Efficacy of 9-cis retinoic acid and mitotane combination in an adrenocortical cancer xenograft model" ***P. Igaz*** *Semmelweis University – Budapest (Hungary)*

10.10 "The novel dosage-dependent SF-1 target gene *VAV2* promotes actin cytoskeleton remodeling and invasion of human adrenocortical cancer cells" ***C. Ruggiero*** *IPMC - Sophia Antipolis (France)*

10.25 "Patched: a new target for the treatment of adrenocortical carcinoma?" ***I. Mus-Veteau*** *IPMC - Sophia Antipolis (France)*

10.40 "Notch1 signaling pathway is activated in adrenocortical tumors and may be related to clinical outcome" ***C. Ronchi*** *University of Würzburg – Würzburg (Germany)*

10.55 – 11.15 coffee break (**Le Carré**)

11.15 – 11.45 ACC communications (II) (**Baie des Anges room**)
Chair: M. Fassnacht
"European conference consensus on adrenocortical carcinoma surgical management - preliminary results" ***S Gaujoux, R Mihai***

11.45 – 12.45 ACC working group meeting (**Baie des Anges room**)

12.45 – 13.45 lunch (**Riviera I**) / ENS@T Steering committee meeting (**Fregate room**)

13.45 – 15.30 guided poster tour (**Mistral room**)
Chairs: *G. Eisenhofer, J. Favier, P. Igaz, B. Opocher*

ENS@T registry tutorial *A. Stell* (**Clipper room**)

15.30 – 16.15 APA communications (**Baie des Anges room**)
Chairs: E. Davies, M.-C. Zennaro

15.30 "Aldosterone suppression on contralateral adrenal during adrenal vein sampling does not predict blood pressure response after adrenalectomy" ***P. Mulatero***
University of Turin – Turin (Italy)

15.45 "Genetic spectrum and clinical correlates of somatic mutations in aldosterone-producing adenoma" ***F.L. Fernandes-Rosa***
INSERM U970 PARCC – Paris (France)

16.00 "Development of a computational, data driven model of steroidogenesis in NCI H295R cells" ***A. Mangelis***
Dresden Technical University – Dresden (Germany)

16.00 – 18.00 European conference consensus on adrenocortical carcinoma surgical management - working group meeting (**Fregate room**)

16.15 – 17.15 APA working group meeting (**Baie des Anges room**)

17.15 – 17.30 coffee break (**Le Carré**)

17.30 – 18.00 NAPACA communications (**Baie des Anges room**)
Chairs: F. Beuschlein, M. Terzolo

17.30 "Armadillo repeat containing 5 gene (*ARMC5*) in a large cohort of bilateral macronodular adrenal hyperplasia (BMAH): genotype-phenotype correlations " ***R. Libé***
Cochin Institute – Paris (France)

17.45 "Functional Study of *ARMC5* (Armadillo Repeat Containing 5), a gene involved in Bilateral Macronodular Adrenal Hyperplasia" ***L. Drougat***
Cochin Institute – Paris (France)

18.00 – 19.00 NAPACA working group meeting (**Baie des Anges room**)

20.30 Dinner and awards announcement (*sponsored by HRA Pharma*) (**Riviera I**)

November 22

9.00 - 11.00 PCC/PGL communications (**Baie des Anges room**)
Chairs: A.-P. Gimenez-Roqueplo, H. Timmers

9.00 "The genomic landscape of pheochromocytomas and paragangliomas" ***L. Castro-Vega***
INSERM U970 PARCC – Paris (France)

9.15 "Germline mutations in FH confer predisposition to malignant pheochromocytomas and paragangliomas" ***A. Buffet***
INSERM U970 PARCC – Paris (France)

9.30 "Overnight collections of urine for biochemical diagnosis of pheochromocytoma provide a simplified alternative to 24-hour collections" ***M. Peitzsch***
Dresden Technical University – Dresden (Germany)

9.45 "SDHB/SDHA Immunohistochemistry in Pheochromocytomas and Paragangliomas: A Multicenter Interobserver Variation Analysis using Virtual Microscopy" ***T. Papathomas***
King's College Hospital (UK)

10.00 "Global promoter methylation analysis as a tool to identify malignant pheochromocytomas" ***S. Backman***
University of Uppsala – Uppsala (Sweden)

10.15 "A xenograft mouse model of *Sdhb*-/- chromaffin cells" ***C. Lepoutre-Lussey***
INSERM U970 PARCC – Paris (France)

10.30 "Spatio-temporal heterogeneity characterizes the genetic landscape of pheochromocytoma and defines early events in tumorigenesis"
J. Crona
University of Uppsala – Uppsala (Sweden)

10.45 "Identification of markers predictive for malignant behavior of pheochromocytomas and paragangliomas"
L. Evenepoel
Erasmus Medical Center - Rotterdam (The Netherlands)

11.00 – 11.15 coffee break (**Le Carré**)

11.15 – 12.15 PCC/PGL working group meeting (**Baie des Anges room**)

12.15 – 12.45 General assembly (**Baie des Anges room**)

12.45 Farewell and lunch (**Riviera I**)

ORAL COMMUNICATIONS

November 21

9.10 – 11.00 ACC communications (I)

Genome-wide identification of genes involved in adrenocortical tumors: towards sequencing-based molecular predictor?

Assié G[1], Letouzé E[2], Jouinot A[3], 4, Barreau O[1], Fassnacht M[4], Luscap W[3], Omeiri H[3], Rodriguez S[3], Perlemoine K[3], René-Corail F[3], Elarouci N[2], Sbiera S[5], Kroiss M[6], Allolio B[7], Waldmann J[7], Quinkler M[8], Mannelli M[9], Mantero F[10], Papathomas T[11], De Krijger R[11], Tabarin A[12], Kerlan V[13], Baudin E[14,], Tissier F[3,15], Dousset B[16], Groussin L[1], Amar L[17], Clauser E[18], Bertagna X[1,19], Ragazzon B[3], Beuschlein F[5], Libé R[1], de Reyniès A[2], Bertherat J[1,19]

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ntroduction Several genes present somatic mutations in cancer, that can be readily identified by exome sequencing. Some genes may also be altered in their expression by homozygous deletion or by gene amplification. ENSAT has recently reported the first exome sequencing of adrenocortical cancer (ACC) as part of an integrated genomic characterization of these tumors. One objective now is to study how specific somatic gene alterations can be used to classify these tumors in order to develop molecular diagnostic tools based on targeted next generation sequencing.
Methods The exome of 45 adrenocortical carcinomas (discovery cohort) was compared to the corresponding non-tumor DNA. New recurring genes were sequenced by targeted next generation sequencing on 77 other tumors (validation cohort). The genome of these tumors was also analyzed by SNP chip.
Results In a discovery cohort of 45 ACCs analyzed by exome and SNP arrays, the adrenocortical carcinomas have a median of 30 somatic mutations, but two tumors were hypermutated (517 and 1364 mutations). The mutation rate correlates with the 5-year survival (Wilcoxon p = 0.003) and the Weiss score (ANOVA p = 0.02)
Among the 45 ACCs of the discovery cohort, 9 genes are recurrently altered according to the exome sequencing and SNP chip analysis: ZNRF3 in 10 tumors (22%), CTNNB1 and TP53 in 9 (20%), RB1 and CDKN2A in 4 (9%), MEN1, TERT in 3 (7%), DAXX and MED12 in 3 (7%). Among these genes, ZNRF3 is a new tumor suppressor gene, identified in this study. ZNRF3 is a putative inhibitor of the Wnt/beta-catenin pathway. In addition, alterations of CTNNB1 and ZNRF3 are mutually exclusive. These recurrent mutations are found mainly in the "aggressive" subtype of carcinomas, as defined by the transcriptome clustering also performed on these tumors.
Among the 77 ACCs of the validation cohort, targeted next generation sequencing and SNP arrays identify alterations of ZNRF3 in 16 tumors (21%, CTNNB1 in 12 (16%), TP53 in 11 (14%), RB1 in 4 (5%), CDKN2A in 10 (13%), MEN1 in 6 (8%), TERT and DAXX in 4 (5%),and MED12 in 3 (4%). None of these prevalences are different between the discovery and the validation cohort (Fisher exact p-values >0.5).
Conclusion There are two types of adrenocortical carcinomas, one with a poor prognosis characterized by specific mutations (p53 and Wnt/beta-catenin pathways mainly), the other without recurrent mutation. These alterations should serve as a basis for designing a molecular prognostic predictor based on SNP array and targeted next generation sequencing techniques.

Genomic landscape of pediatric adrenocortical tumors

Emilia M. Pinto[1], Xiang Chen[2], John Easton[2], David Finkelstein[2], Zhifa Liu[3], Stanley Pounds[3], Carlos Rodriguez-Galindo[4], Troy C. Lund[5], Elaine R. Mardis[6,7,8], Richard K. Wilson[6,7,9], Kristy Boggs[2], Donald Yergeau[2], Jinjun Cheng[2], Heather L. Mulder[2], Jayanthi Manne[2],Jesse Jenkins[10], Maria J. Mastellaro[11], Bonald C. Figueiredo[12], Michael A. Dyer[13], Alberto Pappo[14], Jinghui Zhang[2], James R. Downing[10], Raul C. Ribeiro[14], Gerard P. Zambetti[1] for the St. Jude Children's Research Hospital-Washington University Pediatric Cancer Genome Project

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Tumors of the adrenal cortex in children are very rare and frequently arise in the context of Li-Fraumeni and others constitutive genetic syndromes. Little is known about their molecular characteristics. We performed a comprehensive genomic analysis using whole genome sequencing, whole exome sequencing and transcriptome profiling in a total of 37 cases representing the spectrum of ACT in the pediatric age group including those with Beckwith-Wiedemann and Li-Fraumeni syndrome associated, wild-type *TP53* and cases with the founder R337H *TP53* mutation. The tumors were clonally heterogeneous and chromosomal instability was a hallmark. All informative cases had 11p paternal isodisomy and *IGF2* overexpression. Copy-neutral loss of heterozygosity for chromosomes 11 and 17 was prevalent and observed during early tumorigenesis, indicating likely driver events. ACTs associated with germline *TP53* mutations showed a complex spectrum of genomic alterations whereas those with wild-type *TP53* were more stable. Recurrent somatic mutations were rare, and additional acquired mutation was observed in *ATRX* and *CTNNB1.* A very poor outcome was predicted by concomitant *TP53* and *ATRX* mutations and associated genomic abnormalities, including massive structural variations and a high background mutation rate. These findings provide new insight into key genetic events, and their timing, in the pathogenesis of this pediatric tumor and potentially other embryonal tumors.

An International Ki67 Reproducibility Study in Adrenocortical Cancer:
A Multicenter Interobserver Variation Analysis using Virtual Microscopy
A Multinational Study of the European Network for the Study of Adrenal Tumors (ENS@T)

Pucci E[1*], Papathomas TG[1,2*], Giordano T[3], Lu H[1], Duregon E[4], Volante M[4], Papotti M[4], Lloyd R[5], Tischler A[6], Nose V[7], Erickson L[8], Mete O[9], Asa S[9], Gill A[10], Turchini J[10], Matias-Guiu X[11], Stephenson T[12], Skordilis K[13], Tissier F[14], van Nederveen F[15], Dinjens W[1], Korpershoek E[1], van der Spek P[1], Stubbs A[1], de Krijger R[1,16]

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Ki67 labeling index has proven to be the most powerful tool in terms of prognostic stratification in adrenocortical carcinomas and has been recently integrated in treatment flow charts. Lack of uniformity and consistency in quantification, as well as intratumoral heterogeneity in proliferation rate, might cause limitations and errors in Ki67 labeling index assessment. In our study we aim to determine the interobserver variability for Ki67 labeling index assessment, examining the current clinical practice among expert endocrine pathologists. Seventy-eight conventional adrenocortical carcinomas were analyzed by fourteen pathologists using their method of preference for scoring the Ki67 labeling index. Additionally, we also tested an automated system that uses software, which automatically determines ten hotspot areas and provides the percentage of Ki67 positive cells within those areas in sixty-two tumors. These frequencies were compared to frequencies obtained by a single pathologist, who selected independently ten hot spot areas on the same tumor samples. Statistical analysis was performed to evaluate the interobserver variability in Ki67 labeling index assessment and the degree of concordance between software selection and human selection of hotspots. The interobserver variability among the pathologists was very high, calculated by ANOVA single factor (F=10.45; Fcrit=1.73; p=0). In addition, no significant correlation was seen between the different methods of assessment that were used by the pathologists. With regard to the automated counting, a significant correlation was seen for the choice of the different fields by the automated system and the independent evaluation of the pathologist, as well as for the Ki67 labeling index. The Ki67 labeling index assessment had a strong correlation (rho=0.88, p=0), calculated with Spearman rank order correlation. In summary, we conclude that the interobserver variability is very high between the pathologists, and no significant correlation could be observed between different methods of evaluation of the Ki67 labeling index; software selection and human selection of hotspot areas have a high concordance in the Ki67 labeling index assessment; software selection can be applied in clinical practice to increase the reproducibility of Ki67 labeling index and to reduce the interobserver variability. Preliminary analysis of clinical data of twenty-five cases, to evaluate the correlation between Ki67 labeling index and overall survival, has indicated that the software assessment is more powerful (p=0.014) to discriminate the clinical outcome than the average of the pathologists (p=0.16), taking Ki67 labeling index of 20% as cut-off.

EFFICACY OF 9-CIS RETINOIC ACID AND MITOTANE COMBINATION IN AN ADRENOCORTICAL CANCER XENOGRAFT MODEL

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The current drug treatment options for adrenocortical carcinoma (ACC) are rather limited and intensive efforts are going on to find novel effective agents with favorable side effects profile. In our previous functional genomics study, retinoid signaling via the retinoid X receptor (RXR) was identified as a major pathogenic pathway in ACC and we have demonstrated the *in vitro* activity of 9-cis retinoic acid (9-cisRA) acting via the RXR on NCI-H295R cells and also found that 9-cisRA has antitumoral effects in a small pilot xenograft study. Our aim has been to investigate the antitumoral effect of 9-cisRA and its combination with mitotane in a large xenograft study. 43 male SCID mice xenografted with NCI-H295R cells have been treated in four groups (i. control – corn oil vehicle, ii. 5 mg/kg 9-cisRA, iii. 200 mg/kg mitotane, iv. 5 mg/kg 9-cisRA + 200 mg/kg mitotane) by oral gavage for 28 days. Tumor size follow-up, histological and immunohistochemical (Ki-67) analysis and gene expression microarray (Agilent platform) have been performed. Both 9-cisRA and mitotane reduced tumor growth relative to control, but only the combination of the two agents resulted in significant tumor size reduction. The Ki-67 index was significantly reduced in both 9-cisRA and 9-cisRA+mitotane groups. Gene expression analysis revealed 483 genes with significant differences in expression. Seven genes have been selected for validation by real-time qRT-PCR. Our results show that 9-cisRA might be helpful in the treatment of ACC mostly in combination with mitotane, and these results might pave the way for further studies, including clinical trials.

THE NOVEL DOSAGE-DEPENDENT SF-1 TARGET GENE VAV2 PROMOTES ACTIN CYTOSKELETON REMODELING AND INVASION OF HUMAN ADRENOCORTICAL CANCER CELLS

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Steroidogenic factor-1 (SF-1) overexpression has an important role in adrenal cortex tumorigenesis[1]. Clinical studies have confirmed SF-1 overexpression in childhood adrenocortical tumors (ACT)*2* and revealed its prognostic significance in ACT in adults[3]. We have recently identified the *VAV2* gene as a novel dosage-dependent target of SF-1 in human adrenocortical H295R cancer cells[4]. In a cohort of 47 ACC, *SF-1* and *VAV2* expressions were highly significantly correlated. *VAV2* encodes a guanine nucleotide exchange factor for the Rho small GTPases, participating in actin cytoskeleton reorganization, migration and invasion. We thus investigated SF-1 role in Rho small GTPases activation, actin cytoskeleton rearrangement and invasion in human adrenocortical cancer cells. Our findings indicate that an increased SF-1 dosage promotes Rac1 and Cdc42, but not RhoA activation. This correlates with an increased number of cells exhibiting lamellipodia/ruffles and filopodia, which are classically induced by Rac1 and Cdc42 activation respectively. The increased SF-1 dosage also stimulates adrenocortical cancer cell invasion through Matrigel. *VAV2* knock-down impairs SF-1 dosage-dependent lamellipodia/ruffles and filopodia formation and cell invasive ability, indicating that *VAV2* is critically required downstream of SF-1 to induce adrenocortical cancer cell cytoskeleton remodeling and invasiveness. Importantly, *VAV2* expression was inversely correlated to overall survival in our ACC patients cohort. Altogether, our study shows for the first time that SF-1 overexpression and its target *VAV2* play a relevant role in the establishment of an invasive and metastatic phenotype by adrenocortical cancer cells and suggest that VAV2 might represent a potential novel druggable target to exploit for adrenocortical cancer therapy.

References

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PATCHED: A NEW TARGET FOR THE TREATMENT OF ADRENOCORTICAL CARCINOMA?

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The Hedgehog (Hh) signaling pathway controls cell differentiation and proliferation. It plays a crucial role during embryonic development, but is also involved in cancer development, progression, and metastasis. The Hh receptor Patched is an Hh target gene that is over-expressed in many aggressive cancers. We observed the expression of Patched in adrenocortical carcinoma (ACC) both *in vitro* on the human ACC cell line H295R and *in vivo* on TMA from 99 ACC patients. Adrenocortical carcinoma is a rare cancer which affects about one person in a million whose 5-year survival is only 35% on average. The best treatment available at the present time is composed of a mixture of chemotherapeutic agents combined with the adrenolytic substance mitotane. However, the response to this treatment remains modest. We recently demonstrated that Patched is involved in the efflux of drugs such as doxorubicin, a chemotherapeutic agent used for clinical management of recurrent cancers, suggesting that Patched could contribute to chemotherapy resistance of cancer cells (Bidet *et al*. 2012, patent WO2012-080630). To find small molecules able to inhibit the multidrug resistance activity of Patched and to increase chemotherapeutic treatment efficiency, we developed a screening based on the ability of small molecules to inhibit growth of yeast over-expressing human Patched in medium containing doxorubicin. Two of the hits identified were able to increase doxorubicin cytotoxic effect on ACC cells with EC50 inferior to 10µM. These compounds are drugs approved by FDA for other pathologies, and will be soon tested on mice xenografts in combination with chemotherapeutic agents.

M. Bidet, A. Tomico, H. Guizouarn, P. Martin, P. Mollat, and I. Mus-Veteau, The Hedgehog receptor Patched has a multidrug transport activity and contributes to chemotherapy resistance, *Mol. Cancer Res.* 10:1496-1508 (2012)

Notch1 signaling pathway is activated in adrenocortical tumors and may be related to clinical outcome

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Background: Single nucleotide polymorphism (SNP) array analysis revealed frequent genetic alterations of the Notch pathway in benign and malignant adrenocortical tumors. Dysregulation of the Notch signalling has been implicated in several cancers with oncogenic or tumor suppressor functions. Aim of the study was to investigate in more detail the expression of several components of the Notch signalling pathway in adrenocortical tumors and their correlation with clinical parameters.

Material and methods: the mRNA expression of *NOTCH1*, *JAG1* (ligand of Notch receptor), and some specific target genes (*HES1, HES5*, and *HEY2*) was evaluated in 80 fresh frozen samples (28 normal adrenal glands=NA, 24 ACA and 28 ACC) by quantitative real-time PCR. Immunohistochemistry (IHC) was performed in 221 tissues on paraffin slides (13 NA, 28 ACA, 180 ACC) for the evaluation of JAG1 and HEY2 protein expression.

Results: mRNA expression of *JAG1* was slightly higher in ACC than in NA and ACA (*P=0.13*). Both *HES1* and *HEY2* showed a trend to higher levels in ACC and positively correlated with tumor size (*P=0.008*, r=0.37, and *P=0.049*, r=0.28, respectively). In IHC both JAG1 and HEY2 protein were more frequently expressed (H-score 1-3) in ACC than in ACA or NA samples (74% vs 54% vs 60%, and 91% vs 85% vs 66%, both *P<0.05*). Interestingly, in ACC patients, JAG1 expression was associated with smaller tumor size (*P=0.056*), lower ENSAT tumor stage (*P=0.07*), lower number of metastases (*P=0.08*) and a better prognosis (median overall survival 62 vs 15 months, *P=0.0012,* HR=0.43, 95%CI=0.16-0.63), representing a favourable prognostic factor. In contrast, HEY2 protein expression was higher in advanced ACCs (*P=0.009*) and had a negative prognostic impact, being associated with shorter overall survival (median: 30 vs 86 months, *P=0.047*, HR=1.84, 95%CI=1.0-2.94). Both survival analyses remained significant after adjustment for ENSAT tumor stage.

Conclusion: Notch1 signaling pathway activation might be involved in adrenocortical tumor progression and play a role for the clinical outcome of patients with ACC. The complex regulation of Notch signaling and its impact on prognosis in adrenocortical cancer need to be further investigated.

November 21

11.15 – 11.45 ACC communications (II)

VARIABILITY IN SURGICAL TREATMENT FOR ADRENOCORTICAL CANCER

Radu Mihai, Sébastien Gaujoux

Submitted on behalf of the ACC joint working group from ESES and ENSAT

Background. Majority of patients with adrenocortical cancer (ACC) present with advanced disease and their surgical management requires an individualized approach.

Method. Retrospective analysis of operative notes and histology reports of patients operated over a 5-year period under the care of members of the joint working group of ESES-ENSAT.

Results. Surgeons from nine centres contributed with data regarding patients operated for ACC between January 2009 and June 2014 (3-16 cases/centre, median 6). A total of 73 patients (23M:50F, age 19-84 years, median 51 years) underwent laparoscopic (n=13) or open adrenalectomy (n=60) alone (n=38) or in combination with excision of surrounding viscera, i.e. ipsilateral nephrectomy (n=17), splenectomy (n=7), distal pancreatectomy (n=4).

There was a predominance of right tumours (21L:39R, 3 bilateral, 10-no details). Median diameter was 10 cm. Multi-organ resection were more common in tumours >10 cm. Lymph nodes were dissected in 14 patients. The histological assessment of resection margins was R0 (n=43), R1 (n=6) or R2 (n=3). Histological analysis confirmed ACC, with a Weiss score of 3-9 (median 6). At the time of data collection 45 patients were deceased and 38 patients were alive.

Conclusion. Multi-centre collaborations allow collection of data from large number of patients. There is significant variability of workload in different centres. Size of primary tumour seem to not be the only factor impacting on the extent of resection in such patients. The debate about the feasibility of formal lymph node dissection and the need for multi-visceral resections could be explored in a prospective multicenter cohort study within a reasonable time. Guidelines on several aspects of the perioperative care of such patients will be presented by this multidisciplinary working group.

November 21

15.30 – 16.15 APA communications

Aldosterone Suppression on Contralateral Adrenal During Adrenal Vein Sampling does not Predict Blood Pressure Response After Adrenalectomy

Silvia Monticone[1], Fumitoshi Satoh[2], Andrea Viola[1], Evelyn Fischer[3], Oliver Vonend[4], Giampaolo Bernini[5], Barbara Lucatello[6], Marcus Quinkler[7], Vanessa Ronconi[8], Ryo Morimoto[2], Masataka Kudo[2], Christoph Degenhart[9], Xing Gao[4], Davide Carrara[5], Holger S Willenberg[10], Denis Rossato[11], Giulio Mengozzi[12], Anna Riester[3], Enrico Paci[8], Yoshitsugu Iwakura[2], Jacopo Burrello[1], Mauro Maccario[6], Gilberta Giacchetti[8], Franco Veglio[1], Sadayoshi Ito[2], Martin Reincke[3], Paolo Mulatero[1]

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Background Adrenal vein sampling (AVS) is the only reliable means to distinguish between aldosterone producing adenoma and bilateral adrenal hyperplasia, the two most common subtypes of primary aldosteronism (PA). AVS protocols are not standardized and vary widely between centers.

Objective To investigate whether the presence of contralateral adrenal (CL) suppression of aldosterone secretion was associated with improved postoperative outcomes in patients who underwent unilateral adrenalectomy for PA. Patients and setting- The study was carried out in 8 different referral centers in Italy, Germany and Japan. From 585 consecutive AVS in patients with confirmed PA, 234 procedures met the inclusion criteria and were used for the subsequent analyses.

Results Overall, 82% of patients displayed contralateral suppression. This percentage was significantly higher in ACTH stimulated compared to basal procedures (90% vs 77%). CL ratio was inversely correlated with aldosterone level at diagnosis and, amongst AVS parameters, with lateralization index (p=0.02 and 0.01, respectively). The absence of contralateral suppression was not associated with a lower rate of response to adrenalectomy in terms of both clinical and biochemical parameters and patients with CL suppression underwent a significantly larger reduction in aldosterone levels after adrenalectomy.

Conclusions For patients with lateralizing indices of > 4 (which comprised the great majority of subjects in this study), CL suppression should not be required to refer patients to adrenalectomy, since it is not associated with a larger blood pressure reduction after surgery and might exclude patients from curative surgery.

GENETIC SPECTRUM AND CLINICAL CORRELATES OF SOMATIC MUTATIONS IN ALDOSTERONE-PRODUCING ADENOMA

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Primary aldosteronism is the most common form of secondary hypertension. Somatic mutations in *KCNJ5, ATP1A1, ATP2B3* and *CACNA1D* have been described in aldosterone-producing adenomas (APA). Our aim was to investigate the prevalence of somatic mutations in these genes in unselected patients with APA (n=474), collected through the European Network for the Study of Adrenal Tumors (ENS@T). Correlations with clinical and biochemical parameters were first analyzed in a subset of 199 patients from a single center and then replicated in two additional centers. Somatic heterozygous *KCNJ5* mutations were present in 38% (180 of 474) of APA whereas *ATP1A1* mutations were found in 5.3% (25 of 474) and *ATP2B3* mutations in 1.7% (8 of 474) of APA. Previously reported somatic *CACNA1D* mutations as well as 10 novel *CACNA1D* mutations were identified in 44 out of 474 (9.3%) APA. There was no difference in the cellular composition of APA nor in *CYP11B2*, *CYP11B1*, *KCNJ5*, *CACNA1D* or *ATP1A1* gene expression in APA across genotypes. Patients with *KCNJ5* mutations were more frequently female, diagnosed younger and with higher minimal plasma potassium concentrations compared to *CACNA1D* mutation carriers or non-carriers. *CACNA1D* mutations were associated with smaller adenomas. These associations were largely dependent on the population structure of the different centers. In conclusion, recurrent somatic mutations were identified in 54% of APA. Young women with APA are more likely to be *KCNJ5* mutation carriers; identification of specific characteristics or surrogate biomarkers of mutation status may lead to targeted treatment options.

DEVELOPMENT OF A COMPUTATIONAL, DATA DRIVEN MODEL OF STEROIDOGENESIS IN NCI H295R CELLS

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Background: Steroid hormones are responsible for regulating a variety of physiological functions such as salt balance, stress responses and various metabolic processes. The human adrenocortical cell line NCI H295R is a useful model for adrenocortical steroid synthesis since this line produces steroids originating from different adrenal zones and has shown expression of all enzymes involved in adrenal steroidogenesis. The development of mechanistic computational models that describe steroidogenesis, based on experimental data, may improve understanding of intracellular mechanisms and stimulatory responses. Our aim was to create a model for exploring the metabolic pathways of adrenal steroidogenesis.

Method: We produced a deterministic model that describes the kinetics of steroidogenesis in the absence and presence of three stimulatory factors: angiotensin II, forskolin and K^+. Our model was evaluated by time-course concentration measurements of 14 steroids (pregnenolone, progesterone, corticosterone, 11-deoxycortisosterone, aldosterone, 17α-hydroxy-progesterone, 11-deoxycortisol, cortisol, DHEA, androstenedione, testosterone, estrone) utilizing LC-MS/MS. The concentrations were measured at 7 time points (0, 2, 6, 10, 24, 34, and 48 hours) after stimulation. In addition, a cell proliferation model, also based on experimental data, was used to improve the accuracy of the model.

Results: Our results showed a response of the cells to the three stimulatory factors. Specifically, we observed 6 and 3.5 fold increases in aldosterone concentrations after 48 hours of stimulation with angiotensin II and potassium, respectively. In the Forskolin stimulated samples we observed a 10-fold increase in cortisol and a 6-fold increase in DHEA. In addition, the precursor steroids, pregnenolone and progesterone, showed a 5 to 8-fold increase during the first three hours after stimulation, returning to initial concentrations after 20-24 hours. By fitting our model to the experimental data, we generated the rate constants for all intermediate steps of steroid production for each condition. The produced model parameter estimations corresponded well to the time-course measured concentrations. In most cases, the changes in the predicted rate constants were consistent with the expected changes in the pathways, regarding the alterations in enzyme activity induced by each stimulatory factor.

Conclusions: Our study demonstrates the time-course changes in the concentrations of steroids, showing the dynamics of the steroidogenic pathways, both for precursors and final products. It verifies the potential of computational modelling for the description and definition of metabolic mechanisms and pathways *in vitro*. Such computational models can contribute to a better understanding of the role of metabolomics in adrenal dysfunction/or pathophysiology.

November 21

17.30 – 18.00 NAPACA communications

Armadillo repeat containing 5 gene (*ARMC5*) in a large cohort of bilateral macronodular adrenal hyperplasia (BMAH): Genotype-phenotype correlations

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Context: Bilateral macronodular adrenal hyperplasia (BMAH) is a rare cause of primary adrenal Cushing's syndrome (CS) characterized by bilateral adrenal macronodules. *ARMC5* germline mutations have been recently identified in BMAH.
Objective: to determine the prevalence of *ARMC5* mutations and to analyze the genotype-phenotype correlation in a large cohort of BMAH patients with subclinical or clinical CS.
Patients and Methods: *ARMC5* was sequenced in 98 unrelated BMAH index cases. BMAH was defined by a bilateral adrenal nodular enlargement on CT scan. Clinical and hormonal data were collected: midnight cortisol level, ACTH, androgens, renine/aldosterone ratio, cortisol after overnight dexamethasone suppression test, cortisol and 17-hydroxyprogesterone after ACTH 1-24 stimulation and illegitimate receptor responses. Computed tomography and histological reports were analyzed.
Results: *ARMC5* damaging mutations were identified in 24 patients (26%). The missense mutants and the p.F700del deletion lose the ability to induce apoptosis both in H295 and Hela cell lines. *ARMC5 mutated p*atients showed an overt CS more frequently compared to wild type patients: higher midnight plasma cortisol, lower plasma ACTH and lower plasma cortisol after dexamethasone suppression (p-values 0.019, 0.003 and <0.001 respectively). Adrenals of mutated patients were larger, with a higher number of nodules (p-values 0.001 and <0.001 respectively).
Conclusions: *ARMC5* mutations are common in index cases with BMAH. *ARMC5* mutated patients show a more severe hypercortisolism and larger adrenals. *ARMC5* status should help to better classify the BMAH. *ARMC5* genetic *s*creening may help earlier diagnosis.

Functional Study of *ARMC5* (*Armadillo Repeat Containing 5*), a gene involved in Bilateral Macronodular Adrenal Hyperplasia

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Bilateral Macronodular Adrenal Hyperplasia (BMAH) are adrenocortical tumors (ACT) leading to adrenal Cushing's syndrome. Recently, our laboratory has identified the first gene predisposing to BMAH in adults. The gene named *ARMC5* (Armadillo Repeat Containing 5) is altered in half of studied patients who underwent adrenalectomy[1]. The germline *ARMC5*-inactivating mutations and the second somatic alterations identified in BMAH - a loss of heterozygosity (LOH) of the short arm of chromosome 16 (16p) containing *ARMC5*, or a second ARMC5-inactivating mutation suggest that ARMC5 acts as a tumor suppressor gene. However, the mechanism of action of ARMC5 remains unknown. The aim of this work is to determine the biological role of ARMC5 protein and also to study how mutations affect this function.

The human adrenocortical cells H295R were transiently transfected with expression vectors of wild type ARMC5 or mutants ARMC5. These 8 mutations studied (F700del, C657R, I664S, L754P, Y736S, R362L, R315W, L331P, C139R) have been identified in leukocyte or tumoral DNA of operated patients with BMAH. After 8 and 14 hours of transfection, apoptosis was analyzed by flow cytometry and immunofluorescence analysis (cleaved caspase 3 staining). Cell overexpressing wild type ARMC5 promptly undergo apoptosis compared to cells transfected with mutants ARMC5 that all loose the ability to induce apoptosis. In parallel, co-immunoprecipitation experiments were carried out to identify protein partners of ARMC5 by mass spectrometry. The choice of the partner(s) of ARMC5 identified by mass spectrometry was promoted by the function of the candidates and mainly in comparison to the lists of interacting proteins identified between wild type and p.L548P and p.R898W mutant forms of ARMC5. We identified 16 proteins specifically interacting with wild type ARMC5 involved in AMPc/PKA pathway, the degradation of proteins and the redox system.

These results confirm the role of ARMC5 in the regulation of apoptosis and its abolition by germline and somatic mutations identified in patients with BMAH. Identification of protein partners of ARMC5 will help to identify the signalling pathways involved this new tumor suppressor gene.

1- G.Assié *et al., NEJM*, 2014

November 22

9.00 – 11.00 PCC/PGL communications

The genomic landscape of pheochromocytomas and paragangliomas

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Pheochromocytomas (PCC) and paragangliomas (PGL) are neuroendocrine tumors caused by germline or somatic mutations in known susceptibility genes in up to 70% of cases. We performed the integrated genomic analysis of gene copy number, whole-exome sequencing, DNA methylation, miRNome and transcriptome datasets in the collection of PCC/PGL collected by the French COMETE Network. We found that PCC/PGL are characterized by a very low mutation load with no significantly mutated genes or focal copy number alterations (CNAs). In contrast, we detected recurrent broad CNAs encompassing the biallelic inactivation of the known PCC/PGL susceptibility genes. We also identified DNA methylation and miRNA expression clusters strongly associated with known molecular groups. Of note, high expression of the miRNA cluster 182/96/183 is present exclusively in *SDHB*-mutated tumors and *SDHB* knockout murine chromaffin cells. Moreover, overexpression of miR-183 or miR-96 in mouse chromaffin wild type cells promotes the activation of the epithelial to mesenchymal transition program. On the other hand, a marked silencing of the imprinted *DLK1-MEG3* miRNA cluster, associated with loss of heterozygosity of 14q32.2, was found as a potential driver in a subset of sporadic tumors. This comprehensive analysis illustrates the functional interdependence between genomic and epigenomic dysregulations and sheds lights on the molecular mechanisms underlying PCC/PGLs that ultimately constitutes the basis for the rational design of future therapies.

Germline mutations in *FH* confer predisposition to malignant pheochromocytomas and paragangliomas

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Context

We recently identified in a patient who had developed a malignant pheochromocytoma with an "SDH-like" molecular phenotype, a germline mutation in the *FH* gene. Herein, we investigated the role of *FH* in paraganglioma/pheochromocytoma (PGL/PCC) predisposition, by screening for germline *FH* mutations in a large international cohort of patients.

Patients and methods

598 patients with PCC/PGL without mutations in known PCC/PGL susceptibility genes were screened. We performed direct sequencing and MLPA methods to search punctual mutations and large deletions in *FH* gene. Global alterations in DNA methylation and protein succination were assessed by immunohistochemical staining for 5-hydroxymethylcytosine (5-hmC) and S-(2-succinyl) cysteine (2SC), respectively.

Results

We identified five pathogenic germline *FH* mutations (four missense and one splice mutation) in five patients. Three patients have a malignant PGL and three have multiple PGL. We found a somatic inactivation of the second allele in three PGL of three patients tested. Loss of fumarate hydratase activity was observed in two PGL. Low 5-hmC immunostaining, resembling those of *SDHB*-deficient tumors, and positive 2SC staining were detected in tumors with *FH* mutations.

Conclusion

Germline mutation in *FH* gene, not only confers predisposition to HRLCC syndrome, but also to malignant and/or multiple paraganglioma. We propose screening for *FH* gene mutation in malignant paraganglioma. 2SC immunohistological staining can be used to predict or validate *FH* mutations.

Overnight collections of urine for biochemical diagnosis of pheochromocytoma provide a simplified alternative to 24-hour collections

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Objective: To evaluate differences in day and overnight collections of urinary catecholamines and their free O-methylated metabolites.

Methods: We compared urinary free metanephrines and catecholamines collected separately during day and overnight in subjects with (n=37) and without pheochromocytoma (PCC) (n=589; 260 healthy normo- and hypertensive volunteers, 329 patients in whom PCC was tested and excluded). Levels were determined by liquid chromatography tandem mass spectrometry and expressed as µmol/mol creatinine.

Results: Among volunteers, urinary outputs of normetanephrine, metanephrine, norepinephrine and epinephrine were respectively 49%, 12%, 97% and 327% higher ($p<0.001$) for daytime than overnight urine collections (11.1±6.2 vs. 7.4±3.6, 8.6±4.0 vs. 7.8±3.8, 17.3±8.4 vs 8.1±4.8 and 3.2±2.3 vs. 0.8±0.6). Similarly, among patients tested for PCC but without tumors, urinary outputs of normetanephrine, metanephrine, norepinephrine and epinephrine were 41%, 5%, 63% and 183% higher ($p\leq0.006$) for daytime than overnight collections (12.2±9.1 vs. 8.8±7.8, 7.1±5.4 vs. 6.7±8.6, 16.2±16.4 vs. 9.9±10.4 and 2.6±2.4 vs. 0.8±1.2). In contrast, there were no differences in urinary excretion of metanephrines between daytime and overnight collections for patients with PCC. ROC curve derived diagnostic power, evaluated by comparisons of areas under curves (AUC), showed similar AUC using overnight and daytime excretion of metanephrines corrected by creatinine as well as for total 24h excretion levels of metanephrines without correction for creatinine (0.979 vs. 0.969 vs. 0.967 respectively).

***Conclusions*:** Overnight collections of urine for measurements of free metanephrines corrected for creatinine provide similar diagnostic efficacy to standard measurements in 24h collections without correction for creatinine, but offer a simplified alternative collection method without being compromised by daytime increases in sympathoadrenal activity.

SDHB/SDHA Immunohistochemistry in Pheochromocytomas and Paragangliomas:

A Multicenter Interobserver Variation Analysis using Virtual Microscopy

A Multinational Study of the European Network for the Study of Adrenal Tumours (ENS@T)

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Despite the established role of SDHB and SDHA immunohistochemistry (IHC) as a valuable tool to identify patients at risk for familial succinate dehydrogenase (SDH)-related pheochromocytoma (PCC)-paraganglioma (PGL) syndromes, the reproducibility of the assessment methods has not as yet been determined. The aim of this study was to investigate interobserver variability among seven expert endocrine pathologists using a web-based virtual microscopy approach in a large multicenter, multinational cohort of genetically well-characterized paraganglionic tumors comprising 351 samples: (1) 73 *SDH* mutated (39 *SDHD*, 24 *SDHB*, 4 *SDHA*, 4 *SDHAF2* and 2 *SDHC*), (2) 105 non-*SDH* mutated (37 *VHL*, 25 *RET*, 21 *NF1*, 8 *MAX*, 6 *HIF2A*, 4 *TMEM127* and 4 *HRAS*), (3) 128 without identified *SDH-x* mutations, and (4) 45 samples with incomplete *SDH* molecular genetic analysis. Substantial agreement among all the reviewers was observed either with a two-tiered classification (SDHB IHC κ=0.7338; SDHA IHC κ=0.6707) or a three-tiered classification approach (SDHB IHC κ=0.6543; SDHA IHC κ=0.7516). Consensus, defined as agreement at least among 5 out of 7 pathologists, was achieved in 315 cases (89.74%) for SDHB IHC and in 348 cases (99.15%) for SDHA IHC respectively. Among the concordant cases, 62 of 69 (~90%) *SDHB*-/*C*-/*D*-/*AF2* mutated cases displayed SDHB immunonegativity and SDHA immunopositivity, 3 of 4 (75%) with *SDHA* mutations showed loss of SDHA/SDHB protein expression, while 98 of 105 (93%) non-*SDH* mutated counterparts demonstrated retention of SDHA/SDHB protein expression. Of note, two *SDHD*-mutated extra-adrenal paragangliomas were scored as SDHB immunopositive, whereas 8 of 128 (~6%) tumors without identified *SDH-x* mutations, 6 of 37 (~16%) *VHL* mutated as well as 1 of 21 (~5%) *NF1* mutated tumors were evaluated as SDHB immunonegative. Although 13 out of the latter were non-metastatic, a significant correlation between SDHB immunonegativity and malignancy was observed (P=0.0002). We conclude that SDHB/SDHA IHC is a reliable tool to identify patients with *SDH* mutations at least in the specialized setting and together with *SDH* molecular genetic analysis should be viewed as complementary tests. In this framework, if *SDH* genetics fails to detect a mutation, *VHL*/*NF1* testing with the use of targeted Next-Generation Sequencing (NGS) is advisable. The presence of discordant cases highlights the need for quality assessment programs regarding not only standardized staining protocols, but also SDHB/SDHA IHC evaluation procedures.

Global promoter methylation analysis as a tool to identify malignant pheochromocytomas

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Pheochromocytomas are tumors derived from chromaffin cells in the adrenal medulla. Up to 60% of all pheochromocytomas harbor germline or somatic mutations in one of *SDHA, SDHB, SDHC, SDHD, SDHAF2, VHL, EPAS1, RET, NF1, TMEM127, MAX* or *H-RAS* genes. The role of epigenetic deregulation remains to be elucidated. Bisulfite-treated DNA from 39 pheochromocytomas and 4 normal adrenal medulla was applied to the Illumina Infinium HumanMethylation27 microarray. Unsupervised clustering and principal components analysis (PCA) were performed utilizing standard bioinformatics algorithms. SNP array data was available for 31 of the tumor samples. Unsupervised clustering of the tumor samples utilizing the 2757 most dissimilar probes revealed two distinct clusters. Cluster A (n=28) contained all malignant pheochromocytomas (n=9, sensitivity=1, specificity=0.37) in the cohort, while Cluster B (n=11) consisted solely of benign tumors. PCA results were in concordance with the results from the clustering and revealed shorter spatiotemporal distance between normal tissues and Cluster B than Cluster A. The average methylation index (MI) was significantly lower in Cluster A than in Cluster B (0.2419 vs. 0.2765, p=1.54*). Normal adrenal medulla had an average MI of 0.2739 which was not significantly different from Cluster B. Cluster B was associated with fewer chromosomal aberrations (p=0.004). *VHL* and *SDHB* mutated tumors had equal number of hypomethylated probes as hypermethylated probes, while *H-RAS, RET* and *NF1* mutated tumors had more hypomethylated than hypermethylated probes. In conclusion, unsupervised clustering and principal components analysis were both able to group and identify all malignant tumors, however benign tumors could not be completely separated in one cluster. Together with identification of a subgroup of benign tumors having average methylation levels indiscernible from that of normal tissue our results suggest aberrant DNA methylation being an important but late event in the malignant progression of pheochromocytoma tumors.

A xenograft mouse model of *Sdhb*$^{-/-}$ chromaffin cells

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Purpose

Pheochromocytomas (PCC) are rare neuroendocrine tumours that arise from chromaffin cells of the adrenal medulla. Around 15% of PCC are malignant. *SDHB* mutations are associated with malignancy and poor prognosis. SDH deficiency leads to succinate accumulation creating a cellular pseudohypoxic phenotype that stimulates VEGF expression and angiogenesis. Our objective is to phenotype *in vivo* the microvascularization and the metabolic pathways of a xenograft *Sdhb (Sdhb*$^{-/-}$) knock-out model by using multimodality imaging.

Methods

Sdhb$^{-/-}$ and wild-type (WT) immortalized mouse chromaffin cells previously generated in the laboratory (*Letouzé et al Cancer Cell 2013*) were propagated in the fat pad of NMRI nude mice. Tumour growth, vascular density (CD31 immunostaining on paraffin-embedded tumours) were evaluated and accumulation of succinate (mass-spectrometry) was measured by mass spectrometry. These results were compared with non-invasive *in vivo* multimodality images. High-temporal resolution DCE-MRI was performed. A compartmental analysis was used to calculate tumour blood flow (F), blood volume fraction (Vb), permeability surface product (PS) and interstitial volume fraction (Ve). Measurements of succinate and lactate were performed by magnetic resonance spectroscopy (MRS) at 4.7 T. Imaging of angiogenesis was based on the utilization of Angiostamp®, an RGD fluorescent $\alpha_v\beta_3$ integrin. Metabolic imaging was assessed by ^{18}FDG-PET.

Results

WT tumours were detected about one week before the development of *Sdhb*$^{-/-}$ tumours WT tumours grew faster (220 mm^3 per day) than Sdhb$^{-/-}$ tumours (102 mm^3/d). The vascular density assessed on paraffin sections was significantly higher in *Sdhb*$^{-/-}$ tumours. Global enhancement was dramatically higher in *Sdhb*$^{-/-}$ compared to WT tumours, with a marked increase in F and Vb. Angiostamp® signal was significantly higher in *Sdhb*$^{-/-}$ tumours. MRS showed an equal peak of lactate, indicative of anaerobic glycolysis, in *Sdhb*$^{-/-}$ and WT tumours but an accumulation of succinate specific of *Sdhb*$^{-/-}$ tumours.

Conclusion

Our preliminary results show strong differences between *Sdhb*$^{-/-}$ and *WT* xenografts and suggest that preclinical therapeutic studies could be implemented in this unique model of SDHB-deficient tumour.

Spatio-temporal heterogeneity characterizes the genetic landscape of pheochromocytoma and defines early events in tumorigenesis.

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Pheochromocytoma and paraganglioma (PPGL) patients display heterogeneity both in the clinical presentation and underlying genetic cause. To determine PPGL genetic landscapes and tumour heterogeneity we performed multiregional sampling and analysed DNA with SNP array and targeted deep sequencing (including *SDHA, SDHB, SDHC, SDHD, SDHAF2, VHL, EPAS1, NF1, RET, TMEM127, MAX* and *HRAS* loci). Mutations were found in 61% (n=57/94) of tumours. A total of 1159 somatic copy number aberrations were identified, median number of SCNA per sample 7 (range 0-21). Pseudohypoxic PPGLs defined as *VHL* mutated cases or relatively high VEGFA expression had pronounced contamination of non-tumour cells (p=0.003 and <0.001 respectively). Malignant tumour, old age (>50 years) and large size (>60mm) were associated with increased number of SCNA (p=0.005, 0.023 and 0.004 respectively). Intratumour heterogeneity were observed within 74/136 samples as well as in 19/22 primary tumours with multiple samples. Cases (n=3) with paired distant metastatic lesions showed intertumour divergences (proportion ubiquitous SCNA 14, 16 and 24%). Integrative analysis suggested 11p loss and biallelic *VHL* inactivation (*VHL* cases) as well as oncogenic mutation in *HRAS* or *RET*, bi-allelic *NF1* inactivation and 1p loss (*HRAS, NF1* and *RET* cases) as obligate events occurring early in tumorigenesis. These results define the genomic landscape of PPGL as specific to genetic subtypes and characterized by inter- and intratumour heterogeneity, providing insight into the evolution of these tumours

Identification of markers predictive for malignant behavior of pheochromocytomas and paragangliomas

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Pheochromocytomas (PCCs) and paragangliomas (PGLs) are relatively rare and mostly benign tumours. Approximately 10% of PCC are malignant, as defined by the presence of metastases, i.e chromaffin tissue at a location that usually does not contain chromaffin cells. However, up to 35% of tumours in patients carrying an *SDHB* mutation appears to be malignant. Nowadays, no reliable marker allows to predict whether a PCC/PGL is, or will become malignant. In addition, there are no curative treatments if metastases occur. In order to identify genetic markers allowing to distinguish benign from malignant tumours, 40 benign and 12 malignant PCC and PGL were investigated for differences in mRNA expression with Affymetrix arrays. Expression data were normalized according to Affymetrix recommendations. Then, using Pomelo II (http://pomelo2.bioinfo.cnio.es/), a Limma t-test was performed, to assess which genes were differentially expressed between benign and malignant PCCs. First, a non-clustered analysis was performed and 10 genes with a False Discovery Rate (FDR) below 0.05 and a relative overexpression ratio of at least 4 were found, including Interleukin 13 Receptor α 2 (*IL13RA2*) and Monooxygenase DBH-like 1 (*MOXD1*). Secondly, a supervised cluster analysis was performed (based on HIF target genes), resulting in 2 groups, which were both investigated for differences in mRNA expression between benign and malignant tumours. Five genes showed an FDR below 0.01 and were overexpressed in malignant tumours with a ratio higher than 4, including Contactin 4 (*CNTN4*), Iroquois Homeobox 3 (*IRX3*), and Sulfatase 2 (*SULF2*). These genes were further investigated using qPCR, and immunohistochemistry on Tissue Micro Array including 91 benign and 12 malignant PCCs. Significant overexpression of Contactin 4 was shown in malignant compared to benign tumours, and may therefore contribute to distinguish malignant from benign PCC/PGLs.

POSTERS

November 21, 13.45 – 15.30

ACC

1 - *PRKACA* AND *CDKN2A* MUTATIONS IN A PATIENT WITH AN ADRENOCORTICAL CARCINOMA WITH SUBCLINICAL CUSHING SYNDROME AND PRIMARY ALDOSTERONISM

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Adrenocortical carcinomas (ACCs) are rare malignancies associated with poor prognosis. While a majority of ACCs present with steroid hormonal excess only a few carcinoma with co-secretion of aldosterone and cortisol have been described in the literature. The genetic landscape of adrenal adenomas and carcinomas has been subjected to intense research during the last few years. The most frequent genetic alterations in ACCs are mutations in *ZNRF3, CTNNB1*, *TP53* and *CDKN2A*. *CDKN2A* encodes the tumour suppressor p16, frequently altered in many different tumours. Recently, mutations in *PRKACA* were observed in a large proportion of benign cortisol producing adenomas. However, screening of 40 ACCs did not reveal any mutations. We present a patient with an adrenocortical carcinoma with subclinical cushing syndrome and primary aldosteronism, carrying a *PRKACA* and *CDKN2A* mutation.

The patient was referred to the endocrine unit at Uppsala University hospital because of a blood pressure of 220/120 mmHg and spontaneous hypokalemia (2.3mmol/L). P-aldosterone was 1750 pmol/L with a suppressed PRA of <0.3. The patient had no clinical signs of hypercortisolism, s-cortisol was in the normal range, but dexamethasone suppression test showed unsuppressed cortisol levels (160 nmol/L, reference: <70). The other laboratory tests did not reveal any abnormalities. CT-imaging demonstrated a 5 cm tumour on the left adrenal with suspicion of growth into the left renal vein. Metomidate PET showed a diffuse uptake in the tumour and a suspected lymph node metastasis. Because of the likelihood of malignancy the patient underwent surgery without delay. PAD showed an 8 cm, ACC with intravascular growth and a high mitosis rate. Postoperatively, aldosterone levels normalized and the patient received cortisol substitution as well as streptozocin and lysodren treatment. The ACC recurred two years later, now producing sex steroids. Surgery was performed, removing multiple lymph node metastases and a 4 cm tumour. The patient progressed on different cytostatics and passed away 4 years after the initial surgery. Genetic analysis using a high density SNP array was carried out on the primary tumour and the recurring tumour. This showed that relatively few large CNVs occurred during progression. Further analysis of *PRKACA* revealed a p.Pro102Leu mutation in both the primary tumour and the metastasis. This residue is evolutionary conserved and part of a hydrophobic pocket on the PKA protein. Analysis of the *CDKN2A* gene demonstrated a p.Arg98Gln mutation in both the metastasis and the primary tumour. CNV analysis also revealed copy number loss over the *CDKN2A* locus. No mutations in *KCNJ5*, *ZNRF3*, *CTNNB1*, *ATP1A1* or *CACNA1D* were observed.

We present a case of an ACC with primary aldosteronism as well as biochemically diagnosed subclinical Cushing syndrome. Genetic analysis revealed a novel *PRKACA* mutation and a *CDKN2A* mutation with a corresponding loss of the other allele. Whole genome CNV analysis of the primary tumour and the recurrence showed that a few CNV alterations occurred during progression.

2 - The prognostic value of Ki67 proliferation index in pediatric adrenocortical tumors

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The Ki67 proliferation index has been proposed as one of the most relevant prognostic parameters for adrenocortical cancer (ACC) in adult patients. Although Ki67 index is the most robust microscopic feature to predict outcome in adult ACC patients, this topic has not been so far revisited in pediatric adrenocortical tumors (ACTs). In this study, we investigated the prognostic role of Ki67 proliferation index in a large cohort of pediatric ACTs. Ki67 proliferation index was assessed in 53 adrenocortical tumors (12 histologically benign and 43 malignant) diagnosed in 52 patients (median age at diagnosis 2.5 yrs, from 0 to 18 yrs). Among the histologically benign tumors, only 2 out of 11 (18%) presented Ki67 index ≥ 10%, whereas 21 out of 42 (50%) histologically malignant tumors had Ki67 index ≥ 10% (X^2= 3.6; p= 0.058). At multivariate analysis, a Ki67 index ≥ 10% (HR 36.5, 95% CI 1.16-1142; p= 0.041) and ENSAT-stage 3 or 4 (HR 87.8, 95% CI 2.4-3209; p= 0.015) were significantly associated with reduced overall survival. For disease-free survival, only a Ki67 index ≥ 30% was a significant predictor of recurrence (HR 6.5, 95% CI 1.28-33.06; p= 0.024) at multivariate analysis. In conclusion, we first established a Ki67 index cut-off to discriminate poor outcome in pediatric ACTs. A Ki67 index ≥ 10% and an advanced ENSAT were independent predictors of reduced overall survival. For disease-free survival, only a Ki67 index ≥ 30% was a significant predictor of recurrence.

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3 - BIRC7/livin is overexpressed in malignant adrenocortical neoplasia

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Introduction: BIRC7/livin gene, a member of the inhibitors of apoptosis (IAP) family, plays an important role in cancer development and progression in a variety of human malignancies, mostly through the direct inhibition of caspase-3. The aim of our study was to evaluate the expression of BIRC7 and caspase-3 in normal and neoplastic adrenal glands.

Methods: *BIRC7* and *caspase-3* mRNA expression were detected by quantitative real-time reverse transcription PCR analysis in 77 fresh-frozen tissue samples (30 ACC, 24 ACA, and 23 normal adrenal glands=NA). The correlation between BIRC7 and caspase-3 levels and several clinical parameters was also investigated.

Results: *BIRC7* mRNA expression was similar between ACAs and NA, but significantly increased in ACCs (*P<0.005 vs* ACA and *P<0.001 vs* NA). No significant difference was found between cortisol-secreting and non cortisol-secreting tumors. *Caspase-3* mRNA levels were similar in NA and adrenocortical tumors. There was no correlation between *BIRC7* and *caspase-3* mRNA expression. In the ACC group, we did not observe any significant correlation between *BIRC7* or *caspase-3* levels and clinical parameters, including tumor size, Weiss score, ENSAT tumor stage, Ki67-index and number of metastasis at diagnosis. Of note, both overall survival and progression free survival were shorter in patients with low *BIRC7* expression (*P=0.056* and *P=0.05,* respectively).

Conclusion: Our study demonstrates that BIRC7 is specifically over-expressed in ACC, suggesting that BIRC7 may be involved in adrenocortical tumorigenesis. BIRC7 represents a novel marker for malignancy in adrenal tumors.

4 - Adrenal renal cell cancer metastasis without macroscopic primary tumor in a pregnant patient

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Introduction: Adrenal tumors are rarely discovered during pregnancy. The main question that has to be answered is whether the tumor has to be removed during pregnancy. The indication for surgery is hormonal activity of a tumor that may influence pregnancy outcome and malignancy.

Case report: 38 – year old pregnant woman (27 HBD) was referred to the Department of Endocrinology from gynecologist, for evaluation of a 4,8x4,0x4,8 cm adrenal mass discovered incidentally 8 months ago on CT. The tumor was heterogenic with intensive contrast enhancement of a solid part. The patient offered no complaints. There was no history of chronic diseases but her mother died of renal cell cancer. On admission patient presented no clinical features of hormonal activity of a mass. MRI showed no/slight enlargement of a tumor with a high signal intensity T1-weighted with no loss of signal intensity on the chemical shift, opposed - phase sequences. The possible MR characteristic was metastasis, adrenocortical carcinoma or pheochromocytoma. Laboratory results revealed normal 24h urinary methanephrins, adrenaline, noradrenaline and dopamine, normal plasma chromogranin A, testosterone, DHEAS, concentration. Diurnal cortisol rhythm was normal (8 AM 23.38 ug/dl, 11 PM 6.04 ug/dl), saliva cortisol (11 PM 0.5 ug/dl, 0.6 ug/dl (<0,43)) and 24h urine free corticoids (320 ug/24 h 410 ug/24 h (40-120)). ACTH morning plasma concentration 32.7 pg/ml. There was no clinical data for metastases as well as no data on abdominal MR for primary tumor. As the risk of surgery didn't outbalance the potential profit the patient was referred for laparoscopic adrenalectomy in 30 HBD, which was performed without complication. Histopathological examination revealed adrenal gland with a tumor with marked features of degeneration build up from atypical cells (anisocariosis, pronounced large nucleoli) with a fair cytoplasm. Morphologic picture and immunochemistry was inconsistent but clarocellular renal metastasis was finally diagnosed. The patient delivered healthy child in 37 HBD by natural labour. The chest and abdominal contrast enhanced CT done 3 months later didn't reveal abnormalities. The patient remains under follow-up.

Conclusion: RCC may present as a metastatic tumor without primary renal site.

5 - METFORMIN INHIBITS CELL GROWTH IN AN ADRENOCORTICAL CARCINOMA CELL MODEL

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Adrenocortical carcinoma (ACC) is a rare but aggressive tumor with a poor prognosis. Radical surgery, possibly associated to mitotane (MTT) adjuvant therapy, is the only available treatment. However, the mean 5-year survival rate drops under 10% in metastatic ACC and chemo-resistance often develops. Thus, more specific and effective drugs for ACC treatment are urgently required. The anti-diabetic drug metformin (MET) has been associated with decreased cancer incidence and mortality. Our study is aimed to evaluate the potential anti-cancer effects of metformin *in vitro* on H295R adrenocortical tumor cell line, compared to those related to the reference drug represented by mitotane. Treating cells with MET determines a time- and dose-dependent cell growth inhibition, as observed by MTS assay and cell counting (IC50 after 7days: 23.8±0.9 mM and 10.1±0.3 mM, respectively). Moreover, combining MET and MTT leads to a synergistic effect which associates with a lower IC50 dose (16.7±1.1 mM after 7days). We performed Western Blot analysis to further investigate the molecules involved into cell proliferation signalling pathways: after 6 and 24 hours treatment with 100mM MET, we observed a reduced expression of IGF1-R, which drives the paracrine/autocrine proliferation loop in H295R. Moreover, we observed a dose-dependent decrease of ERK2/ERK1 activation and an increased activation of AMPK, associated to a decreased p-mTOR. In conclusion, our data show that MET interferes *in vitro* with H295R proliferation, with a synergistic effect when combined with MTT. Further studies have to be performed *in vivo* by using animal models to confirm the potential anti-tumor effects of metformin in ACC.

6 - MiR-483-5p and miR-139-5p promote invasion of adrenocortical carcinoma through suppression of N-Myc Downstream-Regulated Gene family members

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Introduction: MicroRNAs have recently emerged as key biomarkers for diagnosis and prognosis of adrenocortical tumors. We have previously shown that miR-483-5p and miR-139-5p were up-regulated in adrenocortical carcinomas (ACC). However, the role of these miRNAs in the pathogenesis of ACC is still unknown.

Aims: To identify target genes of miR-483-5p and miR-139-5p in ACC and to investigate their contribution to adrenocortical tumorigenesis.

Methods: Target prediction was performed using miRWalk database combined with the previously published gene expression profiling of adrenocortical tumors (de Reyniès et al, 2009). Tumor miR-483-5p and miR-139-5p levels were measured by RT-qPCR in a cohort of 30 patients from COMETE Network and correlated with the expression level of their putative targets. The 3'-untranslated regions of target genes were cloned into pMiR-Luciferase vectors to evaluate functional miRNA-mRNA interactions. The role of miR-483-5p or miR-139-5p in the malignant phenotype of the NCI-H295R cell line was investigated using transfection of miRNA inhibitors and measurements of cellular processes.

Results: We identified NDRG2 (N-Myc Downstream-Regulated Gene 2) and NDRG4 as targets of miR-483-5p and miR-139-5p, respectively. In ACC tissues, expression of NDRG2 and NDRG4 was inversely correlated with miR-483-5p and miR-139-5p levels. Downregulation of miR-483-5p or miR-139-5p in NCI-H295R cells induced an increase of NDRG2 or NDRG4 mRNA levels. Moreover, NDRG2 and NDRG4 transcripts were negatively regulated *via* specific miRNA target sites as demonstrated by luciferase assays. Finally, silencing miR-483-5p or miR-139-5p did not affect proliferation or apoptosis of NCI-H295R cells but inhibited their anchorage-independent growth and invasion.

Conclusion: Our data indicate that downregulation of NDRG2 and NDRG4 tumor suppressor genes by miR-483-5p and miR-139-5p might contribute to ACC aggressiveness.

7 - RRM1 MODULATES ACTIVITY OF MITOTANE IN ACC CELLS INTERFERING WITH ITS METABOLIZATION

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Mitotane is the reference therapy for adrenocortical carcinoma (ACC) and should undergo metabolization in o,p'DDE and o,p'DDA to exert its antiproliferative activity. We have previously shown a link between RRM1 expression and mitotane activity in an adjuvant setting. This study assesses whether RRM1 expression is correlated to the bioavailability and cytotoxicity of mitotane and its metabolites in ACC cells.

SW13 and H295R cells were treated with mitotane, o,p'DDE and p,p'DDA, and viability was correlated with RRM1 expression. The intracellular concentrations of mitotane, o,p'DDE and o,p'DDA were evaluated using HPLC-UV method in basal condition and under RRM1 silencing.

In H295R cells, mitotane and its metabolites showed a similar cytotoxic and RRM1 expression was not influenced by any drug. In SW13 cells, o,p'DDD and o,p'DDE were effective at high concentrations, while o,p'DDA showed a greater cytotoxic activity. The lack of sensitivity to o,p'DDE in SW13 cells was associated to RRM1 up-modulation. Conversely, o,p'DDA efficacy was linked by loss of RRM1 modulation. Moreover, RRM1 silencing in SW13 cells increased the intracellular transformation of mitotane into its metabolites.

These data suggest that RRM1 interferes with mitotane metabolisms in ACC cells, and this might be a possible mechanisms of drug resistance.

8 - DIAGNOSTIC PITFALLS IN ADRENOCORTICAL TUMORS: A LESSON FROM 300 CONSULTATION CASES

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The correct histopathological classification of adrenocortical carcinoma (ACC) is relevant to establish an early therapeutic strategy of this rare malignancy. Aim of the study was to assess the most frequent pitfalls in ACC diagnosis. Between 2004 and 2014, a large series of 300 consultations was collected from a single Italian Institution serving as referral center for ACC. All cases were reclassified using the appropriate diagnostic system. Reticulin stain and immunohistochemical markers were applied, if appropriate. After revision, the series included 269 ACC, 14 adrenocortical adenomas (ACA), 7 adrenocortical tumors of uncertain malignant potential, 3 pheochromocytomas (PCC), 5 soft tissue tumors in the adrenal gland and two metastases. The original diagnosis was confirmed in 91% of cases. A major disagreement was recorded in the remaining 9% (26 cases). The most obsserved pitfall was to take ACC apart from PCC (7 cases of ACC originally diagnosed as PCC and 3 cases of PCC originally diagnosed as ACC/ACA). Seven cases diagnosed as ACC, were reclassified as metastases from other primaries and primary adrenal soft tissue tumors (among which 3 angiosarcomas). Finally, five ACA were reclassified into ACC and four ACC converted into ACA. Thus, the original pathological diagnosis of adrenocortical tumors can be changed in up to 10% of cases upon revision. Key issues include: a) high index of suspicion for metastatic and soft tissue tumors; b) synaptophysin may be a confounding marker in adrenal tumors; c) oncocytic cortical tumors are the most problematic entities (for both defining malignancy and distinguishing from PCC).

9 - The anti-proliferative effect of Erlotinib combined with mitotane on H295R adrenocortical cancer cell line and primary tumour cultures

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Introduction: Adrenocortical carcinoma (ACC) is a rare disease associated with a low cure and a high recurrence rates. The prognosis is poor and at the diagnosis 30-40% of cases have already developed metastasis. The current therapeutic strategy (surgery, followed by adjuvant mitotane treatment +/- chemotherapy) remains unsatisfactory and requires search for new targeted therapeutic options. Mitotane is considered as a first-line therapy, but at best 24% of the patients show an objective tumour response [1]. Erlotinib is an EGFR-specific tyrosine kinase inhibitor, FDA-approved for the treatment of NSCLC. EGFR is highly expressed, mutated in various cancers and was found to be a malignancy marker for adrenal tumours [2]. EGFR mutations in exons 18-21 were observed in 5-11% of ACC cases [3, 4] (but not in the H295R ACC cell line) with their functional significance remaining unknown.

Aim: This study aimed to assess in a pre-clinical setting whether erlotinib combined with mitotane inhibits proliferation of ACC cells. The effect was assessed with and without EGF stimulation.

Materials and Methods: The proliferation rate of the H295R cell line was assessed by resazurin assay (96-well culture plates, $5*10^3$ cells/well). Cells were grown for 24h before the treatment. Optimum time points for determination of cytotoxic effect of the inhibitors were 48h and 72h of incubation post treatment with erlotinib and 24h post mitotane treatment. The protein expression was determined by Western blot technique. Primary ACC tumour cultures have proven to be difficult to study, but we successfully cultured 3 ACCs, two after surgical treatment and one after neo-adjuvant chemotherapy (EDP) for metastatic disease (EGFR mutation status is pending). Last tumour was very heterogeneous, so we cultured 2 samples from macroscopically different areas.

Results: We found that H295R cell proliferation was effectively inhibited by erlotinib, resulting in cytotoxic effect of 20.98±1.90% (n=6, p<0.01) 72h post-treatment. Mitotane at a concentration of 10μM decreased the proliferation rate by 26.96±0.90% (n=21, p<0.01). H295R cell proliferation was stimulated by EGF with maximal effect of 10.33±1.66% (n=5, p<0.01). Interestingly, EGF stimulation increased the anti-proliferative activity of 10 μM and 20 μM erlotinib by 6.3% and 9.2%, respectively (n=3, p<0.01). Combination of mitotane (10 μM) with erlotinib (10 μM) and EGF (100 ng/ml) resulted in additive or slightly synergistic effect reaching 41.14±4.87% (n=3, p<0.01). Primary ACC cultures responded to mitotane treatment at a dose of either 10 or 50μM (proliferation decreased by 9% and 100%, respectively). In one of the surgically treated ACCs, erlotinib alone (10μM) decreased proliferation by 24%, whilst in the second, proliferation was unaffected. In the metastatic ACC (both samples) addition of erlotinib resulted in 100% cytotoxicity at a lower mitotane concentration (10 in place of 50 μM). However, erlotinib alone caused opposite proliferative responses in the two samples of the metastatic ACC.

Conclusions: Erlotinib alone has an anti-proliferative effect in H295R cell line and in some primary ACC tumour cultures, and allows for a reduction of mitotane concentration if used in combination. For cases pre-treated with neo-adjuvant EDP therapy, erlotinib may cause opposite responses in different parts of the same tumour; hence its clinical use needs further careful consideration. Given the lack of somatic EGFR mutations in H295R cell line, the potential biomarker(s) that may indicate sensitivity to anti-EGFR TKI is currently under investigation.

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10 - Synchronous versus metachronous metastases in Adrenocortical Carcinoma

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Introduction Adrenal Cortical Carcinoma (ACC) is a rare malignancy with an incidence of 1.0 per million in the Netherlands. Median survival varies according to ENS@T tumor stage. It is unknown whether time of development of metastases is of influence on prognosis, as has been shown in other types of malignancy.
The objective of this analysis is to compare the outcomes of patients with synchronous vs. metachronous ACC metastasis.
Method Data were retrospectively obtained from centers of the Dutch Adrenal Network. Patients that presented with ACC between 1st of January 2004 to 31st October 2013 were included. Date of metastases, number of metastases and affected organs were registered. 160 patients were included in the analysis. . Synchronous metastases was defined as diagnosis of metastasis ≤ 6 months after the initial diagnosis of ACC. Overall survival was calculated from the date of diagnosis of metastasis to death of any cause.
Results Of the 160 patients, 53 were stage IV at diagnosis. Another 64 patients developed metastases during follow up. Of the 117 patients with metastases, 84 patients had synchronous metastases and 33 developed metachronous metastases. 43 patients did not develop any metastases.
Synchronous diagnosis of metastases (p=0,043), more than one affected organ (p<0,001) and four or more metastases (p<0,001) in ACC patients are of negative influence on overall survival after diagnosed with metastases.
Discussion In clinical practice all ACC patients diagnosed with metastases are classified as having stage IV disease while it is known that this is a heterogeneous group when looking at survival. Synchronous vs. metachronous disease in ACC have different prognosis to which therapeutic strategy could be adapted. The clinical characteristics associated with prognosis in this study support the opinion to refine the prognostic classification of IV ACC patients.

11 - METHYLATION STATUS OF VITAMIN D-RECEPTOR GENE PROMOTER IN BENIGN AND MALIGNANT ADRENOCORTICAL TUMORS

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Vitamin D receptor (VDR) and its ligand 1,25-Dihydroxyvitamin D3 play, in general, an inhibitory activity on tumor cell proliferation. We previously showed a decreased expression of VDR mRNA and protein in a group of adrenocortical carcinoma (ACC) tissues (Pilon et al, JSBMB, 2014), suggesting a protective role of VDR against adrenocortical malignant growth. No VDR gene mutations were found by recent whole exome-sequencing analysis in a very large series of ACCs (Assié et al, Nat Genet, 2014). Down-regulation of VDR gene expression may result from epigenetics events, i.e., methylation of cytosine nucleotide of CpG islands in the VDR-gene promoter. We analyzed methylation of CpG sites in the VDR-gene promoter of various adrenocortical tumor samples, including 3 normal adrenals, 15 benign tumors (3 non-functioning adenomas, 10 aldosterone-producing adenomas, 2 cortisol-producing adenomas) and 8 carcinomas (5 cortisol-producing carcinoma and 3 non-functioning carcinomas). Methylation of CpG-rich 5' regions were assessed by bisulfite sequencing PCR using bisulfite-treated DNA from archival microdissected paraffin-embedded adrenocortical tissues. A high methylation level (arbitrary cut-off of 30% or more) was found in the promoter region of VDR gene in 3/8 (2 cortisol-producing and 1 non-functioning) ACCs, while no VDR-gene methylation was observed in normal adrenals and benign adenomas. Expression of VDR mRNA was lower in ACCs than in benign tumors and normal adrenals ($P < 0.05$), and VDR immunostaining was low in all 3 methylated ACC samples. VDR gene methylation was also observed in the adrenocortical carcinoma H295R cell line. CONCLUSION: The association of VDR-gene promoter methylation with reduced VDR gene expression is not a rare event in ACCs, suggesting that VDR epigenetic inactivation may have a role tumorigenesis. Other epigenetic mechanisms in the upstream signaling pathway involved in silencing VDR gene expression, i.e. post-translational histone modifications, should be investigated.

12 - Abiraterone therapy in the management of Cushing's syndrome induced by adrenocortical carcinoma: a case report

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Introduction: Adrenocortical carcinoma (ACC) is a rare disease that in 50% of cases determines hormonal hypersecretion and endocrine syndromes, the most common of which is Cushing's syndrome. Abiraterone acetate is a selective and irreversible inhibitor of cytochrome P450 17A1, currently used in metastatic castration-resistant prostate cancer to reach maximal androgen suppression by inhibiting adrenal glands' hormonal synthesis. The drug also induces a quick reduction of cortisol levels and therefore it is a candidate for the management of Cushing's syndrome. We report the hormonal effects of Abiraterone administration in a patient with Cushing's syndrome induced by metastatic heavily pre-treated ACC.

Case presentation: We report a case of a 50-years-old woman with metastatic ACC heavily pre-treated with Mitotane and Chemotherapy suffering from severe and unmanageable Cushing's syndrome. ECOG Performance Status was poor: 3. The patient received abiraterone 500 mg/daily, while mitotane treatment was interrupted. After 9 days of treatment the patient obtained improved autonomies and deambulation, dramatic reduction of edema with a weight loss of 12,3 kg (from 85 kg to 72,7 kg), better control of blood pressure (120/80 mmHg) and glycemia, serum potassium normalization, restoration of sleep-wake rhythm and disappearance of mental confusion episodes.

Particularly, pre-treatment hormones evaluation pointed out the presence of hypercortisolism associated to increased cortisol urinary excretion and ACTH suppression. It was also found an increased serum level of testosterone and androstenedione, while DHEAS (dehydroepiandrosterone sulfate) was normal. Post-treatment hormonal laboratory tests showed marked and quick reduction of circulating androgen levels and urinary cortisol excretion, while serum cortisol remained stable. Table 1 shows serum and urinary hormones levels before and after abiraterone administration, considering Day 0 as the first day of treatment. The drug was interrupted because of hepatic impairment due to disease progression leading the patient to death 3 weeks later.

Conclusion: Abiraterone can be efficacious in the management of Cushing's syndrome induced by ACC. The benefits observed in this patient after a short time period warrant confirmation in a prospective clinical study.

13 - Predictive biomarkers of chemotherapy efficacy in advanced/metastatic adrenocortical carcinoma

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Introduction

ACC (adrenocortical carcinoma) is a rare cancer that is poorly responsive to chemotherapy and has a poor prognosis. Etoposide, doxorubicin and cisplatin plus mitotane (EDP-M) combination regimen is the standard therapy for metastatic ACC. The efficacy of EDP-M, however, is limited and it is quite toxic. Noteworthy a minority of patients experience exceedingly long survivals with this regimen. This observation points on the importance of predictive factor of efficacy in order to select patients destined to obtain benefit from this treatment from those who do not.

Several markers have been demonstrated to predict efficacy of single cytotoxic agents, among them topoisomerase alpha 2 (TOPO2A) in predictive of efficacy of anthracyclines, thymidylate synthase (TS) for fluoropyrimidines, excision repair cross-complementation 1 (ERCC1) for Cisplatin and RRM1 for Gemcitabine. Recently, RRM1 expression was found to be predictive or mitotane efficacy.

Patients and Method

We retrospectively assessed the role of TOPO2A,TS, ERCC1 and RRM1 assessed at diagnosis in predicting the antineoplastic activity of EDP-M in the management of metastatic disease in 26 ACC patients. The EDP-M activity was assessed in terms of clinical benefit defined as the attainment of complete response (CR), partial response (PR) or stable disease for at least 6 months (SD). RNA isolation was performed by commercially available RNA extraction kits designed for paraffin material according to the manufacturer's instructions. The expression of all the 4 markers was assessed by PCR. The median expression value of the entire population was employed to stratify tumors with high or low marker expression.

Results

Among the 26 ACC patients assessed, four (15%) achieved a PR, nine (35%) SD and 13 (50%) experienced progressive disease.

The clinical benefit (SD or PR) of EDP-M was directly associated with TOPO2A expression: 12 patients attaining SD/PR out of 17 (71%) in high TOPO2 tumors as opposed to 1/9 (11%) in low TOPO2A expressing ones ($p=0.0039$). Similar results were obtained stratifying patients according to TS expression: SD/PR in 9/13 (69%) versus 4/13 (31%) in high and low TS expressing tumors ($p<0.05$). Neither ERCC1 nor RRM1 were associated with clinical benefit of the therapy: SD/PR in 8/15 (53%) versus 5/11 (45%) in high and low ERCC1 expressing tumors ($p=0.69$); SD/PR in 7/16 (44%) versus 6/10 (60%) in high and low RRM1 expressing tumors ($p=0.42$). Data on time to progression will be provided at the meeting.

Conclusions

TOPO2A hyperespression may predict the responsiveness of metastatic ACC to EDP-M This underline the importance of antracyclines for the management of ACC. The correlation of TS with the proliferative activity may explain its predictive role of this marker for EDP-M activity.

14 - Differential activity of EGF in two human adrenocortical carcinoma cell lines

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Adrenocortical cancer (ACC) is a rare and aggressive malignancy. The main therapeutic option is surgery, but due to difficult and delayed diagnosis and to metastases onset, medical therapy, manly represented by Mitotane, is often tried with variable results. It is therefore mandatory to identify new molecular targets for ACC medical treatment.

Preliminary data obtained in our laboratory show that EGF 30 nM increased ACC cell lines proliferation (+20% and +10% vs. control in SW13 and NCI-H295 cells, respectively). EGF receptor (EGFR) expression was higher and ubiquitous in SW13 cell line, while it was weaker in NCI-H295 cell line, where it is presents only on the membrane. The aim of our study is to analyse EGF intracellular signalling in human ACC cell lines. We found that in SW13 cells the main effectors of EGF is ERK1/2, which phosphorylation sharply increase after treatment with EGF. PKC, B-Catenin, STAT, GSK3B and AKT proteins were not affected by EGF. On the other hand, on NCI-H295 cells, treatment with EGF caused a slight increase in ERK1/2 phosphorylation, while it importantly modified AKT/GSK3B and STAT/ Cyclin D1 pathways. These data demonstrate a different action of EGF in the two human ACC cells lines. This different behaviour could be explained by mutations in EGFR gene or by a different pattern in EGFR subtypes expression. Additional experiments are needed to better understand this mechanism that could provide molecular targets useful in the future design of ACC medical therapy.

15 - Mitotane enhances doxorubicin cytotoxic activity by inhibiting P-gp in human adrenocortical carcinoma cells

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Purpose: Mitotane is currently employed as adjuvant therapy as well as in the medical treatment of adrenocortical carcinoma (ACC), alone or in combination with chemotherapeutic agents. It was previously demonstrated that mitotane potentiates chemotherapeutic drugs cytotoxicity in cancer cells displaying chemoresistance due to P-glycoprotein (Pgp), an efflux pump involved in cancer multidrug resistance. The majority of ACC express high levels of P-gp and are highly chemoresistent. The aim of our study was to explore in vitro whether mitotane, at concentrations lower than those currently reached in vivo, may sensitise ACC cells to the cytotoxic effects of doxorubicin and whether this effect is due to a direct action on P-gp.
Methods: NCI-H295 and SW13 cell lines as well as 4 adrenocortical neoplasia primary cultures were treated with Mitotane and doxorubicin, and cell viability was measured by MTT assay. Mitotane effects on P-gp were measured by Calcein and P-gp Glo assays. P-gp espression was evaluated by western blot.
Results: We found that very low concentrations of mitotane sensitize ACC cells to the cytotoxic effects of doxorubicin, depending on P-gp expression. In addition, we found that mitotane directly inhibits P-gp detoxifying function, allowing the activity of chemotherapeutic drugs, such as doxorubicin.
Conclusion: These data provide the basis for the greater efficacy of combination therapy (mitotane plus chemotherapeutic drugs) on ACC patients survival. Shedding light on mitotane mechanisms of action could result in an improved design of drug therapy for patients with ACC.

16 - Sunitinib reduces cell viability of a human non-secreting adrenocortical carcinoma cell line by inhibiting EGFR phosphorylation.

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Adrenocortical carcinoma (ACC) is a rare disease with a poor prognosis. Surgery is the main therapeutic approach. However, nearly 50% of the patients has distant metastases at diagnosis or a relapse after surgery. In those case the therapeutic approach is mainly represented by Mitotane, that show discontinuous results and several side effects. For these reason new molecules are needed for the treatment of ACC. The aim of our study is to evaluate the effects of Sunitinib, a multi-target tyrosin kinase inhibitor, on two human ACC cell lines (SW13 non-secreting ACC cell line, and NCI-H295 secreting ACC cell line) e to evaluate its molecular targets. We found that Sunitinib 10 μM is capable of significantly inhibit both SW13 and NCI-H295 cell viability by activating apoptosis trough Caspase 3/7. Moreover in SW13 cells, Sunitinib is capable of significantly inhibit EGFR phosphorylation and to decrease the effects of EGF on its receptor. In addition, Sunitinib counteracts the increase in ERK1/2 phosphorylation induced by EGF in SW13 cells. Although Sunitinib inhibitis NCI-H295 cell viability, in this cell line its mechanism of action seems to be not related with EGFR. Interestingly, in both cell lines VEGF and its receptor seem not to be involved in Sunitinib activity. These data support the use of Sunitinib in the medical therapy of selected ACC, and suggest novel molecular target in the design of ACC medical treatment.

17 - Mitotane, adrenolitic drug, inhibits cell survival and function of several pituitary cytotypes

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Mitotane is an adrenolytic agent that is widely used for the treatment of adrenocortical carcinoma. We previously demonstrated that mitotane reduces thyrotrope cell viability, inducing apoptosis, inhibits both TSH expression and secretion and blocks TSH response to TRH. These data represent a possible explanation of the biochemical picture consistent with central hypothyroidism in patients undergoing mitotane therapy and opened new perspectives on the direct pituitary effects of this drug. Indeed, further studies showed that mitotane also inhibits corticotrope cell viability by inducing caspase-mediated apoptosis and reduces POMC expression as well as ACTH secretion and ACTH response to CRH. Moreover, we showed that cells originating from tissues different from pituitary are not sensitive to the inhibitory effects of mitotane. Overall, our data suggest that mitotane inhibits cell survival and function of many pituitary cytotypes, acting with a generalized, but specific, pituitary toxic effect.
The majority of male patients undergoing adjuvant mitotane therapy show a clinical picture of hypogonadism, characterized by low free testosterone and high sex hormone binding globulin levels in the presence of unmodified LH concentration. We investigated whether mitotane may directly influence both cell viability and function of gonadotroph cells. We found that mitotane reduces cell viability, induces caspase 3/7 activity, modifies the cell cycle phase distribution and LH/FSH secretion of gonadotroph cells. The present data strengthen previous evidence showing a mitotane hypopituitary effect and represent a possible explanation of the lack of LH increase following free testosterone decrease in patients undergoing adjuvant mitotane therapy.

18 - CURED ADRENOCORTICAL CARCINOMAS

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Adrenocortical carcinoma (ACC) is usually an aggressive disease, with a patient median survival of 2 years. Cure has rarely been reported. Here we present 2 cases with long-term cure of ACC.
Patient 1, female, was diagnosed at 40 years with right ACC. She complained of 15 kg weight gain in 2-3 years, hypertension up to 180 mmHg, low-feet edema, secondary amenorrhea during the last year, hirsutism. An ultrasound exam revealed a 22 cm non-homogenous hepatic tumor. Hormonal evaluation before surgery was not available. During surgery, a 20/13/7 cm right adrenal tumor and a metastasis in the 4th liver segment have been excised. Pathological evaluation diagnosed ACC. The patient underwent local external high-voltage radiotherapy 40 Gy, then chemotherapy with farmorubicin and cysplatin (6 months), followed by aminoglutethimide 500 – 250 mg/day for 10 years, withdrawn 1 year, without any clinical or imaging signs of tumor recurrence or metastasis. The adrenal function was reduced during high-dose aminoglutethimide therapy and was replaced during the first 3 years. Nine years after surgery, she was operated for a toxic thyroid nodule.
Patient 2, female, was diagnosed with Cushing's syndrome at the age of 27 years (secondary amenorrhea for 2 years, weight gain, moon-like face; non-suppressible serum cortisol at 2 days-2 mg and 8 mg dexametasone suppression tests). CT scan showed a large left adrenal mass. She was operated and the pathological examination revealed ACC. One year later the patient had tumor recurrence with local invasion and underwent excision of the spleen, left kidney and a pancreatic segment. She underwent local cobalt-60 radiotherapy, then she received prednisone 5 mg/day for 10 years. Twenty years after diagnosis, the adrenal function was normal and the abdominal CT scan showed no recurrence, but a uterine fibroid.
Conclusion. There are exceptions from the fatal risk of metastatic adrenocortical carcinoma.

19- IMPROVING DATA QUALITY IN DISEASE REGISTRIES AND CLINICAL TRIALS: A CASE STUDY FROM THE ENSAT-CANCER PROJECT

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Background

Trials in translational research are collecting more and more information about genotype and phenotype data (-omics). To handle these increasing amounts of data, bioinformatics and medical informatics provide tools for data collection, data processing and data analysis. To develop new relevant clinical hypotheses and to find significant conclusions in biomedical research, the data needs to be of the highest possible quality to meet the needs of the scientists and for optimal patient outcome. Unfortunately, multicenter trials and collaborations don't always achieve this requirement due to the heterogeneity of data collection, challenges of data to be captured, protocol amendments and a lack of human resources and motivation to either enter or check high quality data in data registries.

Method/Aim

We have performed a comprehensive evaluation of the quality of NAPACA and ACC records in the ENSAT registry in order to determine the data completeness (DC) and data accuracy (DA) with the goal to achieve the quality demands and trials aims of EURINE-ACT, a prospective multicenter clinical trial. A data quality score (DQS) was calculated from the combined DA and DC values representing the efficiency and reliability of data. After the analysis of data, monitoring visits were conducted to assess the reasons for the high/low data quality in seven selected sites in France, Germany, Greece and Italy. Logging and auditing analysis on the use of the ENS@T-CANCER systems has also been undertaken. Detailed information on levels of access and use by the broader community has been captured to help better understand the use of the registry and ultimately, the way it can be improved.

Results

Of all enrolled EURINE-ACT records in the ENSAT-Registry, both ACC and NAPACA records showed a lack of DC in imaging records (ACC 21% and NAPACA 69% DC) and a low DA in pathology records for patients that underwent surgery or in the case of ACC, that had no metastasis (ACC 62% and NAPACA 56% DA). Between April and October 2014 the average DQS increased by 18%. Additionally, since August 2014, a new log analyzer tool is under development to supplement the data quality information by processing registry meta-data. Once this tool is established, we will be able to further analyze quality scores and input specifically for EURINE-ACT. As an interim example of its use throughout the wider registry: the user with most utilization of the registry is from Spain with a total use of 15.8% of the overall use of the registry and 3784 separate activities (e.g. creating/editing/deleting data).

Conclusion

While every visited center had individual barriers to deal with, the feedback about the DQS encouraged centers to improve the data entry and to ask questions about the study protocols. Automated data assessment forms and user feedback systems are planned and expected to improve the data quality and to find significant conclusions in biomedical research.

20 - MUC-1 – a novel preclinical model for adrenocortical carcinoma

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Only two human cell lines are available for adrenocortical carcinoma (ACC) which do not reflect the functional heterogeneity of individual patient tumors and metastases. To overcome the lack of preclinical models for testing of novel therapeutic options in recent years we aimed at the development of patient-individual tumor models for endocrine tumors. Therefore, pieces of surgically excised tumor samples were implanted subcutaneously in the neck of athymic nude mice. To investigate whether morphological and functional characteristics between tumor samples after mouse engraftment in comparison to the original tumor would be comparable, we examined vitality, proliferation, vascularization and endocrine markers of the explanted material and the original patient tumor. During these studies one xenograft (MUC-1), derived from a neck metastasis of an ACC, showed distinct engraftment properties and sustained tumor growth over several passages in the murine host. Genetic analyses of known driver genes (*CTNNB1, TP53, CDKN2A, RB1* and *MEN1*) and of genes recently reported to be of importance in ACC (*ZNRF3, DAXX, TERT* and *MED12*) revealed somatic mutation of *TP53* while the cells were devoid of a *CTNNB1* mutation, as described for the current standard model NCIh295R. Immunohistochemical analysis of explanted tumors revealed furthermore highly vascularized tissues of SF-1 positive cells with Ki67 indices of 37.7±0.8%. In blood samples of transplanted mice cortisol levels of about 2.7µg/dL were detectable. Recently, we were able to utilize MUC-1 tumor bearing mice as an *in vivo* model in addition to the classical NCIh295-xenografts to investigate putative practicability in preclinical therapeutic settings. In ongoing experiments we are currently aiming at the overall characterization of MUC-1 to potentially provide a novel *in vivo* tumor model for ACCs.

21 - Dyslipidemia causes overestimation of plasma mitotane measurements

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Mitotane (o,p'-DDD) constitutes the standardized therapeutic option for advanced adrenocortical carcinoma (ACC). Monitoring of plasma mitotane levels is highly recommended but their positive predictive values (>14 mg/L) remain disputable. To further examine the accuracy of mitotane measurements, we studied the influence of dyslipidemia on plasma o,p'DDD and o,p'DDE levels.

Using normal, hypercholesterolemic and hypertriglyceridemic plasma samples spiked with fixed amounts of o,p'DDD, experimentally o,p'-DDD levels measured by HPLC-UV were compared to the corresponding theoretical measurements in patient plasmas.

A systematic 20% to 30% overestimation of o,p'-DDD and o,p'DDE quantitations was observed in hypercholesterolemic and hypertriglyceridemic plasmas, compared to normolipidemic plasmas. A matrix effect was hypothesized since the quantitation of the internal standard p,p'-DDE exhibited a 12% and 19% decrease in hypercholesterolemic and hypertriglyceridemic plasma, respectively, leading to overestimated and inappropriate values for circulating mitotane levels. Such overestimated mitotane measurements were confirmed using Phree® phospholipid removal cartridges thus reducing by 21% in p,p'-DDE extraction when assaying high lipid containing plasmas.

We provide first evidence for an overestimation of mitotane measurements in dyslipidemic patients that should be taken into consideration when examining this parameter as a predictive index of therapeutic efficacy. Improved accuracy in o,p'-DDD measurements would allow a better assessment of the predictive value of plasma mitotane levels in future clinical trials.

22 - Lipoprotein deprivation enhances mitotane efficiency in H295R cells

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Mitotane (o,p'-DDD), the most effective treatment of advanced adrenocortical carcinoma (ACC), is a lipophilic drug that accumulates into circulating lipoprotein fractions and high lipid-containing tissues.
The aim of our study was to evaluate the impact of serum lipoproteins on mitotane efficiency in human adrenocortical H295R cells.
LDL, HDL and lipoproteins deficient sera (LPDS) were prepared from fetal calf serum (FCS) using separation by ultracentrifugation. H295R cells were then cultured in media containing either lipoproteins (FCS, LDL only or HDL only) or in lipid-free medium with LPDS, and treated with 50 µM o,p'DDD for 48h. Proliferation, apoptosis, expression of genes and proteins involved in steroidogenesis and mitochondrial respiratory chain were analyzed to evaluate o,p'DDD efficiency. Interestingly, as demonstrated by RTqPCR analysis, we showed that o,p'DDD drastically reduced by 15 fold factor *StAR* and *CYP11A1* transcripts in LPDS grown H295 cells compared to other media (reduced by 3 fold factor) and also seriously compromised CYP11A1 protein expression whereas mitotane in all other media had no substantial effect. Dose-dependent curves demonstrated that mitotane in lipid-free medium exerts significant higher anti-proliferative effects than in the presence of lipoprotein fractions. Treatment with 50 µM Mitotane for 48h was unable to induce H295R cell apoptosis but led cell death when H295R cells were exposed to mitotane in the absence of lipoproteins as assessed by caspase 3/7 activation and anti-apoptotic BCl2 expression. Finally, in lipid-free medium, mitotane more efficiently reduced *COX2* gene expression (encoding a subunit of respiratory chain complex IV) by 85% as compared to mitotane in the presence of lipoprotein fractions (-25%).
Collectively, we showed that addition of lipoproteins in the cell culture medium blunted mitotane efficiency in H295R cells while lipid deprivation dramatically enhances its pharmacological properties. These results allow a better understanding of mitotane mechanism of action.

23 - SHORT-TERM VARIATION IN MITOTANE PLASMA LEVELS CONFIRMS THE IMPORTANCE OF TROUGH LEVEL MONITORING

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Objective: Mitotane is the drug of choice in patients with adrenocortical carcinoma. The anti-neoplastic effect is correlated with mitotane plasma levels, which renders it crucial to reach and maintain the concentration above 14mg/L. However, mitotane pharmacokinetics are poorly understood. Aim of the present study was to investigate the variation of plasma mitotane during the day and the influence of a single morning dose.

Methods: Patients who had been treated for at least 24 weeks and had reached the therapeutic plasma level (14mg/L) at least once were eligible. In the first group mitotane levels were determined hourly for the duration of eight hours after administration of a single morning dose. In the second group mitotane levels were assessed similarly without administration of a morning dose.

Results: Ten patients were included, three patients participated in both groups. Median plasma level at baseline was 16.2mg/L (range 11.3-23.3mg/L) in the first group (n=7) and 17.0mg/L (13.7-23.8) in the second group (n=6). Plasma levels displayed a median increase compared to baseline of 24% (range 6-42%) at t=4 after morning dose and a change of 13% (range -14-33%) at t=4 without morning dose (*P*=0.02).

Conclusion: A substantial increase in mitotane plasma levels was observed in steady-state patients within a period of eight hours after morning dosing. Without morning dose, mitotane curves showed a variable profile throughout the day. This implies random sampling could yield incidentally high levels. For this reason, we recommend early-morning trough sampling as standard management in monitoring mitotane treatment.

24 - Inhibition of the TCF7/β-catenin complex impairs adrenocortical tumor cell proliferation and adrenal steroidogenesis

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Background: β-catenin mutations and/or Wnt/β-catenin pathway abnormal activation are common in adrenocortical tumors (ACTs). PNU-74654 is a non-FDA approved that acts as Tcf/β-catenin complex antagonist.
Objective: to investigate the *in vitro* effect of PNU-74654 on β-catenin-dependent transcription, adrenal steroidogenesis and cell viability in NCI-H295 adrenal cell line.
Methods: NCI-H295 cell line was treated during 24 to 96h with vehicle (0.2% DMSO) and PNU-74654 (5, 10, 25, 50, 100 and 200μM) to evaluate cell viability by MTS-based proliferation assay. β-catenin expression and localization by immunofluorescence, mRNA expression of *CTNNB1, AXIN2, MYC*, *TCF7, SF1* and *CYP21A2* by qPCR, and adrenal steroids production by RIA were evaluated at 24 and 48h treatment at 10 and 100μM. β-catenin exon 3 and TP53 mutations were screened.
Results: sequencing confirmed that NCI-H295 cell line carries the p.S45P β-catenin mutation but not *TP53* mutations. The Tcf/β-catenin complex antagonist impaired cell proliferation in a dose-dependent manner. NCI-H295 cell viability 24h after treatment decreased 14%, 60% and 94% at 50, 100 and 200μM PNU-74654 ($p<0.0001$), respectively. This effect was more pronounced after 48, 72 and 96h. Immunofluorescence showed markedly cytotoxic effect as well as reduction of β-catenin expression mostly at nuclear level at 100μM 24h after treatment. These effects were stronger 48h after treatment even at 10μM. PNU-74654 treatment did not affect *CTNNB1* and *AXIN2* mRNA expression; however, 24h after treatment *MYC* expression increased at 100μM PNU-74654 ($p=0.03$) and resumed to basal levels after 48h. *TCF7* expression decreased at 100μM ($p=0.01$) 48h after treatment. PNU-74654 treatment also impaired steroidogenesis: androstenedione, testosterone, SDHEA and cortisol secretion was reduced 24h after treatment with 100μM (66, 73, 55 and 78%, respectively) and almost abrogated at higher doses. Indeed, at 10 and 100μM PNU-74654 treatment impaired *CYP21A2* (12% and 88%, respectively; $p=0.017$) and *SF1* (20% and 66%, respectively; $p=0.001$) expression.
Conclusion: In NCI-H295 adrenal cell line, PNU-74654, a TCF7/β-catenin inhibitor, impaired cell proliferation, β-catenin expression and nuclear localization, and adrenal steroids secretion. Thus, Tcf/β-catenin complex inhibitors might be a new target therapy approach for children and adult ACT patients with Wnt pathway activation.

25 - Frequent *ZNRF3* homozygous deletions in adrenocortical carcinomas with activated WNT signalling pathway

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Adrenocortical carcinoma (ACC) is rare and an aggressive disease with poor prognosis. Activation of the WNT signalling pathway, is the most common aberrance observed in non-syndromic ACC, although only a subgroup of these has been shown to be a consequence of a *CTNNB1*(encoding β-catenin) activating mutation. β-catenin activation has previously been associated with a decreased survival rate in ACC patients.
In this study 68 tumour DNA were subjected to high resolution SNP array and subsequent copy number variation analysis. The mutational status of *ZNRF3* and *CTNNB1* were analyzed by Sanger sequencing and cDNA was analyzed by q-PCR to determine expression levels of ZNRF3 and AXIN2. The hypodiploid and polyploid CNV pattern previously observed in ACC were also found in our cohort in a significant number of tumours. Most frequent homozygous deletions were found in *ZNRF3* (n=14), *LINC00290* (n=8), *TENM3* (n=8), and *CDKN2A* (n=5). However we did not find any *RB1* deletions in our cohort. Recurrent deletion of locus chr4q34.3-q35.1 (including genes: *LINC00290* and *TENM3*), chr3q13.31 (including genes: *LSAMP/LSAMP-AS1*, *TUSC7, LINC00901*) and chr1p36.21 (including genes: *PRAMEF1, PRAMEF11, PRAMEF2*) were also observed. *TARP, SRA1, ABCA13* and *PARP8* were the most significantly amplified genes. We found *CTNNB1*mutations in 9.6% of the tumours, which were mutually exclusive to *ZNRF3* deletion. Expression of ZNRF3 in *ZNRF3* deleted tumours were markedly reduced in comparison to β-catenin mutated and the tumours without both aberrations. AXIN2 expression was higher in *ZNRF3* deleted tumours, but less in comparison to *CTNNB1* mutated tumours, still indicating activation of the WNT signalling pathway in tumours with *ZNRF3* deletion. In conclusion, *ZNRF3* homozygous deletions, but not point mutations are frequent genetic events in adrenocortical carcinomas. This can explain the activation of WNT signalling pathway observed in subgroup of ACCs without any *CTNNB1* mutations.

26 - Microsatellite instability in adrenocortical cancer

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Objective: We elucidated the prevalence, pattern and clinical impact of MSI in adrenocortical carcinoma (ACC).

Method: MSI was investigated using 2 mononucleotide-repeat (BAT-25, BAT-26), 6 dinucleotide-repeat markers (D2S123, D5S346, D17S250, D11S4150, D11S4088, EGFR) and 2 complex markers(MYCL ,SF1), mapped to 9 chromosomal loci(1p, 2p,4q, 5q, 7p,9q, 11p, 11q, 17q). MSI was defined as the presence of allelic shift or additional bands, following the PCR amplification of tumor DNA. Instability in more than 30% of markers including at least one mononucleotide marker was defined as high-level MSI (MSI-H). Any other instability was classified as low-level MSI (MSI-L).

Result: MSI was detected in 16 of 31 patients (51.6%). All instabilities were at dinucleotide levels indicating a distinct MSI-L pattern. The instability was most commonly found at the chromosome 11 (D11S4150 and D11S4088), located at untranslated regions of KCNJ5 and KCNQ1, inwardly rectifying potassium channels. MSI-L correlated with disease-free survival (log-rank test, P=0.0024, HR=6.4, 95% CI: 1.938-21.71). Correlation of MSI-L with overall survival showed a trend, but was not significant (HR: 2.4, 95%CI: 0.91-6.35, P=0.07).

Conclusion: The presence of MSI-L defines a novel genetic subset in ACC and may play role in the molecular pathogenesis of this cancer.

27 - COMPARISON BETWEEN PET-CT AND CT IN THE DIAGNOSIS OF RECURRENCE OF ADRENOCORTICAL CARCINOMA

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Adrenocortical carcinoma (ACC) is a rare tumor characterized by a high rate of recurrence following radical surgery. Surgery of recurrent ACC may increase survival; thus, it is mandatory a timely and accurate detection of recurrence, either to increase the chance of radical extirpation or to avoid unnecessary surgeries. This study investigated the role of PET-CT in the diagnosis of recurrence of ACC during follow-up of disease-free patients and analyzed whether this tool may improve the therapeutic strategy.

A retrospective evaluation of the use of PET-CT was done in ACC patients with suspected recurrence during their follow-up in CT findings. Data of 57 patients followed at our center were retrieved. Recurrence was confirmed by pathology when lesions were removed (23 cases), or fine-needle biopsy (5 cases), or detection of unequivocal tumor progression during follow-up (29 cases).

CT scan of the 57 patients showed a total of 153 lesions while PET-CT showed at least one focal uptake in 40 patients (70.2%) for a total of 99 lesions. For liver lesions, PET-CT showed a significantly higher specificity and a reduced sensitivity (sensitivity, CT 80% vs. PET 50%, p = 0.046; specificity, CT 89% vs. PET 99%, p = 0.057). With regard to local recurrence, the two tests have similar diagnostic accuracy (sensitivity: CT 87% vs. PET 79%, p=ns; specificity: CT 94% vs. PET 94%). The same considerations apply to abdominal recurrences (sensitivity: CT 76% vs. PET 70%, p=ns; specificity: CT 94% vs. PET 99%, p=ns) and bone, in which CT and PET have equal sensitivity (86%) and specificity (98%). Conversely, in the lungs CT scan had non-significantly better diagnostic accuracy (sensitivity: CT 87% vs. PET 53%, p = 0.054; specificity: CT 91% vs. PET 95%, p = ns). In 18 patients (33%), PET findings changed the therapeutic strategy suggested by CT by showing the possibility of a radical surgery or avoiding an invasive treatment with the suggestion of a medical approach.

In conclusion, PET can be considered an useful adjunct to CT for the diagnosis of ACC recurrence, increasing diagnostic specificity for suspected liver or abdominal recurrences, and improving the identification of occult lesions or multiple tumor sites. Use of PET has important clinical implications, allowing a smarter use of surgery due to improved selection of patients who can be radically resected.

28 - A NEAR HOMOZYGOUS GENOME IN SUBSETS OF ADRENAL CORTICAL CANCERS

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We recently showed, by combining multi-parameter flow cytometry and SNP array analysis, that subsets of oncocytic follicular thyroid cancers, oncocytic adrenal cortical cancers (ACC-OV) and oncocytic parathyroid cancers go through a phase of incomplete genomic haploidisation. This “near-haploidisation” is often followed by endoreduplication (a duplication of the near-haploid genome, also termed “masked near-haploidisation” by others (1)), resulting in a near-homozygous genome (NHG) (2, 3). A NHG is a relatively rare form of aneuploidy (4) and has been described in chondrosarcoma, acute lymphoblastic leukemia, chronic myeloid leukemia, plasmocytoma, malignant fibrous histiocytoma and peritoneal mesothelioma. In recent work NHG was observed in > 30% of ACC, although different terminology was used (5).

It is remarkable that near-haploidisation in ACC has not been described before. This might have been caused by a lack of high-throughput, high-resolution, well standardised techniques during the earlier days of molecular genetic analysis of solid tumours. For example, the Hedley method (6) was the most frequently used flow cytometric technique for determining gross aneuploidy in archival formalin-fixed, paraffin-embedded (FFPE) tumour samples. By this method it is assumed that in case of a bi- or tri-model DNA histogram the first (most left) G_0G_1 peak represents normal cells (leucocytes, fibroblasts, normal cortical adrenal cells) and that the additional G_0G_1 peak(s) represent tumour cells with a DNA content higher than that of the normal cells in the sample. This technique was previously widely applied to study DNA aneuploidy in adrenocortical tumours by flow cytometry and indeed, many adrenocortical tumours showed to be DNA aneuploid with an increased DNA content compared to that of normal cells (7-13). However, the Hedley method can lead to misinterpretations of the data (2). Using multi-parameter flow cytometry however, we demonstrated that ACC-OV can be DNA near-haploid with a DNA index ranging from 0.6 – 0.7 and frequently show (sometimes multiple rounds) endoreduplication of their entire genome maintaining a NHG. Secondly, integrating the DNA index in SNP-array data the chromosomal dosages can be determined in ACC (14). It remains to be established whether the NHG f(g)enotype of ACC is related to a different clinico-pathological behaviour comparing to ACC not showing this.

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29 - Pdcd4 and miR21 in adrenocortical tumors

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Adrenocortical tumors (ACT) include benign and malignant forms. Adrenocortical carcinomas (ACC) are highly malignant neoplasms with poor prognosis and strong metastatic potential. Pdcd4 is a tumor suppressor involved in invasion, transformation, intravasation and apoptosis. Among translational regulators of Pdcd4, miR-21 seems to play a fundamental role, as it is frequently overexpressed in many malignancies.

Aim of this study is to evaluate the expression of Pdcd4 and miR21 in ACT samples and their interaction.

Expression of miR-21 was evaluated by qRT-PCR in cell lines (SW13 and H295R cells) and in 39 ACT samples: 21 ACC, 6 Aldosterone Producing Adenoma (APA) and 12 Non Aldosterone Secreting Cortical Tumors (NACA) and 6 Normal adrenal (NA). Expression of Pdcd4 was analyzed in 20 ACC, 4 APA, 8 NACA, 3 NA. Also 2 ACC, 2 APA, 2 NACA with their healthy counterparts were analyzed by Western blot.

miR21 resulted upregulated in 71% of ACC and 27% of ACA (APA+NACA) and more expressed in SW13 cells. In ACC samples, Pdcd4 resulted statistically different if compared to miR-21 expression ($P<0.046$). WB analysis showed that Pdcd4 reactivity decreased in 2 ACC tissues compared to healthy parts, while in other samples no appreciable differences were showed.

Pdcd4 and miR-21 are involved in ACT. Increase in miR-21 and loss of Pdcd4 expression are common in many tumors, such as thyroid, colon, ovarian carcinomas. Our results confirmed this inverse correlation and provided evidence of Pdcd4 role in ACC tumorigenesis.

30 - THE METABOLIC REGULATOR ESTROGEN-RELATED RECEPTOR ALPHA AS A THERAPEUTIC TARGET IN ADRENOCORTICAL CARCINOMA (ACC)

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ACC is a highly aggressive tumor extremely heterogeneous with limited therapeutic options and its pathogenesis involves integration of multiple signals. The study of ACC-associated syndromes has suggested that the IGF-II signaling pathway, p53, or Wnt/β-catenin signaling are currently the most attractive targets to fight ACC. Recently, we demonstrated the existence of a functional cross-talk between IGF-II signaling pathway and Estrogen Receptor α (ESR1), a gene overexpressed in ACC that drives estrogen-dependent proliferative effects. A useful strategy to develop an effective therapy for ACC is to identify a common downstream target of these multiple pathways. A good candidate could be ERRα, an orphan member of the superfamily of hormone nuclear receptors involved in the control of energy metabolism, mitochondrial biogenesis, and cancer progression. Several studies suggested that peroxisome proliferator-activated receptor γ coactivator-1 α and β (PGC-1α or PGC-1β) expression level and/or activity could regulate the transcriptional activity of ERRα and the ERRα/PGC-1 complex is a downstream target of multiple signaling pathways in hormone-dependent cancers. ERRα seems to be more expressed in ACC respect to adenoma and normal adrenal where it regulates steroidogenesis. In this study we investigated the role of ERRα in ACC by using the ERRα inverse agonist, XCT-790 and H295R cell line as experimental model for ACC. We revealed that XCT-790 cell treatment (1-10 μM) determined a dose dependent reduction of ERRα protein content concomitantly with a reduction of adrenocortical cancer H295R cells growth *in vitro* and in H295R xenograft model *in vivo*. Flow cytometric analysis indicated that XCT-790 increases cell population within the G0/G1 phase of the cell cycle, without any detectable sub-G1 cells accumulation. Accordingly, XCT-790 treatment decreases cyclin D1, cyclin-dependent kinase-4 (cdk4) protein expression and the phosphorylation status of pRb. Furthermore, XCT-790 treatment increases autophagic vesicles, concomitant with a reduction of PGC1-α protein expression, which is key player in mitochondrial biogenesis. Indeed, the evaluation of c-cic carrier expression, here used as an index of mitochondrial function and mass, suggests that mithophagy could be involved in such inhibitory effect. In conclusion these results, suggest that ERRα depletion could be a new strategy to control ACC growth.

31 - PHASE 1 STUDY OF ATR-101 IN ADRENOCORTICAL CARCINOMA (ACC): ATR-101-001

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ATR-101 (Atterocor, Inc., Ann Arbor, MI, USA) is in clinical development for the treatment of adrenocortical carcinoma (ACC). ATR-101 is a selective inhibitor of ACAT1 (acyl coenzyme A:cholesterol acyltransferase). ACAT1 catalyzes cholesterol ester formation and, in the adrenals, is particularly important in creating a reservoir of substrate for steroid biosynthesis. ATR-101 is uniquely distributed to adrenal tissues and inhibition of adrenal ACAT1 by ATR-101 disrupts steroidogenesis and leads to selective apoptosis of steroid producing adrenocortical-derived cells. Similar effects have been seen in the human ACC cell line, H295R. ATR-101 has shown pre-clinical efficacy in H295R xenograft mouse models.

ACC is an ultra-rare malignancy, occurring in about 2 per million population annually. ACC is frequently discovered in Stage 4 and the overall disease survival is approximately 17 months. Tumors often overproduce steroids normally produced in the adrenal cortex. Current therapies are toxic, difficult to administer, and poorly effective.

ATR-101-001 is a phase 1, dose escalation “3+3” design study of ATR-101 in advanced ACC patients who have failed or declined standard therapy. An expansion cohort of up to 20 subjects will be enrolled at or below the MTD. The primary objectives are the safety and tolerability of once a day, orally administered ATR-101. Secondary objectives include the determination of MTD and pharmacokinetics, anti-tumor efficacy by RECIST, establishment of a recommended phase 2 dose, and evaluation of pharmacodynamic biomarkers, including steroid hormones and steroid intermediate excretion rates. Initial safety evaluation is after 28 days of therapy; subjects who appear to be deriving benefit may continue on ATR-101 indefinitely. The study is open at three centers in the United States and will be opening at a center in Germany and one more US center to patients age 18 and over with advanced ACC. Patient’s mitotane level must be 5 µg/ml or less; the QTcF 470 ms or less; and if present, CNS metastases must be treated and inactive.

Full study information is available on ClinicalTrials.gov, Identifier NCT01898715.

32 - PROTEOMIC PROFILING OF ADRENOCORTICAL CARCINOMA

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Adrenocortical carcinoma (ACC) is a rare and aggressive endocrine tumor characterized by poor prognosis when metastatic at diagnosis. The biology of the tumor is still mostly unclear, thus justifying the limited specificity and efficacy of the anti-cancer drugs currently available. The present study reports the first proteomic analysis of ACC by using the two-dimensional-difference-in-gel-electrophoresis (DIGE) technique to evaluate a differential protein expression profile between adrenocortical carcinomas and normal adrenals. Mass spectrometry associated to DIGE analysis of carcinoma (n=10) and normal (n=8) adrenal specimens identified 22 proteins which are differentially expressed (fold variation <-2 or >2, $P<0.05$) between pathology and normal condition. All proteins appear to be overexpressed in ACC, except one which was downregulated (thiosulfate sulfurtransferase). Among the overexpressed proteins, the differential expression obtained by DIGE analysis for Aldehyde dehydrogenase 6A1 (ALDH6A1), Transferrin, Fascin-1, Lamin A/C, Adenylate cyclase-associated protein 1 (CAP1) and Adrenodoxin Reductase (ADX-Red) was validated by Western Blot analysis on the tissue samples of the same cohort (fold increase±SE7.5±1.4, 3.6±1.2, 2.9±0.2, 2.6±2.1, 1.9±1.4, 1.6±0.8, $P<0.05$, respectively). Immunohistochemistry of ALDH6A1, Transferrin and Fascin-1 performed on paraffin-embedded ACC and normal adrenal specimens of the same patients showed a marked positive signal in almost all cells in the ACC, while it was negative in normal adrenals.

In conclusion, our preliminary findings reveal a different proteomic profile in adrenocortical carcinoma compared to normal adrenals, identifying 6 proteins significantly overexpressed in the tumor. These proteins could represent novel valid protein ACC biomarkers if further validated in a larger cohort of patients.

33 - ECTOPIC ACTH PRODUCTION IN ADRENOCORTICAL CARCINOMA

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Ectopic production of ACTH in adrenocortical cells has been reported as a rare occurrence in adrenocortical macronodular hyperplasia, but, to the best of our knowledge, has never been investigated in adrenocortical carcinoma. We therefore designed a study on a series of 109 adrenocortical carcinoma samples to test ACTH expression at both mRNA and protein level. Messenger RNA was extracted from formalin-fixed and paraffin embedded material and analyzed by means of real time quantitative PCR using a commercially available assay (TaqMan, Hs01596743_m1), following manual microdissection to exclude the presence of normal adreno-medullary cells which may physiologically express ACTH. ACTH protein expression was investigated in PCR-positive cases by means of immunohistochemistry. All cases had major clinical and pathological information available, including sex, age, histological type, functional status, size and weight, mitotic index, overall Weiss score, Ensat Stage and disease status. Sixteen out of 109 cases (about 15% of cases) showed detectable levels of ACTH mRNA, at a variable extent. In five of these 16 cases, ACTH was detected by means of immunohistochemistry in adrenocortical cancer cells, either as single isolated cells or tumor clusters. The expression of ACTH mRNA was associated with histological type, being present in conventional and myxoid cases but not in oncocytic adrenal carcinomas ($p=0.02$), and with the Weiss score, being more prevalent in cases with a Weiss score <6 ($p=0.09$). No other correlations between ACTH mRNA expression and clinical or pathological parameters were observed. In conclusion, ACTH is produced by cancer cells in a subset of adrenocortical carcinomas, mostly in cases lacking oncocytic features and with a low Weiss score, but does not seem to be associated to specific clinical features including functional status and outcome.

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34 - The Weiss histopathological system and immunohistochemistry assessment for the diagnosis and prognosis of adrenocortical cancer. Retrospective review from a single specialist center

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Adrenocortical cancer (ACC) is orphan malignant adrenal tumor often with poor prognosis. Decision of operation of adrenal mass is based on hormonal and radiological data. Preoperative diagnosis is difficult because of heterogenetic clinical symptoms. ACC can behave as a hormonal active tumor (cortisol-, aldosterone-, androgen-secreting, mixed tumor) and a silence tumor (accident found tumor). Postoperative diagnosis is also not easy. Morphological and immunohistochemistry (IHC) assessment help to determinate the nature of tumor. But despite complex assessment there are a lot of cases with indeterminate meaning and according to this remain difficulties with next treatment and follow up. Earlier histological conclusion was based on experience of morphologist. The aim of our study is to assess practical significance of original and modified Weiss histopathological system (WHS) and expression of Ki-67 for the diagnosis of ACC. We have reviewed 90 cases of operated adrenal tumors from 2000 to 2014 year. The diagnosis of ACC was done in 35 cases (without WHS and IHC), others had considered as incidentaloma, corticosteroma, pheochmocytoma, androsteroma. Revision of material with strong criteria of Weiss and immunohistochemistry assessment have showed a great difference in accurate diagnosis. In cases of adrenal tumors, which should undergo operation (hormonal active tumor or tumors with high risk of malignancy) the use of WHS and immunohistochemistry is strongly recommended. Nevertheless it is required new tools to understanding of nature of adrenal tumor and next follow-up, necessity of additional treatment.

35 - Antisecretory and antiproliferative *in vitro* effects of Abiraterone Acetate in the human ACC cell line H295R

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Adrenocortical cancer (ACC) is a rare aggressive tumor with a poor prognosis mostly due to both the advanced stage of the disease at diagnosis and the limited efficacy of therapies when surgery is not curative. To date, the pharmacological therapy of advanced ACC is based on mitotane and chemotherapy that have a modest efficacy and the combination of the two strategies is toxic. Novel treatment strategies are thus urgently required.

Abiraterone acetate (AA), a small molecule that irreversibly inhibits the key enzyme for steroid hormone synthesis17 α-hydroxysteroid dehydrogenase (CYP17A1), has recently demonstrated marked efficacy in patients with metastatic castration-resistant prostate cancer. The observation that CYP17A1-dependent pathway is operating in the andrenocortical gland and that most of ACC highly produces steroid hormones, led us to investigate whether inhibition of CYP17A1 by AA may exerts antisecretory and antineoplastic activity in ACC cells. For this purpose, H295R cells, established from a patient diagnosed with ACC and secreting active steroid hormones, were used; SW-13 cell line, that likely derives from adrenal cortex metastatic cells and do not produce steroids was used as a control.

We found that AA treatment for 4 days induced a dose-dependent decrease of cell viability of H295R, but not of SW13 cells, an effect that likely depends on CYP17A1 inhibition. By using quantitative RT-PCR, in fact, we found that H259R cells express high levels of CYP17A1 while this enzyme was almost totally absent in SW13 cells. Interestingly, a synergistic effect of AA with mitotane on cell viability was specifically observed in H295R cells. We also found that AA deeply increased the number of apoptotic cells, suggesting that apoptosis throughout its extrinsic pathway could be the main mechanism involved in cancer cell growth inhibition induced by AA. On this line, increased synthesis of pro-apoptotic genes, such as those coding for TRAIL “death receptors”, have been detected by using both Protein Array and Western Blot techniques.

By using Mass Spectrometry, the CYP17A1 inhibition on steroids synthesis induced by AA in H295R cells showed a selective increase of progesterone while cortisol, dehydroepiandrosterone and testosterone were drastically reduced. Combined together, these data indicate that AA exerts both antisecretory and antiproliferative activities, providing for the first time the potential therapeutic role of this compound, alone or in combination with mitotane, in the treatment of ACC.

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APA

36 - THE RESULTS OF ADRENALECTOMY FOR PRIMARY ALDOSTERONISM

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Background. Primary aldosteronism (PA), the most common cause of endocrine hypertension, involves more than 11% of referred hypertensive patients. Unilateral adrenalectomy is highly successful in curing the lateralized forms of PA, resulting from aldosterone-producing adenoma (APA) or unilateral adrenal hyperplasia (UAH). UAH previously considered a very rare disease, is actually documented in 8% to 70% of cases of surgically treated PA, but it remains a controversial entity. The aim of this retrospective study was to analyze the outcomes of the surgical treatment of PA in a single center series.
Methods. Kalemia levels, blood pressure values and aldosterone/renin ratio (ARR) were assessed in 128 consecutive patients undergoing unilateral adrenalectomy for PA, before and after surgery. The role of lateralizing techniques and the relationship between outcome and histopathology findings were also evaluated.
Results. Biochemical cure of PA, defined by AAR and kalemia normalization, was achieved in 122 out of 128 patients (95%). Pathology revealed a single APA in 59 cases (46%), multinodular UAH in 57 (45%) and diffuse UAH in 12 patients (9%). No correlation between histopathology and persistence of PA was found. The use of further lateralizing techniques in addition to conventional imaging (computed tomography or magnetic resonance) was the main predictor of PA cure ($p=0.02$). Adrenal venous sampling (AVS) was more accurate than NP-59 scintigraphy in PA lateralization ($p<0.05$). After adrenalectomy, BP values normalized in 67 (55%) and improved in 44 (36%) of the 122 biochemically cured patients. Female gender, lower number of antihypertensive drugs and shorter duration of hypertension were the main predictors of hypertension cure.
Conclusion. The early diagnosis and the correct lateralization of aldosterone hyperproduction by AVS are essential to achieve surgical cure of PA and PA-related hypertension. This study confirms that UAH is common and PA resulting by UAH may be cured by unilateral adrenalectomy.

37 - OXIDATIVE STRESS IN PATIENTS AFFECTED BY PRIMARY ALDOSTERONISM

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Objective: Primary aldosteronism, an important form of secondary hypertension, is associated with significant increase of cardiovascular risk (ischaemic heart, cerebrovascular events, arrhythmias) (relative risk 4.6). The specific treatment of primary aldosteronism significantly reduces cardiovascular risk. In addition to high blood pressure values and direct action of aldosterone, new mechanisms such as increased oxidative stress are involved in the development of organ damage, metabolic, endothelial and coagulation complications.
Methods: The aim of the study was to evaluate parameters of oxidative stress in 38 patients (21 men, 17 women, mean age 53.3 ± 4.7 years) with primary aldosteronism [11 aldosterone-producing adenoma (APA) (4 men, 7 women, mean age 50.2 ± 4.5 years) and 27 idiopathic adrenal hyperplasia (IHA) (17 men, 10 women, mean age 54.5 ± 5.3 years)] at diagnosis and after specific treatment (surgical or pharmacological), with respect to 50 patients with essential hypertension (26 men, 24 women, mean age 49 ± 7.4 years) and 50 healthy individuals (28 men, 22 women, mean age 48.7 ± 4.4 years).
Results: Patients with primary aldosteronism showed significant increase of NADPH oxidase (Nox2-dp) plasma levels and urinary isoprostanes (34.9 ± 4.3 μg/dl and 216.3 ± 15.7 ng/mg, respectively; $P < 0.05$) than essential hypertensive patients (27.1 ± 3.7 μg/dl and 144.8 ± 9.4 ng/mg, respectively; $P < 0.05$). In APA patients undergoing adrenalectomy, we observed significant reduction of both circulating levels of Nox2-dp (29 ± 2.1 vs. 22,4 ± 1.7 μg/dl; $P < 0.05$) and urinary levels of isoprostanes (221.1 ± 10.5 vs. 132.6 ± 8.7 ng/mg; $P < 0.05$).
Conclusions: This is the first study showing an increased oxidative stress in primary aldosteronism, characterized by increased serum levels of Nox2-dp and urinary excretion of isoprostanes. After APA removal with laparoscopic adrenalectomy, we found reduction of serum Nox2-dp and urinary isoprostanes.

38 - SCREENING FOR KCNJ5 SEQUENCE VARIANTS IN A GROUP OF HUNGARIAN FAMILIAL HYPERALDOSTERONISM PATIENTS

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Primary aldosteronism (PA) includes a heterogeneous group of disorders with sporadic and also familial forms (familial hyperaldosteronism type I, II and III.) PA is the most common secondary form of arterial hypertension, with an estimated prevalence between 6% and 10% and as high as 20% in patients with therapy resistant hypertension. In the majority of cases it shows a bilateral disease of mostly idiopatic origin (70%) or unilateral adrenal aldosterone producing adenoma (APA) (30%). Currently 5% of PA cases are estimated to be caused by familial hyperaldosteronism types I, II and III.

The molecular mechanisms underlying aldosterone hypersecretion and nodulation of the adrenal cortex are still largely unknown, but recently responsible genes could be identified with NGS methods. These genes are KCNJ5, ATP1A1, ATB2B3 and CACNAD1.

Choi et al. reported that recurrent somatic mutations of the *KCNJ5* gene coding for the potassium channel Kir3.4 could account for a substantial proportion of APAs (8 of 22 human APAs studied). It was also shown that germinal *KCNJ5* mutations can cause a very rare autosomal dominant and early-onset form of PAL, characterized by bilateral massive adrenal hyperplasia.

In our current work we evaluated peripherial blood samples of 22 patients with familial hyperaldosteronism. Genomic DNA was isolated and fragments covering coding region of the KCNJ5 gene were amplified using polymerase chain reaction (PCR) and bidirectional Sanger sequencing was performed. The sequences generated were compared to reference sequences of ENSEMBL and NCBI databases. The variants found will be reported in this presentation.

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39 - CASE DETECTION OF PRIMARY ALDOSTERONISM – SCREENING BEYOND THE ALDO/RENIN-RATIO

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Primary aldosteronism (PA) is severe form of hypertension characterized by a strongly increased aldosterone secretion mediated by adenomas or other forms of adrenal hyper-activity. Once detected, PA can be usually cured by either surgical intervention or by appropriate pharmacologic treatments. This is also reflected in clinical guidelines of Endocrine Societies in Europe and the US, suggesting extensive PA screening activities among resistant hypertensive patients. The incidence of PA among hypertensive patients varies strongly between different studies, which is in part caused by the complex state-of-the-art testing procedure that unfortunately is far away from being a versatile PA screening tool. Despite strong limitations regarding selectivity, sensitivity and the interference with multiple anti-hypertensive drugs, the aldosterone-renin-ratio (ARR) is widely used for PA case detection. However, there is still a strong demand for accurate and reliable and patient friendly PA case detection. The use of novel and more accurate technologies for quantification of aldosterone and renin activity might help to improve the power of the ARR as a diagnostic tool for PA. However, there is a big need for a versatile PA screening assay that doesn't interfere with anti-hypertensive treatments and therefore allows the clear identification of PA patients without complex corrections and adaptions being necessary and without increasing the patient's cardiovascular risk in the course of the diagnostic process. Novel methods providing improved accuracy and sensitivity in the determination of aldosterone concentration and renin activity will be presented and novel promising approaches suggesting a strongly improved diagnostic power in PA screening among hypertensive patients will be discussed.

40 - Transcriptome and mutational status of adrenocortical adenomas

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Introduction
Cortisol secretion by the adrenocortical adenomas is variable. This variability may be related to differences in pathophysiology. Transcriptome identifies two different groups of adrenocortical adenomas, with globally distinct cortisol secretion levels. Cortisol secretion is related to the Protein Kinase A (PKA) pathway, and activating mutations in the alpha catalytic subunit of PKA (PRKACA) have recently been identified. Finally 30% of adrenocortical adenomas have an activating mutation of beta-catenin (CTNNB1). The aim is to clarify the relationship between the transcriptome, secretion, and mutation status of adenomas.
Methods
39 adrenocortical adenomas studied by transcriptome (Affymetrix) were included (19 Cushing, 9 and 11 non-secreting subclinical Cushing). *PRKACA* and beta-catenin were analyzed by Sanger sequencing.
Results
Unsupervised classification show two distinct groups of adenomas, with distinct cortisol secretion levels: a group of "overt Cushing" adenomas (15/15), and an "admixture" group of non-secreting (9/24), subclinical Cushing (11/24) and overt Cushing (4/24) (Fisher p <0.001). *PRKACA* mutations are found exclusively in the "overt Cushing group" (8/15) and in none of the 24 adenomas from the "admixture" group (Fisher p <0.001). *CTNNB1* mutations are present in 5 of the 13 adenomas from the "admixture" group, and are not found in the 11 adenomas from the "overt Cushing" group (Fisher p = 0.04).
Conclusion
These results suggest the existence of two types of adrenocortical adenomas, one related to the activation of the PKA pathway and responsible for overt Cushing, the other linked to the activation of the Wnt-beta-catenin pathway and associated with globally low -but variable- cortisol secretion levels.

41 - LEPTIN AND ADIPONECTIN MRNA EXPRESSION FROM THE ADIPOSE TISSUE SURROUNDING THE ADRENAL NEOPLASIA

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Introduction: Context Interplay between adipose tissue and adrenal glands has been recently suggested, without well-founded actions of locally adipose tissue surrounding the adrenal glands.

Objective We hypothesized that the local expression of leptin and adiponectin can be associated with pathological changes of the adrenal glands.

Patients and main outcome measures we evaluated RT-PCR of leptin and adiponectin mRNA expression from the adipose tissue surrounding adrenal glands in 30 patients, collecting adipose tissue surrounding the adrenal neoplasms, peri-renal and subcutaneous depots.

Results Leptin mRNA levels from adrenal neoplasia and peri-renal fat were significantly higher in aldosterone-producing adenoma (APA) than in non-functioning adenomas (NFA) ($p<0.001$ and $p<0.02$, respectively). In patients with Cushing's syndrome (CS) leptin mRNA levels were significantly higher in adrenal fat than in peri-renal ($p<0.05$) and subcutaneous adipose tissue ($p<0.001$). Adiponectin mRNA expression from adrenal neoplasia was significantly lower than that from peri-renal and subcutaneous fat depots ($p< 0.05$). Leptin and adiponectin plasma levels significantly correlated with their mRNA expression from the fat depot surrounding the adrenal neoplasia.

Conclusions Our findings suggest an active role of the fat depot surrounding the adrenal neoplasia, with local secretion of leptin and adiponectin.

42 - CORRELATION BETWEEN CELL CYCLE, AND STEROIDOGENESIS IN ADRENOCORTICAL TUMORS CELLS

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Steroid over-secretion is one of the major characteristics of the adrenocortical tumors (ACT). The cyclic AMP signalling cascade and PKA subunits **PRKARIA, PRKARIIb** and **PRKACA** are involved in the pathogenesis of a subset of cortisol-secreting ACT.
In addition to our studies on the effects of **PRKARIA, PRKARIIb** on the proliferation/apoptosis of the H295R adrenocortical cell line, we have recently reported that the decrease of either R1α or R2β protein in cells controls the cell cycle, enhances the accumulation of cells in the G2 phase, and differentially affects cyclins G2 expression. Majors alterations of genes involved in both cell proliferation and the cell cycle have been described by transcriptome and miRNome analysis in various types of ACT (ACC, ACA, AIMAH, PPNAD). In addition, steroid excess causes morbidity of all types of ACT.
The goal of this study is to find whether there is any correlation between the cell cycle deregulation and the steroid over-secretion in adrenocortical tumor cells.
We used pharmacologic drugs to arrest cells at specific cell cycle check point: (G1 phase), (S phase) and (G2 phase). We have studied the cell cycle distribution (FACS), the expression of the different actors of the cell cycle regulation, as cyclins and cdks, the PKA subunits and cell signalling pathways, the expression of steroidogenic enzymes in H295R cell line, and in primary culture of Primary Pigmented Nodular Adrenocortical Dysplasia (PPNAD) cells.
The synchronization of both the H295R or PPNAD cells at G2 phase increased the expression of the steroidogenic enzymes and steroid secretion. However in PPNAD this increase started at the S phase. Arresting both H295R and PPNAD cells in G1 phase decreased the steroidogenic enzymes expression, resulting in a decrease of cortisol secretion. PKA subunits distribution and cell signalling pathways activity are modulated during the cell cycle progression.
In Conclusion; we have found a correlation between the cell cycle check points and the expression of steroidogenic enzymes and cortisol secretion. Targeting specific cell cycle check points may down regulate the hypersecretion of steroids in these tumors.

43 - TOOLS AND STRATEGY FOR CLASSIFICATION OF VARIANTS OF UNCERTAIN SIGNIFICANCE IN PCC/PGL SUSCEPTIBILITY GENES

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The increasing number of known PCC/PGL susceptibility genes and the development of next-generation sequencing analysis for genetic testing result in the identification of an expanding number of unknown genetic variants. The classification of variants of uncertain significance (VUS) into either pathogenic or benign clinical interpretation is highly critical for patient management and family genetic counseling. Predicting the effect of VUS by an in silico approach alone is challenging and can lead to false positive or negative results. Here, we propose a pipeline for the classification of VUS identified in *SDHx* genes that could be extended to other PCC/PGL susceptibility genes. The framework includes clinical and biological presentation of the patient, segregation analysis, in silico predictions, co-occurrence data, mutation databases and literature reviews, and tumor-based analyses (at RNA and/or DNA and/or protein levels) and aims to determine or rule-out pathogenicity of every new VUS. As variant classification requires examination of all available evidence, international collaboration amongst expert genetic laboratories would greatly improve this approach. The entry of variants in an international PCC/PGL dedicated database would be a powerful tool allowing an appropriate clinical decision-making for patients worldwide.

44 - The effects of Sunitinib in SDHB-malignant paraganglioma

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Here we describe the case of a 35 yr old male with a metastatic abdominal paraganglioma. In the past he had undergone surgery twice: at the age of 10 yr for the removal of a chromaffin tumor localized near the left kidney and at the age of 31 yr for a local recurrence. He also presented a congenital right kidney hypoplasia.

In 2012, because of the recurrence of hypertensive crises, palpitations and headache, urinary normetanephrine were tested and found elevated. A ^{18}FDG-PET showed persistent disease at the site of previous surgery and of the left ischium. A I^{123}MIBG scintigraphy showed an uptake only in the bone lesion.

The patient was referred to us in September 2013 when we performed the following exams:

1. urinary metanephrine (MNu), normetanephrine (NMNu) and methoxytyramine (MTXu) (respectively 87 mcg/24h, 8927 mcg/24h and 460 mcg/24h);
2. thorax/abdomen TC showed a large abdominal lesion (L1: 4.67 cm) in the left lumbar-aortic region and other smaller abdominal lesions (L2: maximum diameter 2 cm) as well as some liver metastases (L3);
3. bone scintigraphy was negative for disease;
4. genetic analysis revealed a germ-line SDHB mutation (c.423+1G>A).

As the recurrent primary tumor was strictly adjacent to the ureter and the renal vessels, its surgical removal was excluded to avoid the risk of chronic renal insufficiency and dialysis.

A radiometabolic therapy with labeled somatostatin analogues was excluded because of a negative Octreoscan.

To control blood pressure, we increased doxazosin dosage up to 12 mg/day and added a beta-blocker (atenolol 50 mg) and a calcium-antagonist (amlodipine 5 mg).

In October 2013 the patient developed acute renal failure because of left ureter compression by the abdominal mass. A pig tail was inserted to avoid the obstruction.

Because of disease progression, documented by a significant increase in the lesion sizes, in November 2013 the patient started Sunitinib therapy at the dose of 25 mg/day which was later on increased at 50 mg/day. After 5 weeks a clear reduction of the main lesion (from 6.91 to 3.74 cm) and disappearance of liver metastases were observed as well as a significant decrease in urinary normetanephrine and a significant decrease in ^{18}FDG uptake but we had to reduce the dosage for the occurrence of side effects such as nausea, vomiting, asthenia, hypothyroidism, hypertriglyceridemia.

To limit side effects and continue Sunitinib administration, we adopted the following schedule:

37,5 mg/day for a week, 25 mg/day the second week and finally a week off of therapy.

In July 2014, after additional 5 months, the disease was found stable at TC and ^{18}FDG-PET thus demonstrating the positive effects exerted by Sunitinib in this SDHB mutated malignant PGL.

45 - Validation of a chip-based semiconductor workflow for the screeening of PHEO/PGL susceptibily genes

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The development of next-generation sequencing (NGS) technologies has provided new and reliable approaches to diagnostic testing demonstrating advantages over Sanger such as the abilities to sequence and analyze multiple genes simultaneously at lower costs. The aim of this study was to develop and validate a NGS based workflow for genetic screening of pheochromocytoma and paraganglioma (Pheo/Pgl) patients. We adopted a chip-based semiconductor sequencing technology, based on the use of the Personal Genome Machine sequencer (PGM – Life Technologies) and AmpliSeq™, to analyze up to 43,65kb of genomic DNA, corresponding to the coding sequence of the genes SDHA, SDHB, SDHC, SDHD, SDHAF2, VHL, RET, TMEM127, MAX, PHDx (1, 2, 3), IDH, FH and NF1 and distributed in 424 amplicons by Ion Ampliseq Designer Software. Due to the excessive extension of the panel, the numerous variants detected and the difficulty of establishing their functional role, expecially in some not well characterized genes, we preferred to design a second panel not including PHDx, IDH and NF1. This custom panel (33Kb, 216 amplicons) covered the 97,46% of the coding regions. Lost sequences due to high GC-rich or homopolymer regions were sequenced by Sanger method to obtain 100% of coverage. Data were generated and analyzed with Ion Reporter 4.0, wAnnovar and IGV2.2 software (Broad Institute) and all the mutations were called in roughly 50% of the reads with a 40x coverage as a minimum. We further performed an inter-laboratory quality control procedure in collaboration with the Laboratory of Tumor Genetics (Nijmegen Medical Centre) that developed and adopted the same techniques with the aim to diagnose Pheo/Pgl. A set of 27 DNA samples (group 1) previously characterized by Sanger method as presenting unique variations distributed along the mainly pathogenic genes were used in order to setup and validate the workflow procedure. In addition, 30 DNAs from Pheo/Pgl/MTC patients (group 2) were analyzed by PGM prior of Sanger sequencing and MLPA. The PGM platform confirmed 100% of the variations (mainly SNPs, missense and small deletion mutations) of group 1 submitted during validation. In addition the system detected, in a patient of group 2, a new variant of unknown significance in the SDHAF2 gene (c.C151G:p.P51A) in a patient with abdominal PGL.
In conclusion we have validated and adopted as diagnostic a method fast and cost effective that help clinical genetics screening diagnosis based on the PGM platform to detect mutations in the Pheo/Pgl susceptibility genes.

46 - MULTIPLE HEAD AND NECK PARAGANGLIOMAS AND COWDEN-LIKE CLINICAL FEATURES IN ASSOCIATION WITH GERMLINE G12S *SDHD* POLYMORPHISM

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Head and neck paragangliomas (HNPGLs) are rare tumors associated with the parasympathetic nervous system. Most are sporadic, but about one third result from germline mutations in succinate dehydrogenase (SDH) genes (*SDHB*, *SDHC*, *SDHD*, *SDHA*, or *SDHAF2*). Pathogenic mutations affecting other genes have been discovered in recent years but their role in the pathogenesis of HNPGLs is very limited.

In a series of 113 HNPGLs patients diagnosed and/or treated in the Spanish Central and Northern regions, we found that about 60% are sporadic cases without known pathogenic mutations. One of these patients, a 60-years old woman with jugular and vagal paragangliomas, referred previously diagnosed uterine leiomyomas and thyroid benign tumors suggestive of Cowden or Cowden-like Syndrome, a PTEN-related disorder. Genetic analysis revealed absence of *PTEN* mutations and presence of a known *SDHD* polymorphism (rs34677591, pG12S) which, according to published data, has been widely accepted as not pathogenic by the scientific community. Whole-exome sequencing of blood DNA showed that this patient had 526 nonsynonymous variations in protein coding sequences, of which, 26 can be considered as pathogenic according to Polyphen. We also found seven no synonymous mutations at the somatic level that were not encountered in an independent validation series. None of these genetic variants were in cancer genes, genes associated with paragangliomas/pheochromocytomas neither genes involved in the pseudohypoxia and kinase pathways. The coexistence of multiple HNPGL and Cowden-like symptoms in a PTEN-negative patient carrying SDHD pG12S polymorphism suggests a functional connection of coding SDH polymorphisms and HNPGL development.

47 - A novel adult human adipose stem cell model from inducible brown fat surrounding pheochromocytoma tumors

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The recent discovery of inducible brown adipose tissue (BAT) in adult humans, in which it is involved in controlling adiposity, is pivotal in the development of treatment strategies for obesity. However, the origin of these adipocytes in white adipose tissue (WAT) is still unclear, and no human brown adipose cell models are currently available.

The objective of the study was to define the origin of inducible BAT (iBAT) by isolating brown adipose stem cells (B-ASCs) from periadrenal fat in adult patients with catecholamine secreting pheochromocytoma.

We demonstrated the presence of iBAT islets dispersed in periadrenal WAT in patients operated for pheochromocytoma. From this fat depot, which expresses brite/classical BAT markers and high levels of uncoupling protein-1, we isolated B-ASCs and compared their properties with precursors from sc WAT (S-ASC) of the same patients. B-ASCs showed mesenchymal, stem, and multipotency features and expression of brite/classical BAT markers. When differentiated toward white phenotype, B-ASCs accumulated lipid droplets smaller than S-ASCs and expressed adiponectin. Upon induction of brown differentiation, brown commitment was found only in B-ASCs and not in S-ASCs, with no mature brown adipocytes.

Our findings demonstrate that iBAT developed in periadrenal WAT derives from adult stem cells, unlike WAT precursors, confirming an independent origin of the two fat depots. These stem cells represent a unique in vitro stem cell model to study brown adipogenesis and develop novel antiobesity therapies targeting WAT-BAT conversion. (***J Clin Endocrinol Metab 99: E1903–E1912, 2014***).

48 - MEDIASTINAL PARAGANGLIOMA WITH CHYLOTHORAX

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Paragangliomas (PGL) develop from extra-adrenal autonomic paraganglia. Sympathetic PGL usually secrete catecholamines and are located in the chest, abdomen and pelvis, while parasympathetic PGL are usually nonfunctional and are found along the glossopharyngeal and vagal nerves in the neck and at the base of the skull. PGL are sporadic (70%) or associated with hereditary syndromes (MEN 2A, VHL in 30% of cases).

We present a rare case of mediastinal paraganglioma in a 30 years old female patient with a 3 year-history of severe paroxysmal hypertension, tachycardia, sweating and hyperglycemia. She had very high levels of plasma and urinary normetanephrines (33 x ULN) and slightly increased metanephrines. Abdominal and pelvic MRI and whole-body 131-I MIBG scan were normal 1 year before admission in our clinic. She developed pleural effusion and cough in the hospital. Chest CT revealed a 6 cm tumor in the posterior mediastinum and also left pleural effusion, which was aspirated and diagnosed as chylothorax. After preoperative treatment with phenoxybenzamine, carvedilol and diltiazem, the patient underwent thoracoscopic removal of the tumor and drainage of 12 L of fluid. During surgery the patient had initially hypertension, then severe hypotension for 1 day and normal blood pressure without treatment afterwards. Chylothorax did not recur. Plasma metanephrines were normal after surgery. Pathological exam confirmed a paraganglioma with Ki67 of 3%.

Conclusion. Mediastinal PGL represent only 2% of PGL, may induce compression complications (as chylothorax) and are more frequently associated with mutations in the succinildehydrogenase gene (B or D) and more aggresive behaviour. Long-term follow-up after surgery is mandatory, due to the metastatic potential.

49 - Thoraco-abdominal sympathetic Paraganglioma: analysis of a series of 14 patients

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Background. Pheochromocytomas are rare tumors arising from thoraco-abdominal sympathetic ganglia. They may arise from the adrenal glands, or from the extra-adrenal sympathetic tissue; in these cases are named Paraganglioma (PGL). This study was aimed to perform an analysis of the main clinical, genetic and long-term outcome of PGL in a single-centre series.
Methods. This study focused on 14 patients (12 males and 2 females; median age 35yrs, range 10-72) suffering from PGL, selected from a series 124 consecutive patients undergoing surgery because of thoracoabdominal sympathetic-derived tumors.
Results. Genetic analysis was available in 10 PGL patients: a putative germ-line mutation was found in 4 patients (1 VHL, 1 SDHB, 1 SDHC, and 1 MAX gene mutation). Among these patients, a positive familial history was clinically evident only in 1 case. No statistically significant differences were found between genetically-determined and sporadic PGL concerning age (median 40.5 vs 35.5 yrs) and Sex ratio. An association with intra-adrenal Pheochromocytoma occurred in 5 patients (75% in genetically-determined variants versus 16.7% of apparently sporadic forms). A recurrent course was found in 3 cases with positive germline mutations (SDHC, VHL and MAX), with a disease free interval of 10, 14 and 16 years after initial surgery, while never in case of sporadic PGL (p=0.03). An evident malignant behavior occurred in 3 patients (2 carriers of germline SDHB and SDHC mutations, respectively, and 1 case with sporadic disease). PGL patients had a significantly lower age than patients with intra-Adrenal Pheocromocytoma (p=0.04) and a higher male/female ratio (6 vs 0.9, p=0.009).
Conclusion: PGL is a rare disease accounting for 11.3% of all Pheochromocytomas; it occurs more frequently in male and younger patients. A genetic background may be found in 40% of PGL patients; in these cases, familial history is negative in 3/4th of cases. PGL may be associated to Pheochromocytoma, especially in case of inherited variants. Recurrences may occur especially in genetically determined variants, also after a long disease free interval; a malignant behavior is present in 21.4% of cases, in most cases related to SDH germ-line mutations. Subsequently, genetic investigations and long-term follow up are suggested.

50 - A FAMILY PRESENTING WITH BILATERAL METASTATIC PHEOCHROMOCYTOMA, ERYTHROCYTOSIS, RENAL ONCOCYTOMA, AND COMPLEX *MAX*-GENE REARRANGEMENTS

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Currently, 13 genes have been causally linked to pheochromocytomas (PCC) and paragangliomas (PGL). Germline mutations in several PCC/PGL-related genes cause syndromes, such as *VHL* (von Hippel Lindau disease), *RET* (MEN2 syndrome), *NF1* (Neurofibromatosis type 1), *SDHB*, *SDHC*, *SDHD*, and *SDHAF2* (PCC/PGL syndromes). The last decade, other genes have been found in PCC/PGL, including *SDHA*, *TMEM127*, *KIF1B*, *FH*, and *HIF2a*. Other tumors that have been associated with mutations in the PCC/PGL susceptibility genes include renal cell carcinomas (*SDHB* and *VHL*), medullary thyroid carcinomas (*RET*), and gastro-intestinal stromal tumors (*SDHA*,*SDHB*, *SDHC*, *SDHD*). In addition to PCC, another feature that has been recently described in patients with *HIF2A* mutations is erythrocytosis.

In this study we have investigated an index patient from a family, of which 4 brothers presented with bilateral PCC. In addition, the index patient also presented with a renal oncocytoma and erythrocytosis, while his full brother developed a composite PCC and distant (lymph node) metastasis. The patient tested negative for germline mutations in the coding regions of *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *VHL*, *RET*, *TMEM127*, and *MAX*. In addition, no exon deletions were found in the *SDH-genes*. To identify what genomic alterations were causal for the tumors in this patient as well as his brothers, we performed SNP arrays and exome sequencing on tumor (PCC) and normal DNA from the index patient, of whom frozen tissue as well as blood was available.

The results showed a large genomic germline deletion of 400kb, partially covering the *MAX*-gene. The large and complex deletion was confirmed by PCR and Sanger sequencing. In addition, the SNP array revealed uniparental disomy of the mutant allele, which appears to be a hallmark of *MAX*-mutated tumors. Absence of immune-staining indicated the loss of expression and function of this gene, both in all PCCs from this family as well as in the renal tumor. The germline deletion was also detected by PCR of paraffin-embedded tumor and normal DNA from two brothers.

Our study shows that the *MAX* gene is inactivated by a large germline deletion in the PCCs and renal tumor of this patient as well as in his brothers. Although no other tumors have been described in previous reported *MAX* germline carriers, this study suggests that these patients should be investigated for renal tumors. In addition, as previously described, malignant disease should always be considered in *MAX* mutation carriers.

51 - The transcription factor Phox2a plays a pro-tumorigenic role in pheochromocytoma

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Introduction
Despite the fact that a number of genes involved in pheochromocytoma (PC) have been identified, the molecular pathways involved in this tumor type are still not well understood. Elucidation of these pathways could generate novel targets leading to improved treatment of affected patients. Rats carrying a germline loss-of-function mutation in p27 (MENX syndrome) develop bilateral PC and these tumors were used for gene discovery to identify novel genetic mechanisms involved in PC tumorigenesis.

Methods
Taqman analysis was performed on rat and human pheos for *Phox2a*, a gene found up-regulated in the rat tumors by mRNA array analysis.
We used cell lines such as MPC (mouse PC) and its aggressive derivative MTT, both with high levels of Phox2a. siRNA-mediated *Phox2a* gene knockdown was performed in MPC/MTT cells using specific oligos. *In vitro* assays assessing proliferation (MTT), migration and invasion (Boyden chamber) were then performed.

Results
By gene expression profiling of rat PC we found an up-regulation of the *Phox2a* gene. Phox2a is a transcription factor usually regulating the expression of genes involved in the differentiation of sympathoadrenal cells. Interestingly, *PHOX2A* was also overexpressed in 91% of human PC. Down-regulation of endogenous *Phox2a* expression reduced the proliferation of MPC/MTT cells when compared with scrambled siRNA-transduced cells. Knockdown of *Phox2a* also reduced the invasive potential of MPC cells whereas no changes in migration and invasion were seen in MTT cells.

Conclusion
In conclusion, we observed that *Phox2a* promotes the tumorigenic phenotype of PC cells by enhancing proliferation and possibly invasion. *Phox2a* represents a new molecular biomarker of PC. Studies of its downstream genes/pathways may help in both elucidating the molecular pathogenesis of PC and in discovering novel therapeutic targets for clinical applications.

52 - SDHB-DEFICIENT CHROMAFFIN CELLS DISPLAY HALLMARKS OF SDHB-MALIGNANT TUMORS ASSOCIATED WITH AN INVASIVE PHENOTYPE

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Pheochromocytomas and paragangliomas are rare neuroendocrine tumors, presenting a high risk of malignancy and poor prognosis when carrying *SDHB*-gene mutations. *SDHB*-malignant tumors are characterized by the induction of epithelial to mesenchymal transition (EMT), and by a hypermethylator phenotype.

We developed and characterized an experimental model of *Sdhb* gene knockout in mouse chromaffin cells. *Sdhb* gene inactivation was responsible for a transcriptional induction of EMT-associated hallmarks, also validated at protein level. SDHB loss induced an invasive phenotype characterized by enhanced individual cell migration associated with faster motility and long travelled distances, high invasive, and elevated adhesion abilities. EMT-associated morpho-physiological changes observed, suggested that *Sdhb* deficient chromaffin cells are a suitable model to study *SDHB*-mediated malignancy. *Krt19*, an EMT-associated gene is epigenetically silenced after SDHB loss. Treatment by demethylating agents, decitabin or entinostat, allowed *Krt19* re-expression. Lentiviral-mediated *Krt19* rescue in *Sdhb* deficient cells, and siRNA-mediated *Krt19* inhibition in WT cells emphasized its involvement in this invasive phenotype, as shown by the modulation of collective and individual migration, and by the impact on adhesion properties.

Altogether, these results highlight a new role for SDHB protein, which loss is directly linked to a neuroendocrine to mesenchymal transition (Neuroendocrine-MT), responsible for *SDHB*-mediated malignancy in PCC and PGL. Our data suggested that *Sdhb* deficient chromaffin cells remain to date, the best model to study the role of SDHB in human PCC/PGL metastatic dissemination.

53 - Overexpression of hypoxia-inducible factor 2alpha stimulates proliferation and migration in pheochromocytoma tumor cells

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Pheochromocytomas and paragangliomas (PPGLs) are highly heterogeneous tumors. Gene expression profiling studies indicate two dominant expression clusters: cluster 1 tumors are caused by *VHL/SDH* mutations, overexpress HIF2α and exhibit a pseudohypoxic phenotype, while cluster 2 tumors are caused by *RET/NF1/TMEM127/MAX* mutations and do not express HIF2α. Cluster 1 tumors, particularly those due to *SDHB* mutations, are associated with more aggressive behavior and increased metastatic potential. We hypothesized that HIF2α plays an important role in permitting the metastatic phenotype. To address this hypothesis we combined observational investigations in PPGLs, gene-manipulation studies in a mouse pheochromocytoma cell line (MPC) and volumetric measurement of xenografted tumors in a nude mouse model. To characterize the key function of HIF2α in tumor development, gene expression profiling was carried out using tumor specimens and HIF2α re-introduction into MPC cells. We compared the profiling data and processed gene ontology analysis and found 18 upregulated genes associated with cell proliferation and migration, including *Ndrg1*, *Hk2*, *Cnn3* and *Lmo4*. In cell lines lacking HIF2α, overexpression of the gene led to increased proliferation, migration and immature phenotypic features. In our subcutaneously injected mouse model, HIF2α overexpressed cells demonstrated significantly faster tumor growth compared to the control group. Our data indicate that overexpression of HIF2α is associated with increased migration and faster tumor growth, which may confer a more aggressive phenotype to PPGLs.

54 - SUCCINATE DEHYDROGENASE B SILENCING DEEPLY AFFECTS CELL METABOLISM AND FUNCTIONS: ROLE OF MICROENVIROMENT IN PHEOCHROMOCYTOMA/PARAGANGLIOMA TUMOR PROGRESSION

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Solid tumors are not exclusively composed by neoplastic cells, but also by fibroblasts, endothelial cells and immune system cells, the so-called tumor microenvironment. Due to the reciprocal interaction between tumor cells and microenvironment, a metabolic reprogramming seems to occur in both types of cells thus creating favorable conditions for tumor growth and metastatic spread.

To obtain an experimental model more closely resembling the in *vivo* conditions of SDHB-mutated paragangliomas, we silenced the succinate dehydrogenase B subunit (SDHB) in the human neuroblastoma cell line (SK-N-AS) and we evaluated the effects of silencing on metabolism and proliferation of SK-N-AS cells, cultured either alone or in association with human fibroblasts.

SDHB silencing was verified by Western Blot analysis and densitometry showing a 70% decreased expression and by an almost complete loss of the complex specific enzymatic activity. As expected,. SDHB silenced cells showed a decrese in O_2 consumption and an increase in HIF expression, resembling the in *vivo* tumor cell phenotype. SDHB silencing was associated to an altered metabolism characterized by an unexpected significant decrease in glucose uptake, a significant increase in lactate uptake, and an increase in cell proliferation and metalloproteinase production, compared to control.

When co-cultured with human fibroblasts, both negative control and SDHB silenced cells showed a significant decrease in glucose uptake, and a significant increase in lactate uptake as well as in proliferation. In co-cultured SDHB silenced cells, these effects were even more strikingly evident. Conversely, co-cultured fibroblasts increased glucose uptake significantly.

Our data demonstrate that SDHB silencing causes a metabolic and functional derangement of tumor cells and that microenvironment, here represented by fibroblasts, strongly affects tumor metabolism and growth capacity. In particular, we demonstrated that primary fibroblasts and tumor cells establish reciprocal metabolic changes. In agreement with other studies (Fiaschi T et al. Cancer Res. 2012), we found that tumor cells favor a Warburg-like glycolytic metabolism in fibroblasts, thus increasing glucose uptake and its conversion into lactate which in turn is uploaded by tumor cells and most likely used for fueling Krebs cycle, as well as anabolic processes and cell proliferation.

We believe that the comprehension of the mechanisms driving this cross-talk between tumor cells and microenvironment might possibly represent not only an attempt of understanding the molecular mechanisms leading to tumor onset and progression, but also the first step towards the development of novel pharmacological approaches aimed at limiting the proliferative effect and the invasive/metastasizing potential of these tumors.

55 - The role of p27 in neuroendocrine tumorigenesis

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The *Cdkn1b* gene encodes the p27 cell cycle regulator which binds and inhibits cyclin-cyclin dependent kinase (CDK) complexes. In adult tissues, p27 is ubiquitously expressed and plays a crucial role in maintaining tissue homeostasis by preventing differentiated cells from re-entering the cell cycle. *Cdkn1b* was also demonstrated to be a tumor susceptibility gene for multiple endocrine neoplasia tumors in both rats (MENX syndrome) and in human patients (MEN4 syndrome). MENX is caused by a germline frameshift mutation in *Cdkn1b* that renders the encoded mutant p27 protein very unstable. Among other neuroendocrine tumors, MENX rats develop bilateral pheochromocytoma with complete penetrance.

Recently, it was reported that p27 indirectly regulates gene transcription in mouse fibroblasts by associating with transcription factors and inhibiting gene transcription at specific promoters. p27 co-localizes with p130, E2F4 and co-repressors such as histone deacetylases (HDACs) and mSIN3A and binds to specific promoter regions. The genes regulated by this complex are involved in important cellular functions such as RNA processing and splicing, mitochondrial organization and respiration, translation and cell cycle. We hypothesize that defective p27 may promote tumor formation specifically in endocrine tissues because of aberrant gene expression regulation. To address this issue we want to determine whether p27 works as a transcriptional regulator in rat adrenomedullary cells. We plan to use adrenomedullary cells from wild-type rats (with normal p27 levels) and a specific anti-p27 antibody to perform chromatin immunoprecipitation (ChIP). To further identify the DNA sequences bound by complexes containing p27 we will perform sequencing (ChIP-Seq). So far, we have established and optimized the ChIP method starting from rat adrenomedullary tissue. We could successfully pull down DNA sequences using magnetic beads coupled with an anti-p27 antibody. This indicates that p27, together with unknown transcription factors or co-factors, can bind the chromatin in rat adrenomedullary cells.
Ongoing sequencing experiments will determine which of the DNA sites are bound by the p27-containing complex and will offer valuable clues of an impact of p27 to expression profiles of specific genes involved in tumor progression. Afterwards the effect of the association of p27-containing complexes to specific promoters will be validated by modulating p27 levels and checking target gene expression. The significance of the p27-dependent gene expression will also be validated by analyzing the expression of these target genes in adrenal medulla of MENX-affected rats (with loss of functional p27).
In conclusion, the unique observation of p27 acting as an indirect transcriptional repressor, may give insight into human carcinogenic mechanisms associated with p27 downregulation specifically in neuroendocrine cells.

56 - Metabolic response to treatment on ^{18}F-FDG PET in a patient with metastatic pheochromocytoma

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Background: 18F-Fluorodeoxyglucose (18F-FDG) PET is very useful for localizing metastatic pheochromocytomas and paragangliomas (PPGLs). In other cancers 18F-FDG PET is used for therapy monitoring using PET Response Criteria in Solid Tumors (PERCIST). Actual tumor shrinkage on computed tomography (CT) according to Response Criteria in Solid Tumors (RECIST) can be preceded by a decline in uptake of 18F-FDG.

Methods: Case report. A 62-years-old female was referred for metastatic PPGL. She was carrier of a pathogenic non-sense SDHA mutation (c.91C>T (p.Arg31*)). At the age of 50, she was diagnosed with inoperable PPGL and initially treated with fractionated 131I-MIBG therapy (with a cumulative administered activity of 59.2 GBq). For many years she was clinically and biochemically stable on α- and β-adrenoreceptor blockade. However, at 61-years, she developed multiple lymph and bone metastases resulting in abdominal and bone pain which were controlled by external beam radiation therapy. CT imaging showed progressive disease. Experimental treatment with sunitinib 37,5 mg or placebo daily in the context of FIRSTMAPPP (ClinicalTrials.gov Identifier: NCT01371201) was initiated. Response in target lesions was evaluated by diagnostic CT and 18F-FDG PET scans using RECIST 1.1 and PERCIST 1.0 criteria.

Results: RECIST qualified for stable disease (SD) whereas PERCIST indicated progressive metabolic disease (PMD) (Table). 18F-FDG PET may detect progressive disease before anatomic changes are observed.

Conclusion: This case illustrates that metabolic response does not necessarily parallel anatomical response when evaluating the treatment of metastatic PPGL. The clinical significance of 18FDG-PET data in addition to CT will become clear from the analysis of ongoing clinical trials such as FIRSTMAPPP.

57 - Evaluation of ^{18}F-FDG kinetics in pheochromocytoma and paraganglioma by dynamic PET/CT scanning

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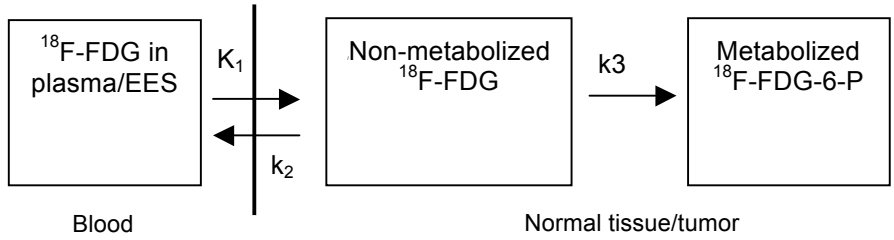
Background: Static single timeframe ^{18}F-FDG PET is very useful for the localization and characterization of both primary and metastatic pheochromocytomas and paragangliomas (PPGLs). The presence of underlying *succinate dehydrogenase* (*SDH*) mutations can be predicted based on high ^{18}F-FDG standardized uptake values (SUV). High SUV has been suggested to be a reflection of tumor biology of *SDH*-related PPGLs, characterized by compromised oxidative phosphorylation and enhanced aerobic glycolysis. The exact determinants of ^{18}F-FDG accumulation in PPGL, however, remain unknown. High SUV might represent accumulation of unmetabolized ^{18}F-FDG in the extracellular and intracellular spaces rather than high glycolytic rate. The aim of this study was to assess ^{18}F-FDG kinetics across sporadic and hereditary PPGLs by dynamic multi-timeframe PET/CT scanning.

Methods: Dynamic FDG-PET/CT scans were acquired in 10 adult patients with biochemical evidence of PPGL who were scheduled for surgery. A two-tissue compartment tracer kinetic model, assuming irreversible FDG metabolism, was used to estimate transfer rates of ^{18}F-FDG between (i) the vascular/extravascular extracellular space (EES), (ii) the intracellular non-metabolized, and (iii) the intracellular metabolized compartments (Figure). The derived transfer rates for transmembranous glucose flux (K_1 (in), k_2 (out)) and intracellular phosphorylation (k_3) along with the fractional blood volume (Vb) were analyzed using non-linear regression analysis. Glucose metabolic rate (MRglu) was calculated using Patlak analysis using venous plasma glucose level and a lumped constant of 1.



Results: Dynamic parameters in PPGLs (Table). SDHD-related pheochromocytoma showed a high glucose metabolic rate (MRglu) compared to other PPGLs.

Sex	Age (yrs)	Tumor location	Genotype	MRglu (nmol/ml/min)	*SUVmax (g/ml)*	SUVmean (g/ml)	K_1 (ml/g/min)	k_2 (/min)	k_3 (/min)	Vb (ml/ml)
F	54	LA	Sporadic	47,2	3,1	1,5	0,010	0,470	0,591	0,017
F	34	RA	Sporadic	63,6	5,0	2,9	0,012	0,467	0,702	0,027
M	65	RA	SDHD	204,0	5,9	3,6	3,245	2,825	0,071	0,598
M	51	EA	Sporadic	14,6	1,9	1,5	0,497	1,153	0,011	0,146
F	32	LA	Sporadic	36,8	2,1	1,5	0,389	0,992	0,027	0,151
M	63	LA	Sporadic	34,9	2,9	1,7	0,184	0,502	0,026	0,084
M	85	RA	Pending	38,9	2,4	1,5	0,863	1,364	0,017	0,072
M	55	RA	Pending	21,8	2,1	1,6	0,696	1,471	0,014	0,216
M	43	LA	Pending	63,2	3,8	2,7	1,516	1,493	0,029	0,284
F	74	LA	Pending	38,5	3,8	1,8	0,366	0,640	0,024	0,348

Abbreviations: LA=left adrenal, RA=right adrenal, EA=extra-adrenal, MRglu=metabolic glucose rate, SUV=standardized uptake value, Vb=fractional blood volume.

Conclusion: Our preliminary data indicate a variable pattern of ^{18}F-FDG kinetics among PPGLs with different underlying genotypes. Compared to other cancers, MRglu was relatively low in PPGLs.

58 - CHARACTERIZATION OF PARAGANGLIOMAS: A SINGLE CENTER COHORT STUDY

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Introduction

Paragangliomas and pheochromocytomas are rare tumours arising from chromaffin cells. However, confrontation between epidemiological data and autopsy series suggest that they are largely underdiagnosed.
Recent advances in genetics highlighted the implication of a dozen genes of predisposition. Mutations of these genes are found in up to 4 cases out of 10.
In this retrospective study, we report a clinical characterization of 30 confirmed paragangliomas.

Population

We collected data of 30 cases that benefit of surgical resection of paraganglioma between 1993 and 2014.

Results

Our population was made of 14 women and 16 men. Median age was 47 year old (29-79 y.o.). Out of these, 20 patients were treated for hypertension and 3 of them had left ventricular hypertrophy. Number of drugs used to control hypertension raged from 1 (n=8) to 4 (n=1).
We report 26 pheochromocytomas and 4 paragangliomas. Pheochromocytomas were unilateral in 22 cases (left n=10 and right n=12). Paragangliomas were mainly located between both kidney (n=3) while one of them arised out of Zuckerkandl's organ.
No significative correlation was found between diameter and total metanephrines, noradrenaline, 5HIAA or VMA values. A positive correlation between tumor diameter and normetanephrine levels was found (p=0,009).
Metastatic disease was found in 3 cases. All of these received I131 MIBG radiotherapy and one of them had chemotherapy. None of these had mutation in *SDHB* gene but one (aged 33 at diagnosis - PASS score = 15) has Von Recklinghausen neurofibromatosis.
MIBG scintigraphy was performed in 26 patients and showed an active uptake in 21 cases.
Genetics evaluation was done in 18 cases. Five had positive results for *ret* mutation and 3 had *VHL* mutation. As usually reported in the literature, patients with *VHL* mutation showed predominantly noradrenaline secretion as one of our *ret* mutated cases. Interestingly, noradrenaline was predominantly produced in our NF case.

Conclusion

Although rare, paragangliomas management can remain challenging. Recent advances in genetics are helpful to better classify this disease and to predict potential aggressivity. However, even if genetics results are negative, precautious attention and follow up should be done.

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