



Project Final Report

Grant Agreement number: 259735

Project acronym: ENS@T-CANCER

Project title: European Network for the Study of Adrenal Tumours - Structuring clinical research on rare cancers in adult

Funding Scheme: Collaborative project

Period covered: From 01-01-2011 to 30-06-2016

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Section 1 – Final publishable summary report

ENS@T-CANCER



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Website: www.ensat-cancer.eu

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1.1 Executive summary

Following the working program of the ENSAT-CANCER consortium yet unmatched progress has been achieved. This is exemplified by a large number of scientific publications, which can be summarized as follows:

Establishment and refinement of a virtual research environment (VRE) has been a major achievement that has welched together the consortium and has provided a common hub for scientific projects and clinical studies. An ever-growing number of patients has been collected with rich clinical annotations and wealth of biomaterial in the common registries and associated decentralized biobanks. The identification of patients with specific clinical characteristics has aided in recruitment of candidates for the clinical interventional trials. Similarly, the biomaterials and associated clinical data have been the basis of biomarker studies from the different work packages. A number of new functionalities have been implemented that include: 1) New search function with advanced notions of timeline representation, 2) Interactive completeness graphs to monitor and improve data quality, 3) Connection with American, Asian, Australian Adrenal Alliance (A5) registry, 4) Imaging support and 5) Robust mobile applications, that have full support of IOS and Android mobile devices.

Within the translational studies, important progress could be achieved towards the elucidation of pathways involved in adrenal tumorigenesis. These studies were able to identify new targets (such as SF-1 or the WNT/b-catenin pathway) that could result in therapeutic strategies. In fact, some of the gathered insights translated in novel treatment modalities, which were tested in studies in dedicated preclinical models.

Researchers forming the pathology platform made particular progress in the standardization of immunohistochemical techniques and interpretation of morphological tumour characteristics (including proliferation marker, extent of vascularization and content of cells with stem cell characteristics) required for comparison of adrenal cancer diagnosis and comparability of biomarker studies in the field across European countries.

For adrenocortical carcinoma, the genomic platform had achieved a major break-through with the first description of an integrative genomic approach. Since the molecular classification was achieved earlier than expected with the success of the integrated genomics of ACC it had been possible to develop molecular markers based on the analysis of methylation alterations in ACC during the last part of the program and to confirm the molecular classification in an independent international cohort. Likewise, epigenetic markers for the prediction of malignancy for pheochromocytomas could be identified which might aid in the differential diagnosis of this rare disease.

Significant progress has been made in our studies related to molecular target expression studies and studies on the relation between functional imaging (18F-FDG PET and MIBG) and genotype-dependent tumour cell energy metabolism, which provide the basis for further biomarker/functional imaging studies. First experience from patients with malignant pheochromocytoma enrolled in the FIRST-MAPPP study indicate potential discrepancy between morphological and functional imaging characteristics following tumour treatment.

Within the clinical study platform, the first randomized prospective trial on malignant pheochromocytoma has been moving forward with 45 centres being activated and 57 patients being randomized into the two study arms. Similarly, for the adjuvant treatment protocol of adrenal cancer patients a significant number of patients could be enrolled. Although the patient recruitment for both studies had been lower than anticipated in both instances the groundwork laid by the consortium made it possible to maintain the study in the recruiting clinical centres. Thereby, a successful completion of the clinical studies can be expected.

1.2 Summary description of project context and objectives:

Background and Aims

Adrenal masses belong to the most prevalent human tumours and comprise a heterogeneous group of pathologies. Tumours of the adrenal can manifest as syndromes of hormone excess and cancer. Among patients with adrenal masses, two entities are found with a low incidence but very unfavourable prognosis:

Firstly, **Adrenocortical carcinoma** (ACC, see Annex A, Glossary) originating from the adrenal cortex has an estimated prevalence ranging from 4 to 12 per million, although in pre-selected patient cohorts on which adrenal tumour surgery had been performed the proportion of ACC may be as high as 12%. The clinical outcome of ACC is poor with an overall 5-year survival of only 15-35%.

Secondly, **malignant pheochromocytomas** (MPH) are tumours derived from the adrenal medulla or other sympathetic ganglia that occur sporadically but also in the context of specific familial disorders. Pheochromocytoma has an estimated prevalence of 1:6,500 to 1:2,500. Approximately 10% of pheochromocytomas (1:65,000 - 1:25,000) are considered to be malignant with a variable but mostly poor clinical course resulting in a 5-year survival of less than 50%.

Due to their **poor prognosis**, concomitant **hormone dysregulation** and **limited treatment options** these two cancer entities are severely devastating for affected patients and their families. Due to the rarity of the diseases the diagnosis is often delayed causing substantial suffering and indicating the **need for enhanced awareness** among physicians and the public. Furthermore, there are very few centres with sufficient expertise to offer optimal treatment and care for these patients. The **rarity of the tumours** also **impedes clinical studies**, which are affected by fragmentation and usually involve too small case numbers to reach conclusive results. This contributes to the relatively **poor state of funding** for clinical and translational research in this area and consequently also limits progress in the development of new diagnostic or prognostic markers and treatments for these rare cancer entities.

The concurrence of the earlier achievements of an **evolving European Network**, the progress in the **understanding of molecular mechanisms** and increasing **availability of specific diagnostic and therapeutic tools** for adrenal cancers and, finally, the support by public, industrial and private partners had provided the unique opportunity to achieve unmatched progress in the implementation of both translational and clinical research dedicated to ACC and MPH. To realise this opportunity, the newly formed ENS@T-CANCER consortium had addressed the following objectives:

- Structuring European clinical and translational research through implementation of a virtual research environment (VRE)
- Improving clinical outcome of patients with adrenal cancer by conducting interventional trials carried out by European centres of excellence
- Improvement of differential diagnosis and risk stratification of adrenal cancer
- Identification and validation of tools for follow-up of patients with adrenal cancer
- Identification of novel biomarkers for treatment response
- Screening for molecular mechanisms as the basis to improve treatment response

These general objectives are further specified below.

Work strategy and general description

Prior to the start of the consortium, the **European Network for the Study of Adrenal Tumours** (ENS@T; www.ensat.org) had implemented a collection of adrenal tumour related databases and defined an associated European network of Biological Resource Centres devoted to research on adrenal tumours. **Common standardized operational procedures** (SOPs) were developed for the collection of biological material from adrenal cancer patients that can be subjected to integrated biomarker approaches. This pre-existing infrastructure was further optimised, homogenised and, importantly, virtually linked via **web-based Clinical Record Forms** (eCRF) as part of the Research Networking Programme. During the lifetime of ENS@T-CANCER FP7 funding allowed considerable enhancements to these initiated projects, in a complementary and synergistic way, thereby providing significant

added value.

During recent years a multitude of novel technological approaches had become available with the potential to functionally characterize tumour samples based on biomaterial derived from patients with malignant diseases. These technologies have started to revolutionize also the way translational and clinical research in the field of adrenal oncology can be assessed. Overcoming fragmentation of research efforts through ENS@T-CANCER has allowed for the combination of multiple morphological, molecular and functional markers which have the potential to further describe tumour biology and clinical behaviour. Independent of the available state-of-the-art assays.

Management structure and procedures

The Project Coordinator ensured the smooth operation of the project and guaranteed that all efforts were focused towards the objectives. He submitted all required progress reports, deliverables, financial statements to the European Commission, and, with the assistance of GABO:mi/ARTTIC . He was responsible for the proper use of funds and their transfers to participants. The ENS@T-Cancer office was established by and based at the coordinator in Munich and at GABO:mi/ARTTIC in Munich. The Project Office at the Coordinator was concerned with the scientific management and the co-ordination of all research activities. The Project Office at GABO:mi/ARTTIC was responsible for administrative, financial and contractual management and the organisational co-ordination of the project activities.

The General Assembly (GA) was composed of all participants each of whom had one representative with the authority to vote. All other non-voting researchers working for this project joined the meetings and discussions. The GA met at least once per year during the funding period and the main tasks were to grant proper implementation of the participant's rights and obligations always in accordance with the contractual framework of the project and the Consortium Agreement. To facilitate the organisation and management, the scientific programme of the project was structured in work packages (WP) which together comprised the project. Each work package has been headed and coordinated by an experienced principal investigator as work package lead and a deputy leader. They were responsible for the management of their WPs. The Steering Committee (STC) was chaired by the Coordinator and comprised of all work package leaders, supported by the representative of the Project Management Office at GABO:mi/ART, the Training Panel and the Scientific Advisory Board. The STC was in charge of monitoring all activities towards the objective of the project in order to deliver as promised, in due time and in the budget and met every six months during the funding period. The Scientific Advisory Board was implemented to ensure a high standard of research and monitor the progress of the project by participating as evaluators at the GA meetings.

Objectives of ENS@T CANCER:

Objective 1: Structuring European clinical and translational research through implementation of a virtual research environment (VRE)

An important limitation to structured and formalized clinical research between European centres had been the lack of a common IT platform that allows access, linkage and analysis of clinical and genetic datasets held in respective participant centres. This limitation was now addressed through implementation of a state-of-the-art virtual research environment (VRE, **WP01**) that allows integration and linkage of clinical and biomedical data sets comprising the ENS@T network of biobanks and data that are generated by the various partner platforms (**WP02-06**).

Objective 2: Improving clinical outcome of patients with adrenal cancer by conducting interventional trials carried out by European centres of excellence

For both tumour entities addressed by this consortium (adrenocortical carcinoma, ACC and malignant pheochromocytoma, MPH) an interventional trial was supported. These clinical studies were used as important anchor points that feed in material and clinical information for translational (**WP03+04**) and basic (**WP02**) research projects and provided the clinical setting for implementation of novel imaging strategies (**WP06**) and identification of hormone biomarkers (**WP05**). Conversely, registries and biobanks were utilized to screen for suitable patients with well-defined and deep clinical annotation (**WP01**).

Objective 3: Improvement of differential diagnosis and risk stratification of adrenal cancer

An increasing number of adrenal masses are detected incidentally during imaging (“adrenal incidentalomas”), but the assessment of the malignant potential of these tumours by imaging procedures is difficult. Furthermore, reliable and sensitive screening tools for early detection and risk stratification of adrenal cancers were lacking, which makes the development of such tools a strategic priority.

Progress in the understanding of molecular mechanisms involved in adrenal tumourigenesis together with insights from recent clinical studies provided the opportunity to improve pathological (WP03), hormonal (WP05) and imaging techniques (WP06) to achieve superior accuracy in initial differential diagnosis and risk stratification of adrenal masses. Newly applied genomic techniques further refined the set of markers that identify subgroups of tumours with defined biological behaviour (WP04). All of these data sets and associated expertise were supported through a targeted VRE providing a unique platform for joint analysis and evaluation of the generated data sets (WP01). Importantly, standardized examination of patients enrolled in the proposed intervention trials (WP07) allowed prospective evaluation of the performance of the applied tests.

Objective 4: Identification and validation of tools for follow-up of patients with adrenal cancer

A significant problem in the follow-up of patients with ACC and MPH is the timely detection of persistent or recurrent disease following an apparently complete surgical resection. Delay in detection can often translate into postponed initiation of treatment and, thus, worsening of prognostic outlook. Biomarkers for detection of persistent or recurrent disease were identified on the basis of hormone metabolomics (WP05) and functional imaging (WP06) obtained during longitudinal follow-up within the implemented clinical trials (WP07), again drawing from the linking of systematic information provided by the VRE (WP01).

Objective 5: Identification of novel biomarkers for treatment response

Similar to the assessment of prognostic risk markers, we screened for biomarkers that would predict the response to a specific treatment. The initiated clinical trials (WP07) were utilized to categorize subgroups of patients with pre-defined treatment responses. These were compared with respect to their transcriptomic (WP04) and metabolomic (WP05) profiles to identify biomarkers predicting the clinical behaviour and the response of individual tumours to specific therapeutic approaches. Candidate markers were further evaluated retrospectively on archived tissue array material (WP01+03) and prospectively using *in vivo* models (WP02). Furthermore, functional imaging techniques were utilized to predict the response to pharmacological intervention (WP06).

Objective 6: Screening for molecular mechanisms as the basis to improve treatment response

Responses to established treatments for both ACC and MPH had been far from being satisfactory. In both instances, understanding of molecular mechanisms relevant for treatment failure and identification of pathways that overcome these limitations is needed (WP02). To achieve this goal, screening strategies in suitable *in vitro* and *in vivo* models were applied on biomaterial available through virtual linkage (WP01) of the biobanks of ENS@T-CANCER participants and supplemented with material collected during the proposed interventional trials (WP07).

1.3 Description of the main S&T results/foregrounds of ENS@T-CANCER

Following the working program of the consortium significant progress in the field of adrenal research has been achieved. This is highlighted by the detailed progress reports of the work packages, which are summarized below.

WP01: ENS@T-CANCER Virtual Research Environment

Establishment and refinement of a virtual research environment (VRE) has been a major achievement that has welched together the consortium and has provided a common hub for scientific projects and clinical studies. An ever-growing number of patients has been collected with rich clinical annotations and wealth of biomaterial in the common registries and associated decentralized biobanks. The identification of patients with specific clinical characteristics

has aided in recruitment of candidates for the clinical interventional trials. Similarly, the biomaterials and associated clinical data have been the basis of biomarker studies from the different work packages. A number of new functionalities have been implemented that include: 1) New search function with advanced notions of timeline representation, 2) Interactive completeness graphs to monitor and improve data quality, 3) Connection with American, Asian, Australian Adrenal Alliance (A5) registry, 4) Imaging support with advanced features of high-resolution detail when sharing images over low-latency networks between continents, 5) Robust mobile applications integrated with online visualisations to support clinical studies (NAPACApp) and biomaterial inventory management (BioApp), that have full support of IOS and Android mobile devices.

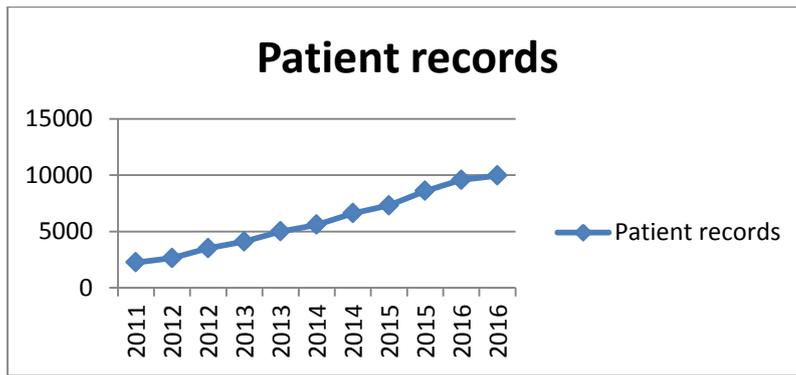
Usage – Figure 1 outlines the current clinical data held within the registry (13th July 2016). As noted last reporting period, for a rare condition such as adrenal cancer, these numbers are impressive and continue to grow. Table 1 shows number of records, user accounts and active users. Figure 2 shows the current “league tables” of contributing centres.

	ACC	Pheo	NAPACA	APA	Total
Records	2530	2704	2927	1798	9962
Biosamples	5085	1973	7409	5669	20136
Clinical Annotations	28914	14664	8295	5460	57333
Annotations Per Patient (Mean)	11.42	5.42	2.83	3.03	5.75
Biosamples Per Patient (Mean)	2.00	0.72	2.53	3.15	2.02
Active Centers	54	60	35	23	91

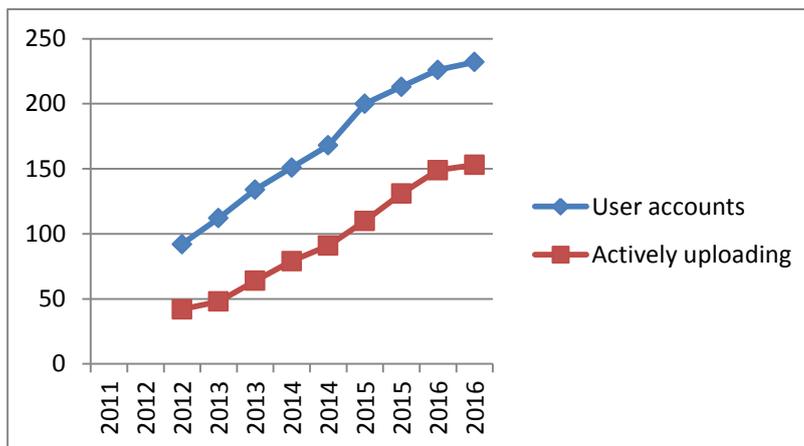
- Figure 1: Summary of current clinical data held in the registry (as of 13th July 2016)

ACC			Pheo			NAPACA			APA			Total		
#	Center	Patients	#	Center	Patients	#	Center	Patients	#	Center	Patients	#	Center	Patients
1	GYWU	1039	1	FRPA1	364	1	CRZA	336	1	FRPA1	554	1	GYWU	1546
2	FRPA3	275	2	ITFL	232	2	GYWU	267	2	GYMU	539	2	GYMU	1053
3	ITTU	216	3	GYMU	221	3	GRAT	251	3	GYBN	161	3	FRPA1	918
4	FRPA2	165	4	GBLB	220	4	ITPD	235	4	GYWU	126	4	GYBN	419
5	ITBR2	70	5	NLNI	218	5	GBBI	234	5	NLNI	81	5	CRZA	395
6	GYMU	68	6	SPMA	165	6	PLWW2	233	6	GYDF	81	6	FRPA3	388
7	GBBI	63	7	GBLG	129	7	GYMU	225	7	ITTU3	64	7	ITPD	351
8	NLEI	63	8	GYWU	114	8	GYBN	180	8	ITPD	62	8	ITTU	340
9	ITPD	53	9	FRPA3	113	9	USMN	178	9	GBLB	52	9	GBBI	335
10	ITFL	47	10	USMN	107	10	BGSO	178	10	NYBE	20	10	ITFL	312

- Figure 2: Rankings of record contribution from particular centres (identified by code) per tumour type in the database



▪ **Figure 3: Growth of patient record numbers from November 2011 to July 2016**



▪ **Figure 4: Number of user accounts versus those actively uploading from November 2012 to July 2016**

Figures 3 and 4 show the number of patient records and user accounts from November 2011 and November 2012 respectively (dates chosen as this was when active tracking of the metrics began). Growth of patient records has yet to (definitively) plateau, as is expected in any population metric, suggesting that the full potential of the registry has yet to be reached. Figure 4 shows one line of registered user accounts (individuals provided with login details) and another line of those actively using the registry (defined as uploading more than one record since receiving their login information). The goal has been to collapse the difference between the two lines by rigorously enforcing user account validity (through direct connection with subscription information in the ENSAT network database), but the opposite has appeared to happen (in Q1 2015). This is still a positive outcome and suggests increased awareness initiatives have been successful, along with a “legitimising” effect by tying the access to a paid subscription (the perceived value of owning a registry account has apparently increased).

Supporting development infrastructure and technical administration have been documented in the last reporting period. These are still regularly updated and used and do not require explanation in this document, again showing the stable nature of the development process of this project.

Clinical trial support – study and trial support continues as before, with submission of project descriptions leading to identification of the study in “Associated Studies” section. The NAPACApp study makes use of mobile technology, with a daily capture of patient symptoms that is then communicated through secure means to the relevant patient forms in the online registry. This information is displayed and visualised to specific pages available to the clinician, allowing a trace of symptoms to accurately followed over a set period (compared to errors of recall that would occur when a patient is asked “how they’ve felt over the last few months” at a single clinical appointment).

Bio-banking support – building upon the biomaterial management pages developed in the last reporting period, two versions (IOS and Android) of the BioApp have been developed and deployed to facilitate easy transfer of materials between participating centers. From the biomaterial management pages in a specific center, samples are identified

and compiled into a manifest, which can be exported as an Excel spreadsheet and printed so that it can be physically included in the sample transfer box. The printout includes a QR image, which encodes the ID of that particular manifest. Once the box is successfully received by the other center, the app can be used to scan that QR code, and the details of that manifest are shown within the app. The device holder can then confirm the receipt of all or some of the samples (“damaged” or “missing” can also be recorded) and automatically update the status of these samples. In this way the history of exchanged samples can be tracked across participating centers more accurately.

WP02: Translational Adrenal Cancer models

Within the translational studies, important progress could be achieved towards the elucidation of pathways involved in adrenal tumorigenesis. These studies were able to identify new targets (such as SF-1 or the WNT/b-catenin pathway) that could result in therapeutic strategies. In fact, some of the gathered insights translated in novel treatment modalities, which were tested in studies in dedicated preclinical models. The following report will highlight a few examples:

Induction of adrenal tumors through SF-1 overexpression

SF-1 (NR5A1) overexpression can induce adrenocortical tumour formation in transgenic mice (Figure 5) and is associated with more severe prognosis in patients with ACC. We have identified Vanin-1 (Vnn1), an SF-1 target gene, as a novel modulator of the tumorigenic effect of Sf-1 overexpression in the adrenal cortex. Vanin-1 is endowed with pantetheinase activity, releasing cysteamine in tissues and regulating cell response to oxidative stress by modulating the production of glutathione. Sf-1 transgenic mice developed adrenocortical neoplastic lesions (both dysplastic and nodular) with a frequency increasing with age. Genetic ablation of the Vnn1 gene in Sf-1 transgenic mice significantly reduced the severity of neoplastic lesions in the adrenal cortex. This effect could be reversed by treatment of Sf-1 transgenic / Vnn1 null mice with cysteamine. These data show that alteration of the mechanisms controlling intracellular redox and detoxification mechanisms is relevant to the pathogenesis of adrenocortical neoplasia induced by SF-1 overexpression.

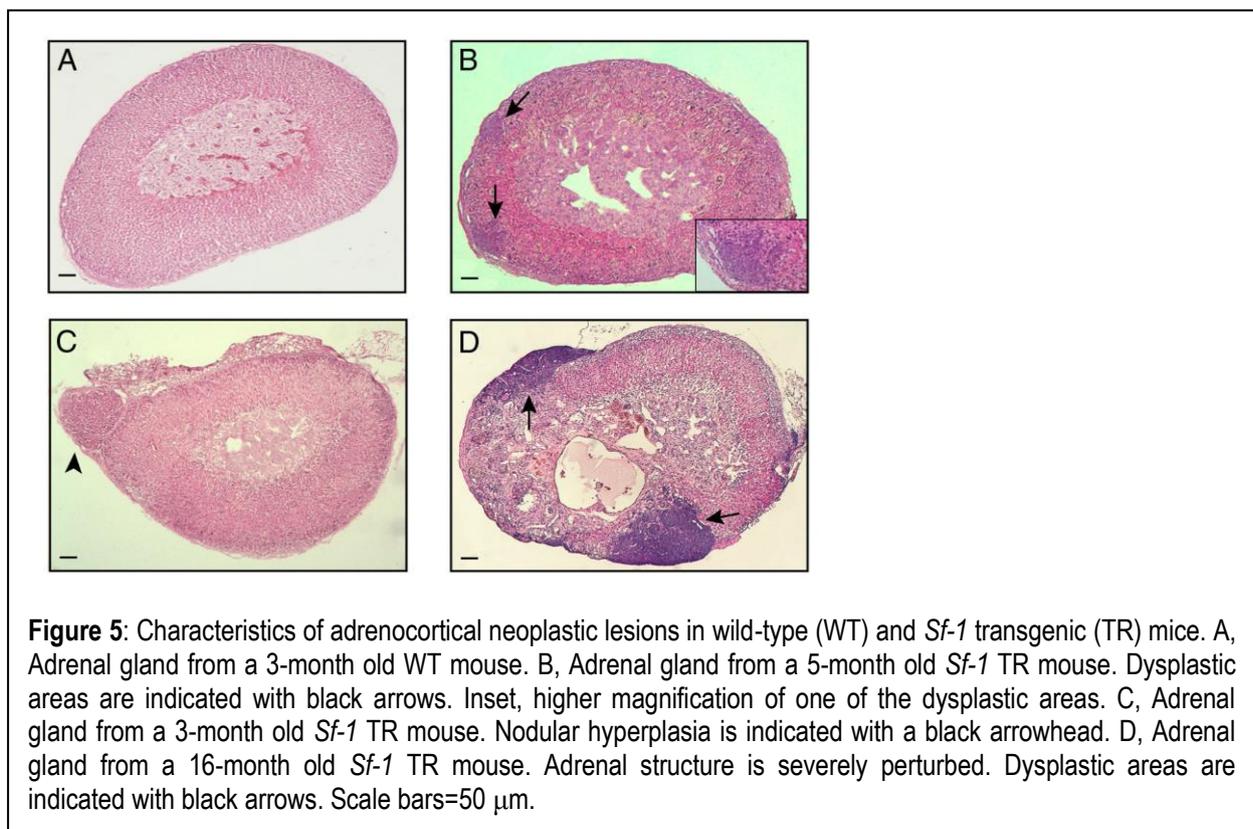
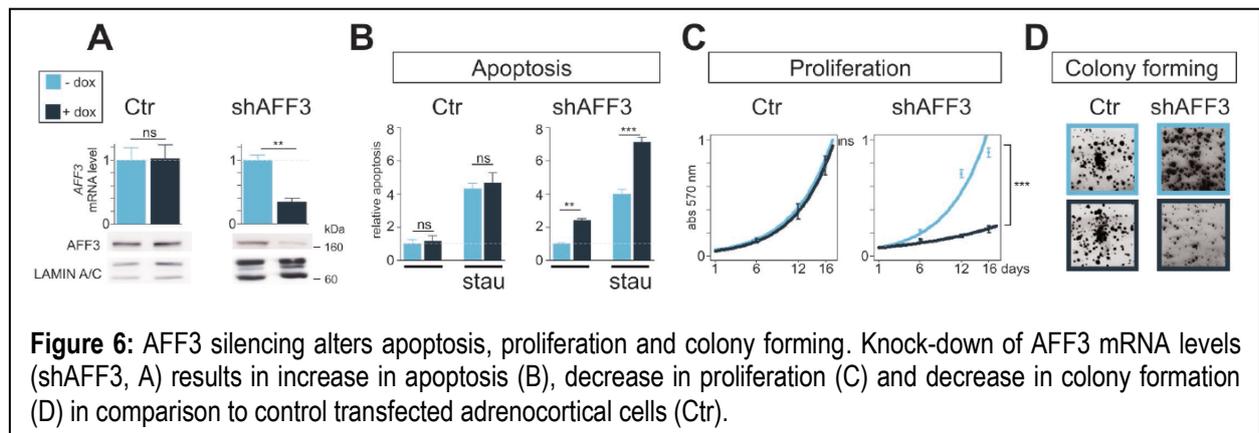


Figure 5: Characteristics of adrenocortical neoplastic lesions in wild-type (WT) and *Sf-1* transgenic (TR) mice. A, Adrenal gland from a 3-month old WT mouse. B, Adrenal gland from a 5-month old *Sf-1* TR mouse. Dysplastic areas are indicated with black arrows. Inset, higher magnification of one of the dysplastic areas. C, Adrenal gland from a 3-month old *Sf-1* TR mouse. Nodular hyperplasia is indicated with a black arrowhead. D, Adrenal gland from a 16-month old *Sf-1* TR mouse. Adrenal structure is severely perturbed. Dysplastic areas are indicated with black arrows. Scale bars=50 μ m.

AFF3 as a mediator of oncogenic effects of β -catenin in adrenocortical carcinoma

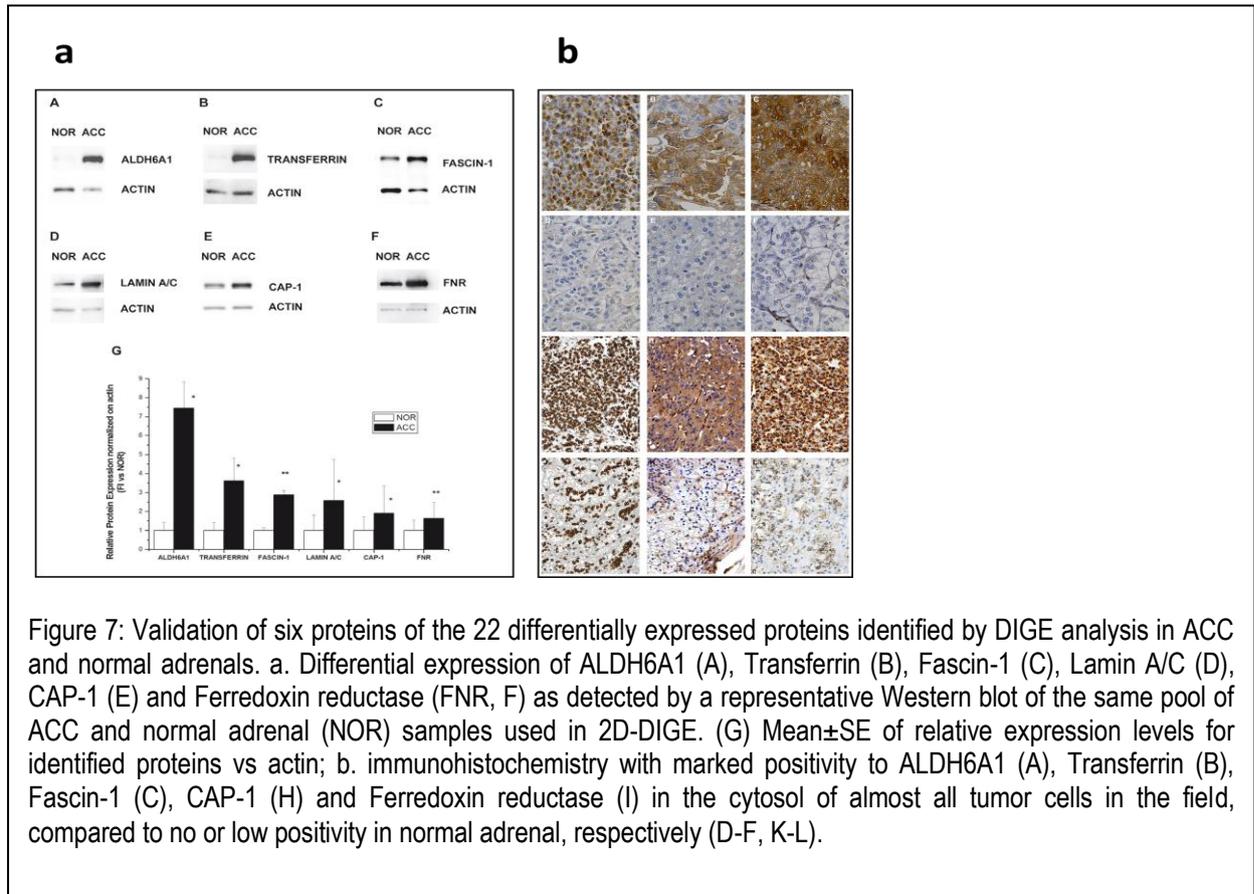
Genomics studies have demonstrated that the most frequent alterations of driver genes in ACC activate the Wnt/ β -catenin signalling pathway. However, the adrenal specific targets of oncogenic β -catenin mediating tumorigenesis had not been established. A combined transcriptomic analysis from two series of human tumours and the human ACC cell line H295R harbouring a spontaneous β -catenin activating mutation identified Wnt/ β -catenin targets with seven genes consistently found in the three studies. Among these genes, AFF3 was found to mediate the oncogenic effects of β -catenin in ACC. Accordingly, AFF3 silencing decreased cell proliferation and increased apoptosis in the ACC cell line H295R (Figure 6). AFF3 was found to be located in nuclear speckles, which play an important role in RNA splicing. Along the same line, AFF3 overexpression in adrenocortical cells interfered with the organization and/or biogenesis of these nuclear speckles and altered the distribution of CDK9 and Cyclin T1, such that they accumulate at the sites of AFF3/speckles. In summary, herewith we demonstrate that AFF3 is a new target of Wnt/ β -catenin pathway involved in ACC, acting on transcription and RNA splicing.



Bruno Ragazzon, Lucile Lefèvre, Hanin Omeiri, Ludivine Drougat, Constanze Hantel, Matthieu Giraud, Pierre Val, Stéphanie Rodriguez, Karine Perlemonne, Corinne Blugeon, Felix Beuschlein, Aurélien de Reyniès, Marthe Rizk-Rabin, and Jérôme Bertherat. Combined transcriptome analyses identified AFF3 as a mediator of oncogenic effects of β -catenin in adrenocortical carcinoma. *Oncogenesis*. 2015.

Proteomic analysis of adrenal cancer samples

This study reports the first proteomic analysis of ACC by using two-dimensional-differential-in-gel-electrophoresis (2D-DIGE) to evaluate a differential protein expression profile between ACC and normal adrenal. Mass spectrometry, associated with 2D-DIGE analysis of ACC and normal adrenals, identified 22 proteins in 27 differentially expressed 2D spots, mostly overexpressed in ACC. Gene ontology analysis revealed that most of the proteins concurs towards a metabolic shift, called the Warburg effect, in ACC. The differential expression was validated by Western blot and immunohistochemistry for Aldehyde-dehydrogenase-6-A1, Transferrin, Fascin-1, Lamin A/C, Adenylate-cyclase-associated-protein-1 and Ferredoxin-reductase (Figure 7). These findings reveal a different proteomic profile in ACC compared with normal adrenal cortex, which may represent promising novel ACC biomarkers and potential therapeutic targets if validated in larger cohorts of patients.



Poli G, Ceni E, Armignacco R, Ercolino T, Canu L, Baroni G, Nesi G, Galli A, Mannelli M, Luconi M. 2D-DIGE proteomic analysis identifies new potential therapeutic targets for adrenocortical carcinoma. *Oncotarget*. 2015 Mar 20;6(8):5695-706.

Establishment of a patient derived ACC tumour model

During the funding period our workgroup established, characterized and implemented the first tumour model for ACC providing both a human cell line and tissue-based tumour model. In an attempt to overcome the lack and insufficiency of preclinical endocrine tumour models, we aimed in a first step at the general development of PDTX-models for ACC. Overall, implanted and subsequently analysed pieces of endocrine tumors remained vital and proliferating and retained specific patient characteristics in the murine host. However, in many instances these tumors showed no relevant increase in tumour size, limiting the applicability of PDTXs for preclinical therapeutic trials. In contrast, during these studies one xenograft (MUC-1), derived from a neck metastasis of an ACC, showed extraordinary engraftment properties and sustained tumor growth over several passages in the murine host (Figure 8 A-H).

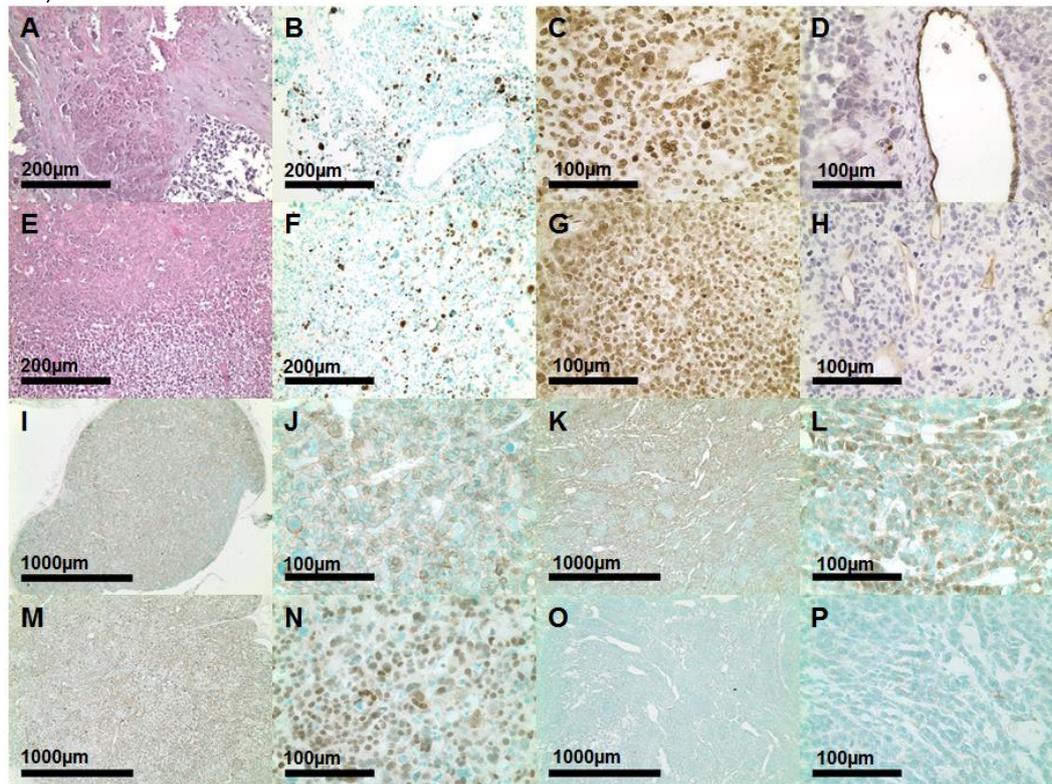


Figure 8: H&E, Ki67, SF-1 and CD-31 tumour analysis from the original patient tumour (A-D) and of MUC-1 xenograft (E-H) derived tumour slides from passage 2. Immunohistochemical β -catenin (I-L) and p53 stainings (M-P) of MUC-1 (I, J, M, N) and NCI-H295R (K, L, O, P) tumour slides.

WP03: Adrenal cancer pathology platform

Researchers forming the pathology platform made particular progress in the standardization of immunohistochemical techniques and interpretation of morphological tumour characteristics (including proliferation marker, extent of vascularization and content of cells with stem cell characteristics) required for comparison of adrenal cancer diagnosis and comparability of biomarker studies in the field across European countries.

Establishment of a tissue micro-array of adrenomedullary and adrenocortical tumours to facilitate high-throughput validation of newly identified prognostic markers

Tissue micro-arrays (TMAs) had been prepared before from series of adrenocortical carcinomas (ACC), and from pheochromocytomas (PCC) and paragangliomas (PGL). While the creation of the TMA is the task of partner 5 (Erasmus MC), this is truly a collaborative effort of many of the ENSAT partners. Contacts have been established with Munich (partner 1), Paris (partners 3 and 4), Florence (partner 6), Madrid (partner 7), Dresden (partner 9), Wurzburg (partner 10), Nijmegen (partner 11), Turin (partner 12), and Padova (partner 14), for the procurement of retrospectively sampled specimens of pheochromocytomas (PCC), paragangliomas (PGL) and adrenocortical carcinomas (ACC).

Implementation of SDHB immunohistochemistry to guide genetic analysis of pheochromocytomas

The conclusion of this work is that SDHB/SDHA immunohistochemistry is a reliable tool to identify patients with SDHx mutations with an additional value in the assessment of genetic variants of unknown significance. If SDH molecular genetic analysis fails to detect a mutation in SDHB-immunonegative tumor, SDHC promoter methylation and/or VHL/NF1 testing with the use of targeted next-generation sequencing is advisable. This work has been described in a scientific publication (Papathomas T et al, Mod Pathol 2015).

Establishment of vascular pattern analysis for the prediction of clinical behaviour in pheochromocytomas

A series of 200 cases from participants 3 and 5 has been selected for this study and stained with conventional histochemistry (Elastic von Gieson stain) or with CD34, to highlight the vascular structures in relation to the tumour cells. The slides have been checked for their tumour content and subsequently scanned by the slide scanner of the virtual microscope. These slides have been scored by a panel of 6 pathologists. There was significant agreement between the 6 observers (mean $\kappa = 0.796$). Mean sensitivity of vascular pattern analysis was higher in tumours >5 cm (63.2%) and in genotype cluster 2 tumours (100%). In conclusion, vascular pattern analysis cannot be used in a stand-alone manner as a prognostic tool for the distinction between benign and malignant PCC, but could be used as an indicator of malignancy and might be a useful tool in combination with other morphological characteristics. This work has been described in a scientific publication (Oudijk L et al., Plos One 2015).

Improvement of current classification systems for the prediction of clinical behaviour in adrenocortical tumours

Following an expert meeting, it was felt that Ki67 labelling index was and is the most interesting marker for risk stratification and for the distinction of clinical behaviour in adrenocortical tumours. This has been the focus of two studies within the consortium. Both these studies involved multiple participants of the consortium as is reflected by the authors list of both scientific publications that have resulted. The study by Beuschlein F. et al (J Clin Endocrinol Metab 2015) had provided important information on the prognostic value of Ki67 labelling in patients following complete ACC resection. The other study has focussed on the technical aspects of Ki67 immunohistochemistry and on multi-observer variation in relation to counting techniques used, all against the background of meaningful stratification of patient subgroups. This has been reported in two scientific publications (Lu H et al., Diagn Pathol 2014; Papathomas T et al., Am J Surg Pathol 2016).

Validation of previously established diagnostic and prognostic markers for risk stratification of adrenocortical tumours

Based on earlier studies it was concluded that current practices in Ki67 scoring assessment vary greatly, and inter-observer variation sets particular limitations to its clinical utility, especially around clinically relevant cutoff values. Novel digital microscopy-enabled methods could provide critical aid in reducing variation, increasing reproducibility, and improving reliability in the clinical setting. Furthermore, another study has been performed with regard to the molecular background of a subgroup of ACC, so-called sarcomatoid ACC, which have a very unfavorable course. The results from this study have been prepared in a scientific publication, which is now under peer-review at Human Pathology.

Establishment of novel immunohistochemical markers derived from genomics approaches to predict clinical behaviour of adrenocortical and adrenomedullary

This task has been accomplished as a collaborative effort between several partners of the ENS@T consortium, including partners 1, 5 and 7. This is exemplified by several studies, 2 of which have already resulted in a scientific publication (Leinhauser I et al, Oncotarget 2015; Oudijk L et al, Eur J Endocrinol 2015). Another study is in preparation and an abstract has been published (Evenepoel L et al., J Hypertens 2015). The study by Leinhauser will be reported elsewhere. The one by Oudijk showed that immunohistochemical expression of stem cell markers was found in a subset of PCCs/PGLs. Further studies are required to validate whether some stem cell-associated markers, such as SOX2, could serve as targets for therapeutic approaches and whether NGFR expression could be utilized as a predictor of malignancy. In the study by Evenepoel significant overexpression of Contactin 4 was shown in malignant compared to benign tumours, and may therefore contribute to distinguish malignant from benign PPGL. This is currently the subject of further collaborative analysis with participants 5 and 7, including functional analysis of this gene and its protein product.

WP04: Adrenal Cancer genomics platform

For adrenocortical carcinoma, the genomic platform had achieved a major break-through with the first description of an integrative genomic approach. Since the molecular classification was achieved earlier than expected with the success of the integrated genomics of ACC it had been possible to develop molecular markers based on the analysis of methylation alterations in ACC during the last part of the program and to confirm the molecular classification in an independent international cohort. Likewise, epigenetic markers for the prediction of malignancy for pheochromocytomas could be identified which might aid in the differential diagnosis of this rare disease.

Integration of results obtained from the different OMIC platforms in adrenocortical tumours.

A series of 45 ACC were extensively investigated by various omics approaches: exome, snp arrays, methylation, transcriptome and miRNome. A second set of 77 ACC were then investigated by snp array and targeted next generation sequencing. Exome sequencing and SNP array analysis revealed recurrent alterations in known drivers (CTNNB1, TP53, CDKN2A, RB1, MEN1) and genes not previously reported to be altered in ACC (ZNR3, DAXX, TERT and MED12). This was validated in second cohort of 77 ACC. The cell-surface transmembrane E3 ubiquitin ligase ZNR3 was the gene the most frequently altered (21%), and appears as a novel tumor suppressor gene related to the β -catenin pathway. SNP array clearly shows major chromosomal alterations in ACC. The study of the mapping of homozygous deletion was important to identify new tumor suppressor gene as ZNR3, as shown below

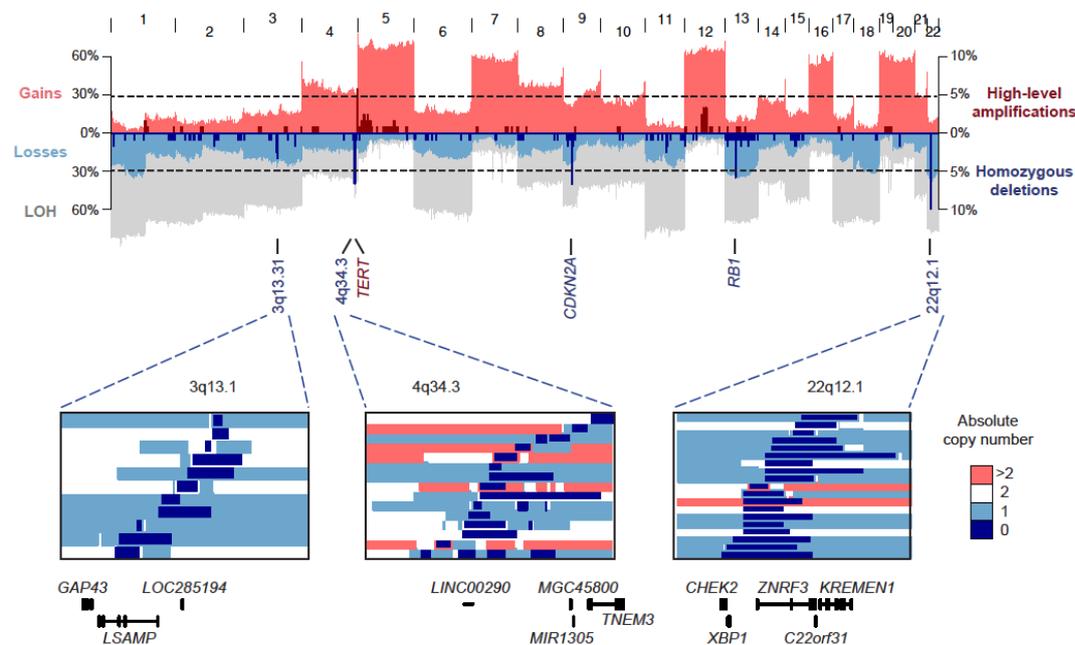


Figure 8: Mapping of the chromosomal alterations by SNP array study of ACC.

Our integrated genomic analyses led to the identification of two distinct molecular subgroups with opposite outcome. The C1A group of poor outcome ACC was characterized by numerous mutations and DNA methylation alterations, whereas the C1B group with good prognosis displayed a specific deregulation of two miRNA clusters. Thus, aggressive and indolent ACC correspond to two distinct molecular entities, driven by different oncogenic alterations. These results clearly point to a new molecular classification of ACC, (Figure 9).

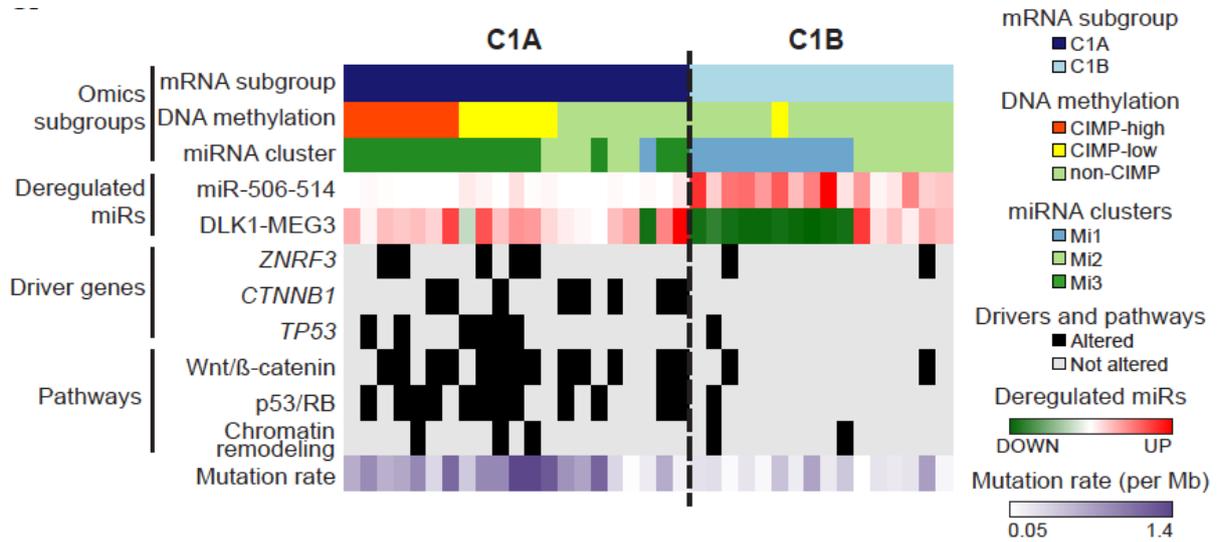


Figure 9: Molecular classification of ACC based on integrated genomics.

This classification perfectly correlates with overall survival, as shown in Figure 10. From these results, we are developing a molecular prognosis tool for ACC based on SNP array and targeted next generation sequencing of the drivers genes identified in this study.

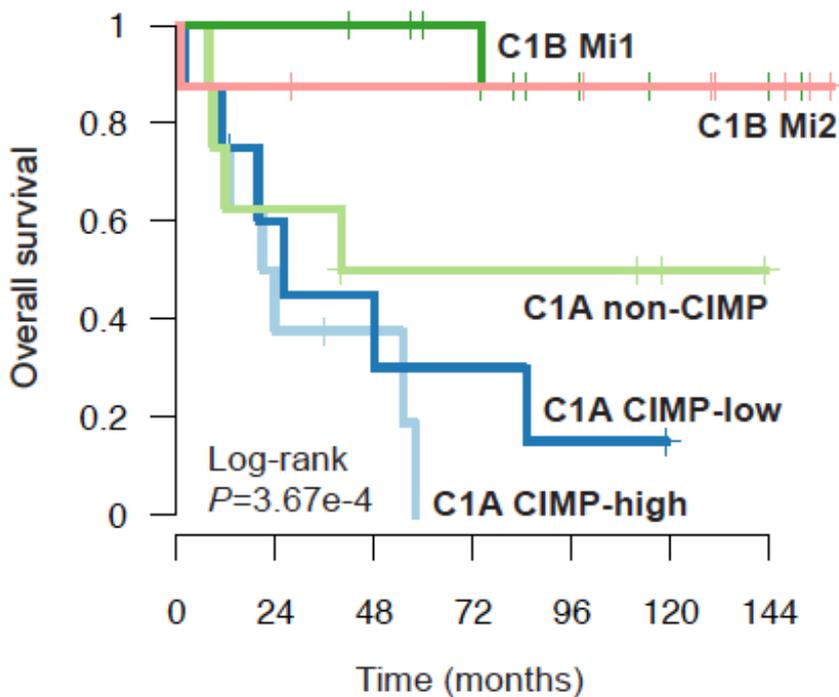


Figure 10: Overall survival of ACC according to the molecular classification based on integrated genomics.

WP05: Hormone biomarker platform

Prospective validation of steroid and catecholamine metabolite profiling for prediction and diagnosis of malignancy in patients with adrenocortical tumours and pheochromocytomas

The differential diagnosis of adrenocortical tumours is often very difficult and in particular the early detection of adrenocortical carcinoma and its differentiation from harmless benign adrenal nodules pose specific challenges. Survival of patients with adrenocortical carcinoma is enhanced by early diagnosis, therefore the sensitive detection of adrenocortical malignancy in small adrenal tumours is very desirable. Similarly, many larger adrenal tumours (>4cm) undergo surgical removal but in at least half of them histology demonstrates a benign cause, exposing a large number of these patients to unnecessary surgery and associated morbidity and mortality.

Imaging is routinely used for the differential diagnosis of adrenal masses, employing multiple modalities including computed tomography, magnetic resonance imaging and positron emission tomography. These examinations are very costly and, whilst relatively sensitive, lack clinically sufficient specificity, resulting a large number of false positives, as recently examined and reported by us in a systematic review and meta-analysis (Dinnes J. et al., *Eur J Endocrinol* 2016).

To address this problem, we have embarked on the development of a novel, sensitive and specific biochemical test for the diagnosis of adrenocortical malignancy.

We have developed a urinary multi-steroid profiling method that allows for concurrent identification and quantification of 32 distinct steroid metabolites by gas chromatography/mass spectrometry (GC-MS). We used this approach to analyse 24-h urines from 102 patients with confirmed benign adrenocortical adenomas and 44 patients with adrenocortical carcinoma. Using machine learning approaches for data analysis, we identified abundant hypersecretion of steroid precursor metabolites rather than end products of adrenal steroidogenesis in the urine of adrenocortical carcinoma patients, in keeping with our hypothesis that a dedifferentiated carcinoma would only be capable of immature steroidogenesis, restricted to the early elements of the steroid pathways. Based on this result, we developed a machine learning based algorithm, specifically employing generalized matrix learning vector quantisation (GMLVQ), and we found that this method allows us to diagnose adrenocortical carcinoma in a 24-h urine of an affected patient with 90% sensitivity and specificity. We published this proof-of-concept study in 2011 (Art W. et al., *J Clin Endocrinol Metab* 2011), and also patented the diagnostic approach, the combination of GC-MS urinary steroid profiling and machine-learning based data analysis.

We subsequently used a similar approach analysing the impact of mitotane, a drug used for adjuvant therapy and treatment of advanced disease in adrenocortical carcinoma. We measured steroid metabolite profiles in urines from patients with and without metastatic ACC and patients with and without mitotane treatment and analysed the data by both statistical and machine learning approaches. The results revealed that mitotane strongly induces the drug-metabolizing enzyme CYP3A4, resulting in rapid inactivation of cortisol to 6beta-hydroxycortisol, which explains why mitotane-treated patients need to increase their hydrocortisone dose by at least 50% as compared to other patients with adrenal insufficiency. We could also show that mitotane strongly decreases 5alpha-reductase activity, which activates testosterone to the more powerful 5alpha-dihydrotestosterone. This explains why male patients with mitotane-induced hypogonadism do not show clinical improvement despite testosterone replacement and treatment with dihydrotestosterone gel might be more helpful, which is currently being explored. We also could show that mitotane treatment does not impact of those steroid metabolites that we use for diagnosing ACC, which suggested that our method could also be used to monitor ACC patients after complete, R0 removal of their primary tumour for the detection of disease recurrence (Chortis V et al., *J Clin Endocrinol Metab* 2013).

Following on from these discoveries, we have carried out an international, multi-centre test validation study to demonstrate that our test performs equally well when used prospectively in a large consecutively recruited cohort that was collected with the smallest amount of bias possible. This study for Evaluation of Steroid Metabolomics in the differential diagnosis of AdrenoCortical Tumours (EURINE-ACT) was carried out with support from ENSAT-CANCER and participation of multiple clinical ENSAT centres from across Europe and also including two American centres. Power calculations prior to study initiation had suggested that we would need to include at least 2000 patients with adrenal tumours including 5% adrenocortical carcinoma. We have recently completed recruitment (30 June 2016) and have achieved the recruitment of 2190 patients including 7% adrenocortical carcinoma.

In parallel to patient recruitment, we have worked on transferring our method from the relative time-consuming and therefore costly GC-MS platform to the high-throughput liquid chromatography-tandem mass spectrometry (LC-MS/MS) platform. We have now successfully developed and validated an LC-MS/MS method that can identify and

quantify 16 steroids of our 32 steroid profiles including seven of the nine most informative steroids differentiating benign from malignant adrenocortical tumours. We have demonstrated in comparison between GC-MS and LC-MS/MS, utilizing 24-h urines from 39 ACC patients and 99 benign adrenocortical adenoma patients, that both methods have comparable performance. These results are currently being prepared for publication (Taylor AE, Bancos I et al.).

As LC-MS/MS is equivalent to GC-MS performance, we have decided to use LC-MS/MS for the analysis of the prospective EURINE-ACT samples, which is currently under way, with 1600 of 2200 samples already measured. We anticipate completion of the biochemical data analysis by end of September 2016 and completion of statistical and computational analysis by the end of December 2016, followed by finalization and submission of the manuscript (Bancos I, Taylor AE et al.). We will also be able to provide unique insights into the epidemiology of the largest ever prospectively recruited adrenal tumour cohort and following the publication of the assay results we will be able to develop diagnostic models, combining clinical phenotype characteristics (e.g. sex, age, tumour size) together with the biochemical urine assay for developing even higher performing diagnostic assays.

Furthermore, embedded into the EURINE-ACT study, we performed a study in adrenocortical carcinoma patients with histologically confirmed complete surgical removal of their primary tumour. In this so-called R0 ACC study, we have prospectively included 162 patients with adrenocortical carcinoma and followed them with 3- to 6-monthly 24-h urine collections. We have established that our GC-MS assay can sensitively detect disease recurrence. These results are currently being prepared for publication (Chortis V et al.) and we are working on the design of a prospective study that allows to test this approach in a systematic fashion, with monthly collection of overnight urine for realtime biochemical analysis, alongside routine 3-monthly imaging. This study, ACC PROTECT, has been endorsed by the ENSAT Network, and we are currently seeking funding for its initiation.

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Metabolomic analysis of novel pathways involved in the development of malignant adrenal tumours

Pheochromocytomas and paragangliomas (PPGLs) have a rich genetic background and derive in over 35% of cases from mutations in at least 15 identified tumor susceptibility genes, with more still to be discovered. Somatic mutations of many of these and other genes have also been identified in tumor specimens, indicating roles of the same downstream pathways in sporadic forms of the tumors. One of the most important pathways contributing to PPGL tumorigenesis involves a common feature of mutations in genes that lead to stabilization of hypoxia-inducible factors (HIFs) via reduced proteosomal degradation. The result is activation of hypoxia-angiogenic pathways that contribute to growth and proliferation of tumor cells. Among tumor susceptibility genes involved in activation of this pathway are those encoding enzymes involved in energy metabolism. Mutated genes include those for 4 subunits of succinate dehydrogenase (SDHA, SDHB, SDHC and SDHD), an associated assembly protein (SDHAF2), fumarate hydratase and malate dehydrogenase. Mutations lead to change or loss of function of encoded enzymes, accompanied by alterations in specific energy pathway metabolites that then result in inhibition of alpha-ketoglutarate dependent enzymes. These enzymes include prolyl hydroxylases (PHDs), which hydroxylate proline residues in HIFs to promote proteosomal degradation. High risk of metastatic disease in patients with PPGLs due to mutations of the aforementioned genes appears due combined inhibition of PHDs and other alpha-ketoglutarate-dependent enzymes involved in epigenetic regulation of gene expression.

We first developed an LC-MS/MS method for quantitative profiling of energy pathway metabolites in tumor specimens. Metabolites in the panel include the established oncometabolites, succinate, fumarate and 2-hydroxyglutarate. The panel also includes other metabolites of the Krebs' cycle (malate, citrate, cis-aconitate, isocitrate, alpha-ketoglutarate) as well as lactate, pyruvate and several other energy pathway metabolites.

One of the first projects utilizing the LC-MS/MS method involved characterization of energy metabolite levels of

PPGL tumor specimens, with an underlying hypothesis that differences in metabolite levels might not only be useful for functional identification of underlying mutations, but also for stratification of disease according to development of malignancy. In a study involving ENSAT-CANCER partners at Dresden, Madrid, Munich, Florence and Nijmegen we showed that tumors with mutations of SDHB, SDHD and SDHC mutations had 25-fold higher succinate levels and 80% lower fumarate levels than other PPGLs [1]. The succinate:fumarate ratio was identified as an extremely accurate functional biomarker to indicate presence of mutations of SDH subunits. Moreover, patients with SDHB mutations or malignant tumors had higher succinate:fumarate ratios than patients with SDHD and SDHC mutations or those with tumors and no metastatic involvement. This not only was consistent with the higher metastatic risk associated with SDHB than SDHC/D mutations, but also indicated that the extent of functional impairment was related to that risk.

Although diagnostic accuracy of the test for identification of SDHB/C & D mutations was high there were nevertheless several false positives involving tumors showing high succinate:fumarate ratios, but no identifiable mutation. In follow-up of these cases, involving ENSAT-CANCER partners at Madrid and Florence, we identified hypermethylation of the SDHC promoter as responsible for one case involving two separate paragangliomas with high succinate:fumarate ratios and other evidence of severely reduced SDH activity, but no mutation [2]. This case, previously designated a false-positive, became a true-positive.

In related collaborative work, led by ENSAT-CANCER partners at Nijmegen, differences in energy pathway and other metabolites in tumor tissue were examined by targeted and untargeted metabolomics approaches to establish further genotype-related abnormalities in mitochondrial energy metabolism and related catecholamine pathways [5,6]. In another related collaborative project headed by ENSAT-CANCER partners at Madrid, the functional significance of newly identified mutations of malate dehydrogenase 2 was established through measured differences in malate relative to other metabolites in both tumor samples and cell lines transfected with the mutant gene [5].

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WP06: Functional Imaging Platform

Significant progress has been made in our studies related to molecular target expression studies and studies on the relation between functional imaging (18F-FDG PET and MIBG) and genotype-dependent tumour cell energy metabolism, which provide the basis for further biomarker/functional imaging studies. First experience from patients with malignant pheochromocytoma enrolled in the FIRST-MAPPP study indicates potential discrepancy between morphological and functional imaging characteristics following tumour treatment.

Establishment of functional Iodometomidate based adrenal imaging

Completion and full evaluation of the current results of Iodometomidate imaging was established based on the data from 51 patients with adrenal lesions and 60 patients with metastatic adrenocortical carcinoma that underwent

SPECT imaging with [¹²³I]Iodometomidate. Patients received 175 ± 20 MBq ¹²³I-IMTO as an intravenous injection. Planar scans of the whole body were performed 5 min, 45 min, 90 min, 4 h, 6 h and 21-24 h post injection using a standard technique. Both qualitative and semiquantitative image analysis was performed. For semiquantitative analysis of the SPECT/CT data, up to 5 spherical volumes of interest (VOIs; \varnothing 2 cm) were assigned to the 5 most prominent tumor lesions visualized by SPECT/CT. One further VOI was placed to the liver adjacent to the right adrenal (box 2 x 3 x 3 cm). Mean voxel counts were calculated for the determination of ratios. Specific tracer uptake was observed in adrenocortical tumours starting 45 min after tracer injection with best target to background ratios after 24 hours (Examples are provided in Figure 12).

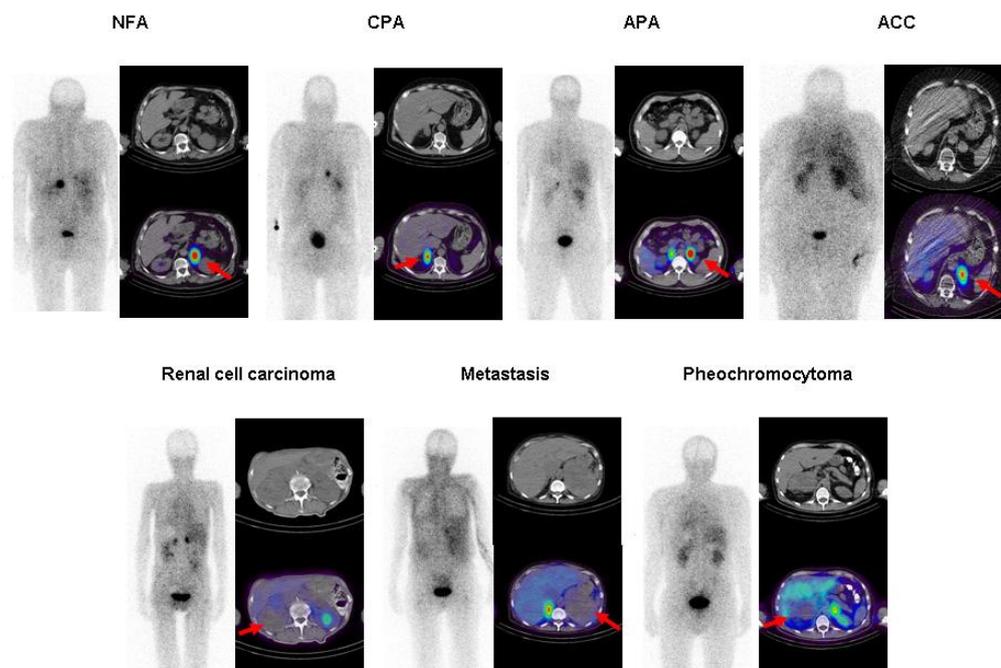


Figure 12: Representative imaging results with ¹²³I-IMTO for different adrenocortical and non-adrenocortical tumours. NFA=non-functioning adrenocortical adenoma, CPA=cortisol producing adenoma, APA=aldosterone producing adenoma, ACC=adrenocortical carcinoma. Left panels planar images 5h post injection, posterior view. Right panels CT (upper image) and SPECT/CT (lower image), transversal view. Tumour lesions are indicated by red arrow.

Qualitative visual inspection revealed high and specific tracer uptake in the tumours of all 16 patients with benign adrenocortical lesions and in 9 of 14 patients with adrenocortical carcinoma. 3 of 21 non adrenocortical lesions were characterized false positive (sensitivity 89%, specificity 86%). ROC analysis of tumour /liver ratios was performed. Primary objective was a cut off value with high specificity for correct characterization of a tumour lesion as being of adrenocortical origin. A tumour/liver ratio above 3.8 identifies lesions of adrenocortical origin with 100% specificity and 61% sensitivity.

Tumour cell energy metabolism

We investigated the effects of genetic alterations in pheochromocytomas and paragangliomas on metabolic networks in tumour cell by the application of proton nuclear magnetic resonance spectroscopy on ex-vivo tumour tissue. We have shown that this technique gives a comprehensive picture of alterations in energy metabolism in SDH- and VHL-related tumours and establishes the interrelationship of energy metabolism and amino acid and purine metabolism. In addition, untargeted metabolomics by proton nuclear magnetic resonance spectroscopy allows highly accurate fingerprinting of pheochromocytomas and paragangliomas of different genetic backgrounds. Rao et al. *J Clin Endocrinol Metab.* 2015;100(2):E214-22, see below.

In collaboration with the Dresden partners, we have shown that targeted metabolomic studies using LCMS for Krebs cycle metabolite enables identification and stratification of tumours caused by succinate dehydrogenase deficiency. Richter et al. *J Clin Endocrinol Metab.* 2014;99(10):3903-11, see below.

Functional characterization of tumours by 18F-FDG PET and 123I-Metaiodobenzylguanidine scintigraphy

We have gained insight in the correlation between glucose uptake and metabolism by tumor cells on one hand and in vivo 18F-FDG PET characteristics of pheochromocytoma and paraganglioma on the other. With immunohistochemical studies, we have shown that activation of aerobic glycolysis in SDHx-related compared to other tumors is associated with increased 18F-FDG accumulation due to accelerated glucose phosphorylation by hexokinases rather than increased expression of glucose transporters. Van Berkel et al. *J Nucl Med* 2014;55(8):1253-9, see below.

In contrast to 18F-FDG PET, we found that with 123I-MIBG scintigraphy such differentiation between genotypes is not possible, but that semi-quantitation of MIBG uptake allows better distinction between pathological uptake by pheochromocytoma and physiological uptake by normal adrenal glands. Van Berkel et al. *J Nucl Med.* 2015;56(6):839-46, see below.

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

Patients with pheochromocytomas and paragangliomas (PPGLs) may have brown adipose tissue (BAT) activation induced by catecholamine excess. 18F-fluorodeoxyglucose (18F-FDG) PET/CT can be used for the localization of both PGLs and BAT. It was unknown whether BAT is specifically affected by altered cellular energy metabolism in patients with SDHx and VHL-related PPGLs. In our study we determined endocrine and paracrine effects of catecholamine excess on BAT activation in patients with PPGLs as detected by 18F-FDG PET/CT, taking into account genetic variation. Patients with PGLs who were fully genetically characterized underwent pre-surgical 18F-FDG PET/CT imaging for tumor localization and to quantify BAT activation in the setting of a single Dutch tertiary referral centre. We investigated 73 patients, age 52.4 ± 15.4 yr, BMI 25.2 ± 4.1 kg/m², mean \pm SD, grouped into sporadic, cluster 1 (SDHx, VHL) and cluster 2 (RET, NF1, MAX) mutations. 18F-FDG mean standard uptake values (SUV_{mean}) were assessed in predefined BAT locations, including perirenal fat. The main results were as follows: 21/73 (28.8%) patients exhibited BAT activation. BAT activation was absent in all six patients with non-secreting PPGLs. No difference in 18F-FDG uptake by perirenal fat on the side of the pheochromocytoma and the contralateral side was observed (SUV_{mean} 0.80 vs. 0.78 respectively, $P=0.42$). The prevalence of BAT activation did not differ between sporadic (28.9%), cluster 1 (40.0%) and cluster 2 patients (15.4%), $P=0.36$. From these results we conclude that patients with PPGLs exhibit a high prevalence of BAT activation on 18F-FDG PET/CT. This is likely due to systemic catecholamine excess. Previous suggestions of 'browning' of peritumoral fat due to paracrine effects of catecholamines could not be confirmed. BAT activation is not associated with specific germline mutations. *Puar et al. J Clin Endocrinol Metab.* 2016;101(1):224-32.

18F-FDG PET kinetics

In a pilot study we have investigated the use of dynamic versus static 18F-FDG PET scanning in patients with pheochromocytoma and paraganglioma. Dynamic scanning allows the investigation of 18F-FDG kinetics using a 3 compartment model. We have shown that increased metabolic rate in SDHx-related tumours detected by dynamic PET facilitates the in vivo stratification of these tumors. Oral presentation at International Symposium on Pheochromocytoma, Kyoto, Japan, 20-9-2014.

Metabolic response to treatment

Our initial results regarding treatment response monitoring in our local participants in the FIRSTMAPPP trial we

suggest that tumour response on anatomical imaging (according to RECIST criteria) does not necessarily parallel the metabolic response assessed by 18F-FDG-PET (according to PERCIST and EORTC criteria). Whether metabolic response can predict anatomical response awaits further analysis at the conclusion of the FIRSTMAPP trial. Oral presentation at the Dutch Endocrine Days, Noorwijkerhout, the Netherlands 16-1-2015.

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WP07: Clinical study platform

Within the clinical study platform the first randomized prospective trial on malignant pheochromocytoma has been moving forward with 45 centres being activated and 57 patients being randomized into the two study arms. Similarly, for the adjuvant treatment protocol of adrenal cancer patients a significant number of patients could be enrolled.

Although the patient recruitment for both studies had been lower than anticipated in both instances the groundwork laid by the consortium made it possible to maintain the study in the recruiting clinical centres. Thereby, a successful completion of the clinical studies can be expected.

1. Report on patient recruitment and first interim analysis of ADIUVO (Task #1-3)

ADIUVO is the first trial assessing the efficacy of mitotane as adjuvant therapy in patients with radically operated adrenocortical carcinoma. The results of this trial are crucial for the future management of this rare disease. The conduction of this trial has proven to be a difficult task due to the extreme rarity of the disease and the strict inclusion criteria.

Due to the rarity of Adrenocortical Cancer (ACC), its epidemiology is largely unknown. At the time when we designed the study, we did not have evidence that patients at low risk of recurrence, the patients targeted by the ADIUVO, were so infrequently observed. Experience at our center and other referral centers involved in ADIUVO demonstrated that approximately only 1 out of 3 patients seen after radical extirpation of the tumor fulfill the inclusion criteria of ADIUVO. The ADIUVO study is providing for the first time solid epidemiologic prospective data to stratify patients for prognosis.

The current status of Ethical approval of the Study is:

Italy Coordinating centre, Internal Medicine 1, S. Luigi Hospital, Orbassano, E.C. Approval: Jan 2008, EudraCT Number: 2007-007262-38. Italian centers activated: 9.

The Netherlands Coordinating centre, Maxima M. Centrum, Eindhoven, E.C. Approval November 2010, Holland centers activated: 2

Germany Coordinating centre, University Hospital Wuerzburg: E.C. Approval December 2010, German centers activated: 6

France Coordinating centre, University Centre Cochin, Paris: E.C. Approval on 29 April 2011, French centers activated: 14.

US University of Michigan, E.C. on 17 Feb 2012

Croatia, University Hospital Center Zagreb; Approval on 19 July 2012

Canada, Department d'Endocrinologie du CHUM Montreal; Approval on 24 August 2012

U.S.A. NCI Bethesda Maryland; Approval on 02 May 2013

UK Coordinating centre, University of Birmingham, E.C. Approval on 20 May 2013 English centers activated: 10

The following countries are actively enrolling patients:

Italy, The Netherlands, Germany, France, Canada, Croatia, UK

Each Country has designated its own study monitor. Monitoring visits take place regularly in Italy and Germany. All data and study procedures can be entered into electronic CRF at the web-site: www.adiuvo-trial.org.

2. Progress for FIRSTMAPP Study

The FIRSTMAPP study is a randomized, double-blind, phase II, international, multicenter study which aims to determine the efficacy of Sunitinib on the progression-free survival at 12 months in subjects with progressive malignant pheochromocytoma and paraganglioma treated with sunitinib at a starting dose of 37.5 mg daily (continuous dosing).

Primary objective:

To determine the efficacy of Sunitinib on the progression-free survival at 12 months in subjects with progressive malignant pheochromocytoma and paraganglioma treated with sunitinib at a starting dose of 37.5 mg daily (continuous dosing).

The following countries are actively enrolling patients:

France, The Netherlands, Germany and Italy.

- France : 8 centers / 27 patients

French Center	Initiation of Project
IGR Villejuif	14/11/2011
CHU Bordeaux	12/07/2012
CHU Toulouse	22/10/2012
Centre Léon Bérard Lyon	28/08/2012
CHU Strasbourg	08/10/2012
HEGP Paris	17/07/2012
CHU Angers	19/04/2012
CHU Timone Marseille	03/10/2012

- Italy : 3 center / 6 patients

Italy Center	Initiation of Project
University of Padova	04/11/2013
University of Brescia	20/11/2013
Federico II University of Naples	19/12/2014

- The Netherlands : 1 center / 2 patients

Netherlands Center	Initiation of Project
Nijmegen Medical Centre	27/05/2013

- Germany : 4 centers / 5 patients

Germany Center	Initiation of Project
Universitätsklinikum Würzburg	14/06/2013
Universitätsmedizin Berlin	13/08/2013
Universitätsklinikum Schleswig-Holstein, Innenstadt	30/09/2013
Universität München	04/12//2013

Total number of centers: 16

All data and study procedures can be entered into electronic CRF at the online web-site: <https://macrorde.igr.fr/macro/Login/LoginForm.aspx?status=%27Logout%27>

Each Country has designated its own study monitor. Monitoring visits take place regularly in France, Germany and the Netherlands.

Significant results

1. ADIUVO trial:

Total number of centers currently activated in Europe and United States for ADIUVO trial is 45.

Up to 30th June 2016, a total of 69 patients have been enrolled in the ADIUVO study with 34 patients in arm A and 35 patients in arm B. The whole recruitment in the ADIUVO trial according to countries is displayed in Figure 13.

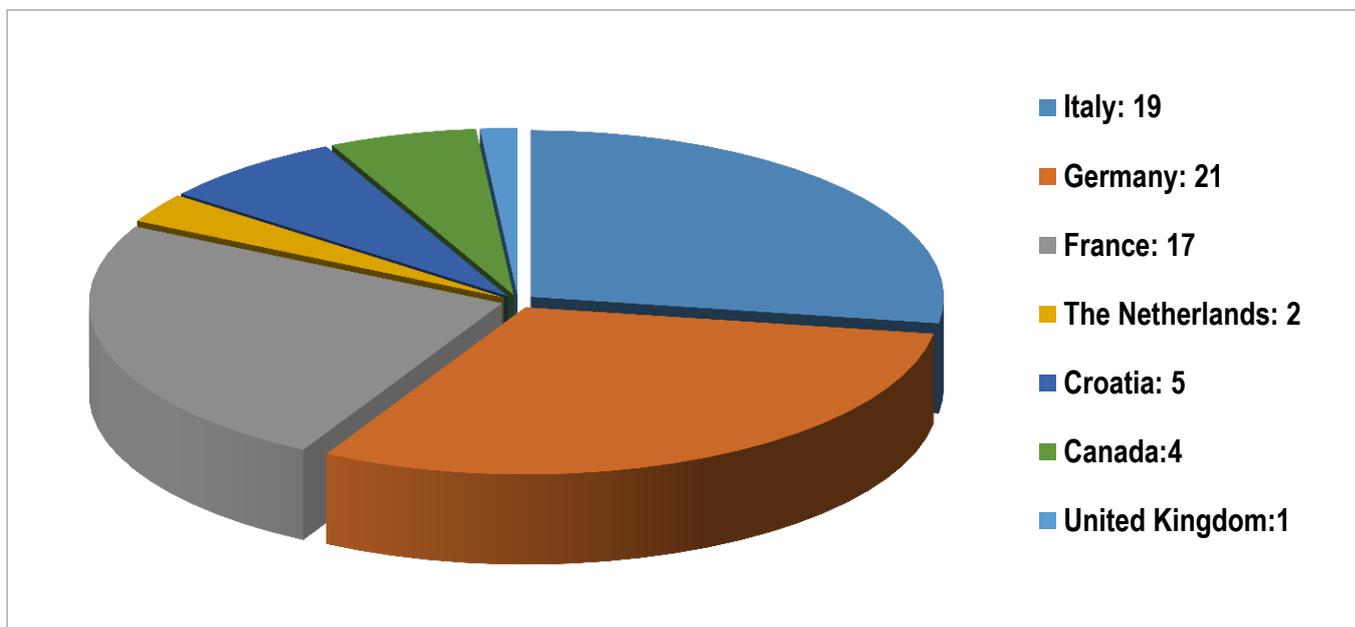


Figure 13: Patient recruitment of the ADIUVO trial by country.

The ADIUVO trial has provided the first ever prospectively collected data on the prognostic characteristic and survival of patients who had undergone surgery for ACC. These data will be key for better understanding the natural history of ACC and for defining new tailored treatments in adjuvant settings.

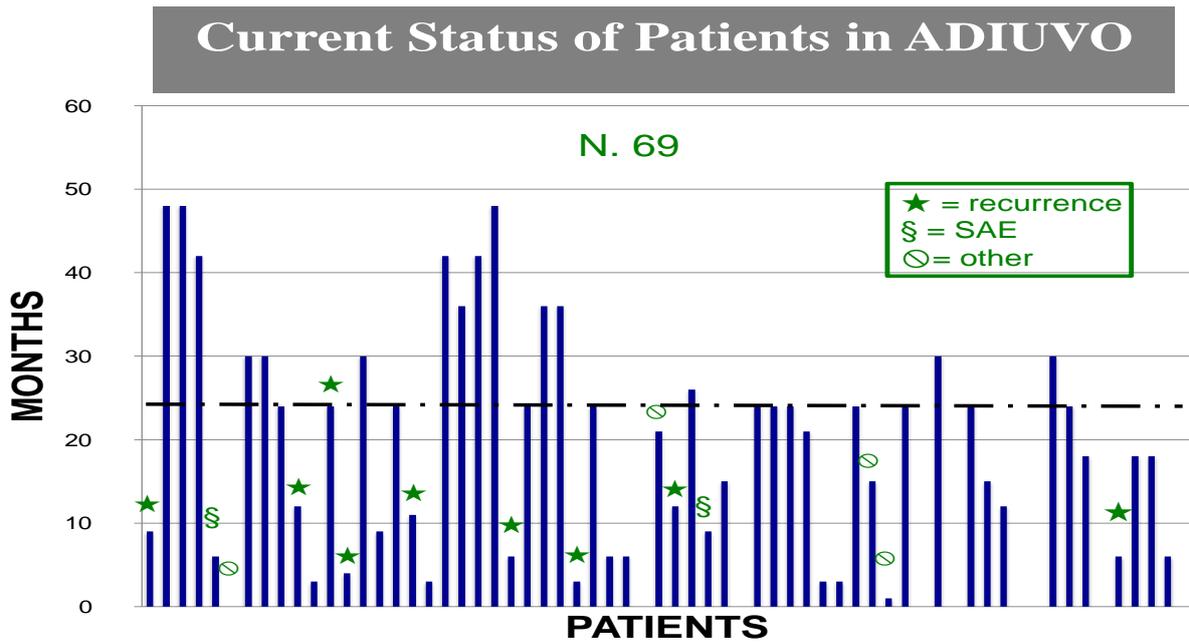


Figure 14: Summary of the clinical status of individual patients recruited into the ADIUVO trial

Patient chart reporting, patient number and their living status is given in Figure 15. We have yet not observed the expected number of recurrences (n=19) that would have triggered the performance of the interim analysis. However, we have included a survival analysis of the overall ADIUVO cohort irrespective of the treatment arm. This analysis demonstrates that recurrence-free survival is better than expected from previous available data that were obtained in a retrospective manner.

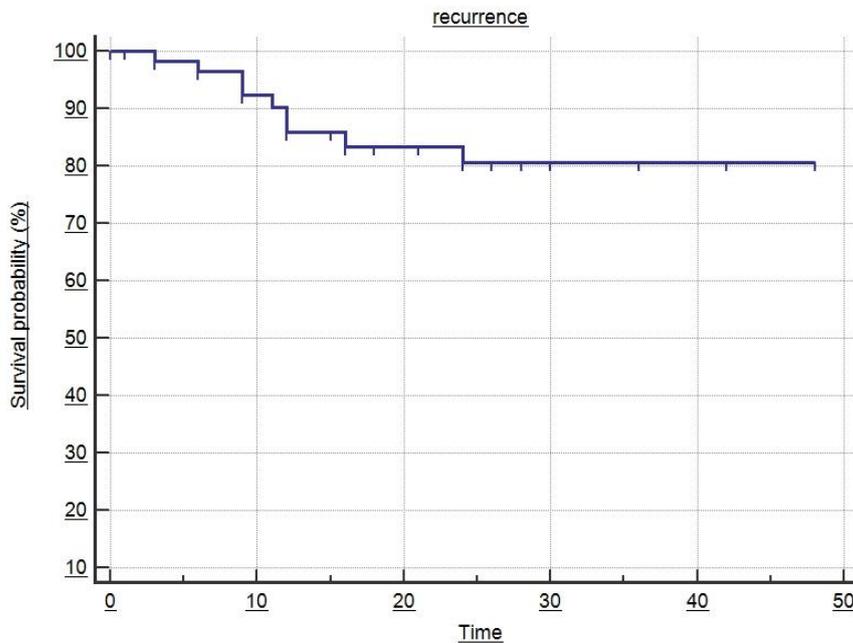


Figure 15: Recurrence-free Survival (of overall ADIUVO patient cohort)

2. FIRSTMAPP trial:

2.1. Patient enrollment in the FIRSTMAPPP Study

At the time of this final reporting (18/07/2016) a total of 57 patients were included. The last patient was enrolled by the Center Wurzburg (Germany) the 02/06/2016. Enrollment is ongoing beyond the lifetime of this working program.

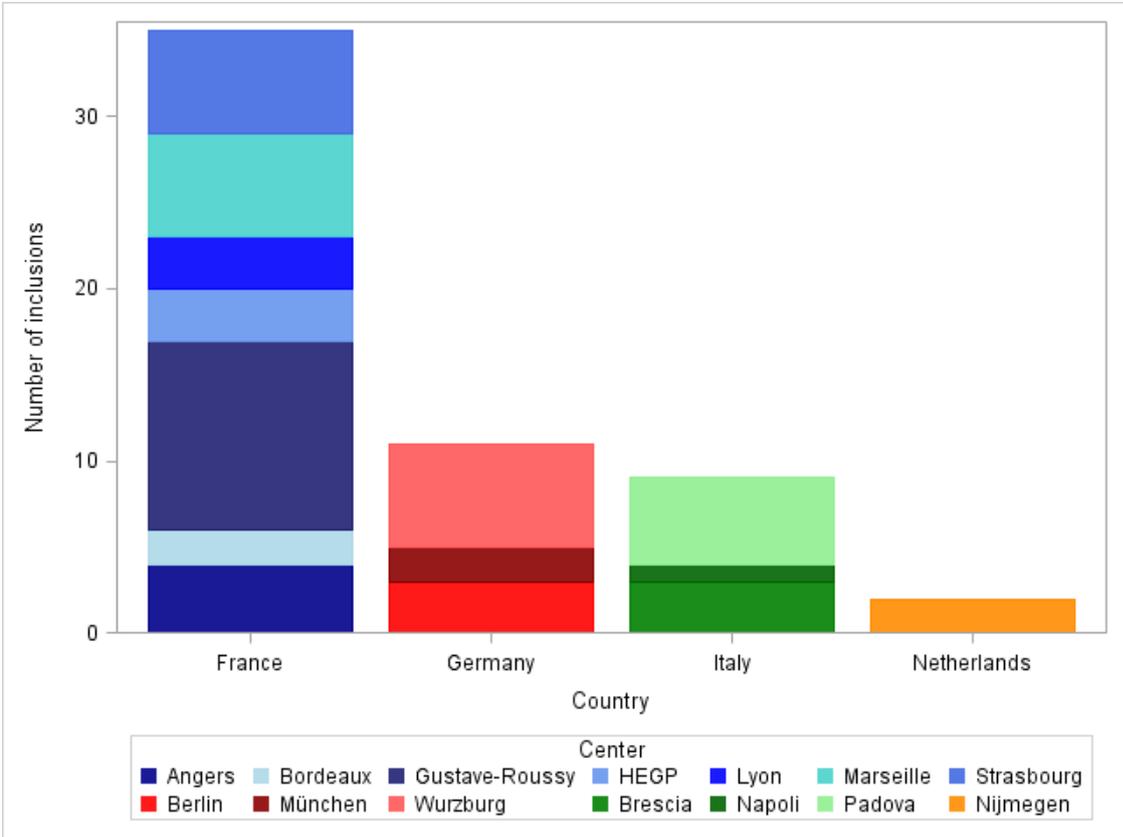


Figure 16: FIRST-MAPPP Inclusion by country

2.2. The rate of the inclusion for FIRSTMAPPP Study

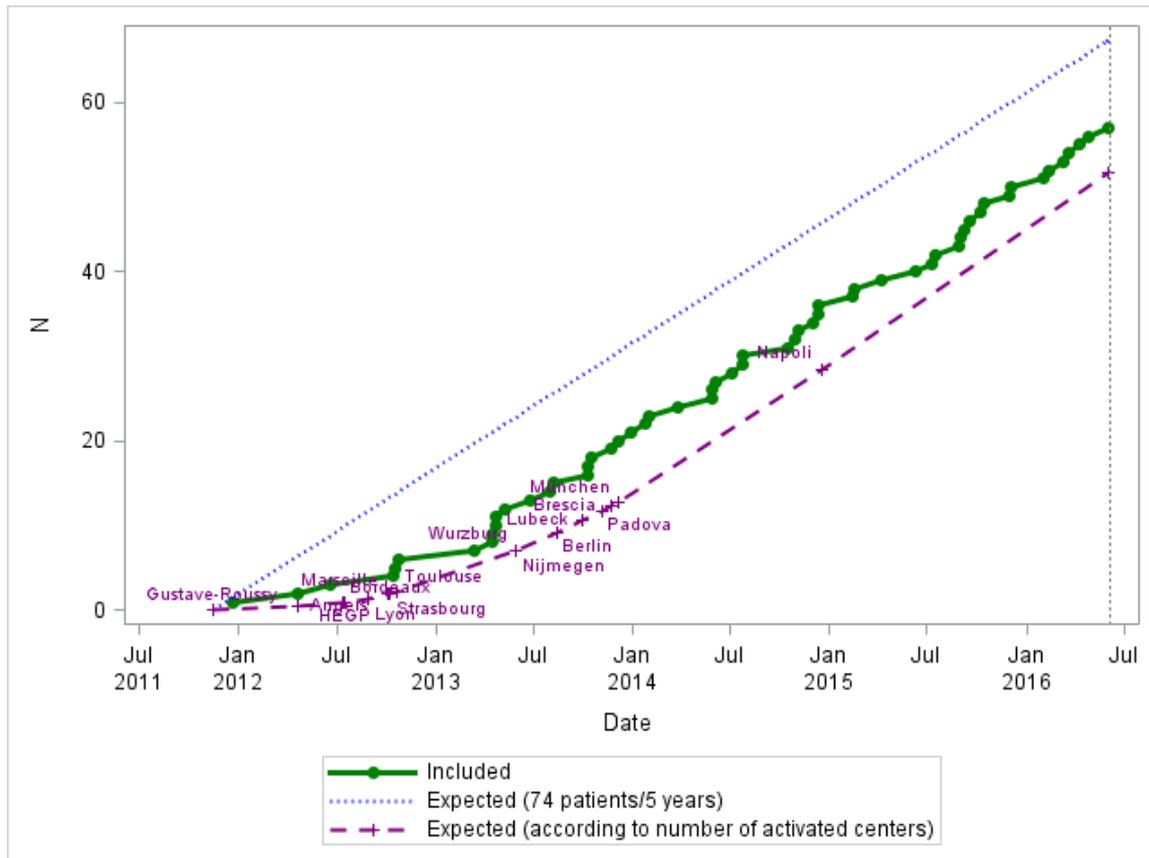


Figure 17: Overview on the expected and projected patient recruitment into the FIRST-MAPPP trial

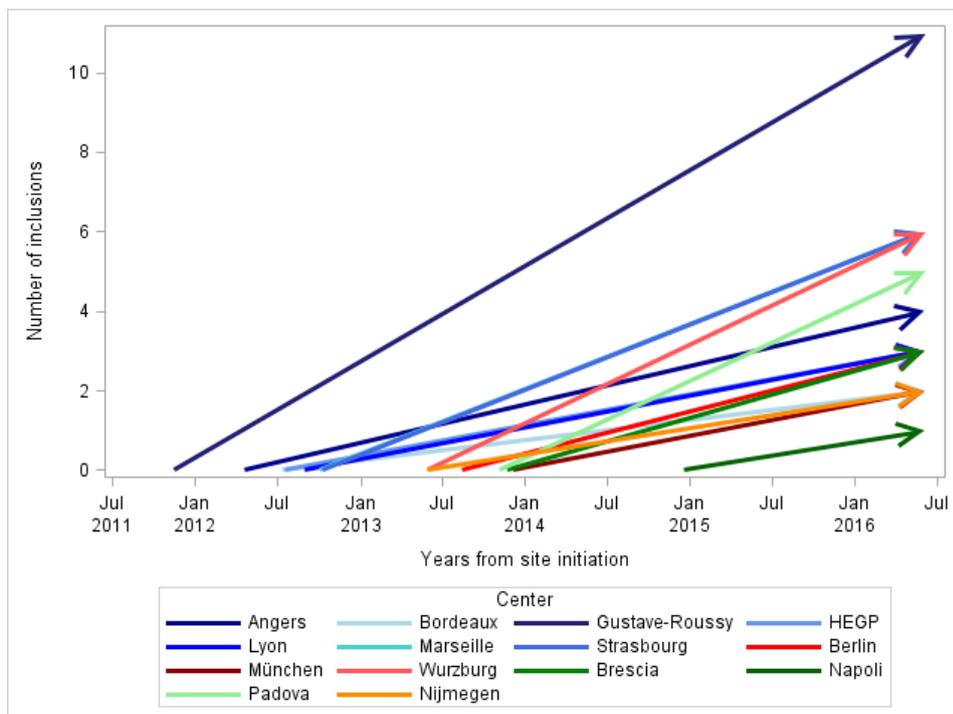


Figure 18: Patient accrual by center

The initial goal had been to reach 50 patients by the end of calendar year 2014. This enrollment was in fact accomplished in December 2015.

This delay in patient recruitment can be explained by the fact that not all clinical centers were opened at the same time. As highlighted in Figure 6 all initiated centers are actively involved in patient recruitment by including on average one patient per year. Based on the rarity of the disease, this had been the projected rate of recruitment.

2.3. First Interim analysis of the FIRSTMAPP Study

Data Accrual:

On May, 27th 56 patients are included. 16 centers are activated with 13 recruiting patients. At this time, all the planned centers are activated. 18 patients are still expected. Due to the delay to open most of the centers, the accrual curve is under the expected curve, but the estimation of one patient per center and per year is correct. The accrual will end on October, 2016. The investigators propose the following protocol amendment: increasing of 18 months the accrual duration. According to the current accrual curve, at least 18 patients could be included. So IDMC members agree with the proposed amendment.

Safety data:

The treatment has been unblinded for 31 patients, 30 for disease progression after review by the steering committee, 1 for death by disease progression. No toxic death occurred. 25 patients experienced at least one SAE, 14 patients at least one SADR. Data about the SADR in each arm are presented: all the SADR in the sunitinib arm are expected except one: amyotrophic lateral sclerosis in one patient who died of the disease. This patient has been treated previously with interferon. Sunitinib has been given approximatively during 4 months (treatment was stopped due to progression), diagnosis of amyotrophic lateral sclerosis occurred more than 6 months after the last dose of sunitinib. The most commonly reported SADR are left ventricular ejection fraction decreased, arterial hypertension and bone pain. Conclusion of IDMC member: the safety data of sunitinib in the Firstmapp study is consistent with the known safety information of the drug. No new safety signals at this time could be identified.

Major protocol deviations:

Major protocol deviations were seen for all the patients (5 patients) included in the same center (Padova Center) especially missing data of the mandatory exams. So evaluation of the efficacy of sunitinib at 12 months could not be assessed. Sponsor decided to stop the inclusions in this center and to meet the local investigator the 10 June to discuss the protocol and the deviations. IDMC members approve the decision of the sponsor to close temporarily the center at least until the discussion between the sponsor and the local investigator.

Strategies of the missing data:

Four strategies for missing data imputation are proposed by the sponsor:

- Strategy 1: maximum bias strategy : missing data : failure in sunitinib arm and missing data: success in placebo arm,
- Strategy 2: missing data = failure in both arms,
- Strategy 3: missing data = success in both arms,
- Strategy 4: imputed data follow binomial distribution with the probability of success observed in the overall sample.

IDMC members do not agree with proposal strategy 1 and proposal strategy 3. IDMC members suggest using as the best strategy for missing data imputation strategy 2, to analyse as well the 4th strategy and to remove the others.

Trial continuation:

According to the presented data, IDMC members recommend trial continuation.

1.4 The potential impact

Socio-economic impact and the wider societal implications of the project

Contribution to Community and social objectives

The established registry and biobanks and the IT structure that defines the VRE have resulted in a lasting structure that will promote future research in the field. Overall, the initiation of the common hub, the biomarker studies and the clinical trials has resulted in improved quality and harmonization of clinical care for patients with rare adrenal cancers. A prominent role for the ENS@T-CANCER virtual research environment has been to provide a centralized biobanking facility that aids the exchange of sample and tissue data across international boundaries. It is to be foreseen that these technical and development improvements will increase the pace of translational research in the area of adrenal cancer in the future.

As summarized above a number of important discoveries and developments could be achieved within the preclinical adrenal cancer work package that could well translate into novel treatment options in the future. Accordingly, findings from the integrated genomic studies have been able to define sub-groups of patients with adrenocortical cancer with poor and intermediate prognosis. This information will be translatable into clinical care and might result in a more individualized therapeutic decision process. Furthermore, yet unknown driving mutations could be identified in adrenal cancer that can be tested as novel therapeutic targets in future translational and clinical studies. Likewise, for patients with pheochromocytoma genomic biomarkers could be identified which have the potential to define novel in diagnostic algorithms and follow-up strategies. Implementation of standardized operational procedures for pathological examinations has proven to be of particular importance to provide harmonized diagnostic tools for clinical care. As pathological samples from patients with adrenal cancers have been set up as part of the pathological work package these can be used to train pathologists for these rare diseases and to provide access for future biomarker studies.

Two interventional trials have been set up: The ADIUVO trial is dedicated to answer the question whether adjuvant treatment with the adrenolytic substance mitotane is able to prolong disease free survival in patients after complete resection of an adrenocortical carcinoma. In addition the FIRST-MAPPP trial is the first to provide prospective data on the usefulness of sunitinib treatment of patients with progressive malignant pheochromocytoma. It is clear that these well-designed studies - when finalized – will change the clinical landscape and gold standard in the area.

While the clinical trials have set out to implement new standard treatments for patients with adrenal cancer the currently built network has already resulted in alignment of clinical practice and standards between European clinical centres. Establishment of the overall structure of ENS@T-CANCER including its dedicated registry and network of clinical and research centres that support it will have impact on research in the area well beyond the lifetime of the proposal. The funding of the consortium has resulted in significant scientific output. As these results are published in scientific journals and presented in national and international conferences this dissemination actions increase the visibility of the scientific and clinical centres. Thereby, access of patients to these specialized centres already has increased which by itself will improve quality of care.

Main dissemination activities and exploitation of results

International visibility and recognition of the ENS@T-CANCER work by the wider community was a key factor and measure of the overall success of the working program. ENS@T-CANCER actively communicated the results of its projects using several platforms to cancer researchers, the international community, and more generally to the wider public. This included the proactive dissemination of research results through standard channels, including presentations at major international scientific conferences, publications and invited talks. Specific actions to support the dissemination activities are summarized as follows:

Dissemination through establishment of the Virtual Research Environment

As explained in detail above the consortium has established a Virtual Research Environment (VRE) comprising a clinical registry for patients with ACC/MPH and a collection of tools for security-oriented data access, linkage,

analysis, annotation and storage for the associated work packages. Where appropriate a variety of information was made available to specific non-ENS@T-CANCER researchers, to the wider research community and to the wider public. This information was realised through targeted auto-extraction of meta-information from the VRE. This includes for example, statistical information on the number of cases and biomaterials available through the VRE. This information was made available through **targeted research environments** where discretionary access control measures are defined and employed. Each iteration of the VRE provided refinements and improvements on the kinds of information that were made available, their personalisation to wider communities, and the technologies that were used to support this.

As expected the VRE itself became a model for how ethically and security-driven research environments can be supported. As such, the work on development of the VRE itself was published widely at related security-oriented, clinical software-oriented and wider biobanking journals and conferences.

Dissemination through a dedicated Website

With the start of the project ENS@T-CANCER set up its own dedicated website that served as an important communication platform both internally and externally. It consisted of public areas for disseminating ENS@T-CANCER activities and a password protected area open to all ENS@T-CANCER scientists for the exchange of confidential project management data and information. The ENS@T-CANCER website had provided the **lay public, medical professionals and scientists with authoritative up-to-date information**. It facilitated regular dissemination of data, publications and review articles by ENS@T-CANCER investigators. The website also provided links to web pages of patients' organizations and the participating institutions.

ENS@T-CANCER activities, news and results of the clinical trials were also disseminated by the ENS@T website (www.ensat.org) which had recently been re-launched after extensive feedback and subsequent updating and restructuring.

Dissemination through meetings and publications

Members of ENS@T-CANCER had presented their research findings at national and **international scientific meetings** in Endocrinology, Oncology and basic life science. Furthermore, the ENS@T-CANCER partners held an **annual meeting** to discuss the latest developments of projects of the consortium, which also served as the primary platform to present research activities and to exchange information with the scientific community. These symposia provided a forum in which a variety of stakeholders did participate and network. They also **raised public awareness** of the achievements of the program. Speakers on the symposia included ENS@T-CANCER partners and leading experts external to the project.

In addition to this, consortium members **published the results in journals** of the highest impact related to the subject. Furthermore, ENS@T-CANCER produced a **newsletter**, which in addition to the information available on the website, was sent to members of the national Endocrine Societies. Furthermore, the consortium directly engaged with the wider lay public through **press releases**.

Participants of the ENS@T-CANCER consortium were actively involved in a number of boards of national and international societies and scientific journals, which open the possibility to foster the awareness of the scientific community for the needs of adrenal cancer research.

Dissemination through medical education

A large number of members of the consortium are renowned not only for their research achievements but also for their clinical expertise in the targeted areas. Thus, the consortium holds key positions for dissemination of scientific results within national **continuing medical education**, which will allow implementation of new diagnostic and therapeutic approaches into clinical practice. Educational activities will be executed on a local (Hospital, University), national (National Endocrinology and Oncology meetings) and international level (e.g. "Meet the professor" sessions during European Society Meeting and Endocrine Society Meeting). It is to be expected from earlier experience that close scientific collaboration between consortium members will also foster exchanges that result in mutual invitations to provide lectures on a specific topic.

Dissemination through patient organization groups

Through their scientific work and their clinical duties, members of this consortium are fully aware of the medical and associated social problems that can confront individuals with adrenal cancer, as well as their families and others that care for them. In particular, many of affected patients are faced with an urgent need to be informed about ongoing research activities, recent developments and prospects for future therapy of their condition. To this end, participants in ENS@T-CANCER **actively promoted public awareness** of novel developments in the prevention, diagnosis and care of adrenal cancer, and through the clinical trials provide documented evidence of hitherto untested approaches for targeted adrenal cancer treatment. During the lifetime of ENS@T-CANCER, patient organizations had been contacted and informed about the current initiative and the associated clinical trials.

Dissemination of knowledge through training

A dedicated exchange programme was utilized as an important instrument to disseminate specific expertise between consortium centres, to enhance collaboration and, importantly, to further training and career opportunities of young scientists. This **fellowship programme** was made possible as part of the Research Networking Programme 07-RNP-067 funded by the European Science Foundation (running period: July 2009 to June 2014), part of which was dedicated to the organization of Exchange Visits.

Specifically, scientists from the participating centres, and from other laboratories, were encouraged to perform “short-term-“ (up to 15 days) or “exchange” visits (between 15 days and six months) to share expertise and new techniques in multidisciplinary collaborative projects between ENS@T – and other - research teams. Applications for PhD students and post-doctoral fellows were proposed to the research community in the field via the project web-site and at conferences.

Exploitation

The ultimate aim of the consortium is to develop research in the field of adrenal cancers to improve our diagnostic and therapeutic abilities. As adrenal cancers can be regarded as “orphan diseases” financial and technical support from the pharmaceutical industry had been negligible in comparison to other malignant tumour entities. Nevertheless, strategic associations with specialized pharmaceutical companies has been aimed at including HRA Pharma and Millendo for the adjuvant treatment of adrenocortical carcinoma patients with mitotane and ATR101, respectively, and Pfizer for the treatment of malignant pheochromocytoma patients with sunitinib. Furthermore, partners of the consortium have explored the possibility of multi-steroid analyses in the differential diagnosis of adrenal tumors which is likely to be available as a diagnostic tool in the future (Assay for detection of adrenal tumour, patent US 20120040332 A1).

Currently, members of the ENSAT-CANCER consortium are actively involved in setting up an adjuvant treatment trial on high risk adrenal cancer patients using liposomal cisplatin (Regulon Inc., Greece) for which funding will be sought through the current call “**PM-08: New therapies for rare diseases**”.

Outlook and future research

A number of scientific questions have evolved from the work of the ENS@T-CANCER consortium:

Biomarker research in adrenal tumour patients

The overall concept of this endeavour is to replace the current costly imaging strategy with a targeted work-up that only requires giving a blood and a urine sample at the point of access, the doctor caring for a patient with adrenal incidentaloma. Through the integration of large scale omics data by computational approaches we will develop an algorithm that will decide on the choice of omics assays applied to individual patient samples, thereby providing a personalised management tool for adrenal incidentaloma. In an initial **exploratory phase** we will utilise extensive existing omics data from ENSAT-AIM participants (metabolomics, genomics, transcriptomics, miRNomics) that will be mined and scoped by the computer scientists for the **generation of an integrated model**. Thus, in multi-disciplinary collaboration of clinicians, biologists and computer scientists, we will create **optimised algorithms for diagnostic pathway prediction in adrenal incidentaloma**. In the subsequent **validation phase** we will test the resulting

algorithm(s) in clinical practice through prospective recruitment of a large cohort of adrenal incidentaloma patients with the help of the ENSAT network. The **implementation and exploitation phase** will be led by the participating specialist diagnostic SMEs with input from health care providers, end users (patients, clinicians) and specialist societies. Importantly, all stake holders will provide continuous input from the start of the project, rather than commenting on the final product. This approach will ensure the **creation of a novel personalised medicine tool fit for purpose and suitable for rapid implementation across diverse European health care systems.**

Targeted therapies for patients with adrenocortical cancer

Overall, the proposed working plan aims to **improve the clinical outcome of patients with adrenal cancer**. As currently available therapies have low efficacy and substantial toxicity, this consortium gathers internationally leading experts in the field to bring forth the most promising therapeutic approaches. The work packages and underlying tasks cover all aspects from proof of concept studies to clinical trials aiming at drug market authorization. Through ENS@T-CANCER a collection of adrenal tumour related databases and defined an associated European network of Biological Resource Centres had been established devoted to research on adrenal tumours. For the proposed clinical trial ENS@T brings together **16 clinical expert centres from seven European countries** that have the enrolment capabilities necessary to successfully accomplish the study protocol. **Novel treatment concepts for ACC patients** will be identified and evaluated in well-defined models and within a structured network. The implementation of preclinical studies that accompany the proposed clinical trial takes into account the common development of treatment resistance, which necessitates alternative or combinatory therapeutic approaches. The consortium will follow **the most promising targets against adrenal cancer resulting from recent break-through achievements** that have provided insights into critical mechanisms in adrenal biology (Assie Nat Genet 2014, Beuschlein NEJM 2014, Assie NEJM 2013, Louiset NEJM 2013).

Biomarker studies in adrenal related hypertension

Arterial hypertension is a major cardiovascular risk factor affecting up to 45% of the general population in industrialized countries (*ESH/ESC Task Force for the Management of Arterial Hypertension, J Hypertension 2013*), Although a large therapeutic arsenal exists targeting a variety of systems involved in blood pressure regulation, **the control of hypertension is still sub-optimal** with 10 to 30% of hypertensives being resistant to the combination of three or more medications (*Calhoun et al, Circulation 2008*). Yet, even small increments in blood pressure are associated with a clearly increased risk for stroke and coronary events (*Lewington et al, Lancet 2002*). Detection of secondary forms of hypertension is key to targeted management of the underlying disease and prevention of cardiovascular complications. In this context, **endocrine forms of hypertension represent major targets for stratified approaches of health promotion**. They include a group of adrenal disorders resulting in increased production of hormones affecting blood pressure regulation: **primary aldosteronism (PA), pheochromocytoma/functional paraganglioma (PPGL) and Cushing's syndrome (CS)**.

In ENS@T-HT, members of this consortium will move from tissue derived genomics developed in previous programs to circulating genomics on fluid biosamples to develop biomarkers defining endocrine forms of hypertension suitable for clinical implementation. This should allow for improved identification of endocrine causes of hypertension for curative treatment and prevention of cardiovascular and metabolic complications and the stratification of primary forms of hypertension for effective and cost efficient therapy.

Finally, members of ENS@T-CANCER are actively involved in an application on the call "**PM-08: New therapies for rare diseases**" with the main focus on clinical studies on adrenal carcinoma. As such, this working program will be a seamless continuation of aims followed during ENS@T-CANCER. In fact, many of the biomarker and translational studies advanced during ENS@T-CANCER will provide the basis for the current clinical protocols.

Section 2 – Use and dissemination of foreground

Please see ECAS.

Section 3 – Report on societal implications

Please see ECAS.