LSHC-CT-2005-518200

TCAC in Cancer

Defects in the tricarboxylic acid (Krebs) cycle genes in tumorigenesis

Specific Targeted Project

Priority 1: Life sciences, Genomics and Biotechnology for Health

Publishable final activity report

Period covered: from 1/1/2006 to 31/12/2009 Date of preparation:10/2/2010

Start date of project: 1/1/2006 Duration: 48 months

Project coordinator name: Lauri A. Aaltonen
Project coordinator organisation name: University of Helsinki
Revision: Final version

Index

1. Project execution…….……3
   1.1 Summary description of project objectives…….……3
   1.2 Contractors involved……..5
   1.3 Work performed and results achieved…….……....5

2. Dissemination and use…….……27

1 Project execution

1.1. Summary description of project objectives

For this effort top European cancer research groups working on the association between defects in the tricarboxylic acid cycle (TCAC) and cancer formed a consortium to 1) profoundly characterize the human phenotypes to enable identification and efficient cancer prevention, and 2) to unravel the underlying molecular mechanisms. Previous breakthrough findings by the consortium participants and others had shown that defects in at least four TCAC genes – fumarate hydratase (FH, fumarase), and succinate dehydrogenase (SDH) B, C, and D - can confer susceptibility to cancer. We took these studies forward by characterizing the natural history of the syndromes in large European cohorts. Simultaneously, we performed functional studies to elucidate the cellular events induced by these defects by systems biology approaches including functional studies in cell lines, model organisms, and transcription profiling of TCAC deficient and proficient tumors and models. TCAC defect associated expression patterns were utilized to examine other cancer types for such defects. We had evidence that modifying genes play a key role in TCAC defect associated tumorigenesis, and candidate regions had been identified. Vigorous efforts were conducted to identify the responsible genetic changes.The rationale to form this consortium was simple and strong. The consortium brought together the key European cancer researchers studying TCAC associated tumorigenesis. Studying separately the tumorigenic effects of FH and the different units of SDH would have been ineffective, and formation of the consortium enabled Europe to maintain the initiative in this new and exciting field of research. The deliverables arising from the workpackages contribute to the common goals; prevention of TCAC associated cancers, and learning the lessons these lesions can teach to cancer research.

This project had one major objective: improved prevention and management of TCAC associated hereditary cancers. The two key tasks to achieve this goal are

1) Characterizing the natural history and prevalence of TCAC deficient cancers.
2) Unravelling the molecular mechanisms driving TCAC associated tumorigenesis.
3) To achieve these goals the work was broken into the following workpackages:

Work-package No[1]	Workpackage title	Lead part. No[2]	Start month[3]	End month[4]
1	**Day-to-day management of the consortium**	1	1	48
2	**Natural history of FH associated tumors**	1	1	12
3	**Natural history of SDH associated tumors**	5	1	48
4	**Prospective collection of 360 fresh-frozen myometrium-myoma sample pairs**	1	1	48
5	**Collection and analysis of population based series of uterine leiomyosarcomas diagnosed under 45 years of age**	1	1	12
6	**Basic molecular characterization of collected sample materials**	4	1	48
7	**Expression profiling of SDH and FH lesions. Creation of a classifier for TCAC defect, to examine other tumor types.**	3	12	36
8	**Identification of the FH modifier gene**	1	1	36
9	**Identification of the SDHD modifier gene**	3	1	36
10	**Identification of novel genes predisposing to paragangliomas/pheochromocytomas**	3	1	48
11	**FH Knockout mouse model**	2	1	48
12	**SDH Knockout mouse models**	3	1	48
13	**Functional studies on TCAC deficiency**	6	1	48

[1] Workpackage number: WP 1 – WP n.
[2] Number of the contractor leading the work in this workpackage.
[3] Relative start date for the work in the specific workpackages, month 0 marking the start of the project, and all other start dates being relative to this start date.
[4] Relative end date, month 0 marking the start of the project, and all ends dates being relative to this start date.

1.2. The contractors involved

Partic. Role*	Partic. no.	Participant name	PI	Participant short name	Country
CO	1	University of Helsinki	Lauri Aaltonen	UH.MCB	FI
CR	2	Institute of Cancer Research	Richard Houlston	ICR	UK
CR	3	Leiden University Medical Center	Peter Devilee	LUMC	NL
CR	4	Universitaetsklinikum Freiburg	Hartmut Neumann	UKL-FR	DE
CR	5	National Institute of Cardiology	Andrzej Januszewicz	NIC	PO
CR	6	Cancer Research UK	Ian Tomlinson	CR-UK	UK

*CO = Coordinator
CR = Contractor

Co-ordinator contact details

Academy Professor Lauri A. Aaltonen
Genome Scale Biology Research Program / Biomedicum Helsinki
P.O. Box 63 (Haartmaninkatu 8), FI-00014 University of Helsinki, Finland
Tel. +358-9-1912 5595, Fax +358-9-1912 5105, e-mail: Lauri.Aaltonen@helsinki.fi

1.3. Work performed and results achieved

WP1 Day-to-day management of the consortium

This WP was on management of the consortium. The project was managed without any significant difficulty, and the appropriate reports have been approved by the EU.

WP2 Natural history of FH associated tumors

To gain comprehensive insight into potential cancer phenotype in *FH* mutation-positive families, the risk of cancer and the tumor spectrum was examined in seven Finnish HLRCC (FAM1-7) and one FH deficiency kindred (FAM8). (Table 1) Genealogical and cancer data from church parish registries, death certificates, the Population Register Centre, and the Finnish Cancer Registry were obtained from 868 family members. To avoid selection bias, only individuals of the completely traced generations (n=256) were included in the cohort analysis of the standardized incidence ratios (SIR). No selection according to mutation status was done. Moreover, all cancers in the index patients (5 cancers in 8 patients) and other patients with RCC or ULMS used for identification of the families (7 cancers in 6 patients) were excluded. The risk analysis provided statistically significant results with 6.5-fold risk (CI 2.1-15.0) for RCC and 71-fold risk (CI 8.6-260) for ULMS compared to the general population (Table 2). The excess was concentrated in the age groups of 15-29 and 30-44. Moreover, the overall risk of cancer was

statistically significantly increased in the age group of 15-29 years (SIR 6.60, CI 1.40-19.0) (Table 2), although of note, this group included only three cancers (RCC, uterine leiomyosarcoma, and Hodgkin's lymphoma). The risk for other tumor types did not reach statistical significance.

Table 1. Family data.

Family	*No. of family members*	*Phenotype exploited in family identification*	*Germline mutation*
FAM1	137	RCC	541delAG
FAM2	97	RCC	541delAG
FAM3	59	Leiomyomas	R300X
FAM4	208	ULMS	H153R
FAM5	37	RCC	H153R
FAM6	42	Leiomyomas	H153R
FAM7	6	ULMS	541delAG
FAM8	282	FHD	Q333P

RCC = renal cell cancer; ULMS = uterine leiomyosarcoma; FHD = FH deficiency.

Table 2. Risk of cancer in *FH* mutation-positive families.

	Observed	Expected	SIR	95% CI
RCC				
All	5	0.78	6.50	2.10-15.0
Ages (years)				
0-14	-	0.01	0.00	0.00-260
15-29	1	0.00	230	5.90-1300
30-44	2	0.04	45.0	5.50-160
45-59	-	0.22	0.00	0.00-17.0
60-74	1	0.35	2.80	0.07-16.0
75+	1	0.14	7.00	0.18-39.0
ULMS				
All	2	0.02	71.0	8.60-260
Ages (years)				
0-14	-	0.00	-	-
15-29	1	0.00	2100	52.0-11000
30-44	1	0.00	180	4.60-1000
45-59	-	0.01	0.00	0.00-540
60-74	-	0.00	0.00	0.00-840
75+	-	0.00	0.00	0.00-2100
TOTAL CANCER				
All	34	24.73	1.40	0.95-1.90
Ages (years)				
0-14	-	0.23	0.00	0.00-16.0
15-29	3	0.45	6.60	1.40-19.0
30-44	5	1.97	2.50	0.82-5.90
45-59	6	6.16	0.97	0.36-2.10
60-74	15	10.40	1.40	0.81-2.40
75+	5	5.51	0.91	0.29-2.10

We next investigated the tumor spectrum in *FH* mutation-positive individuals. Fifty-four out of 98 tested patients harbored a germline *FH* mutation, and approximately half of these patients had been diagnosed with cancer (Table 3). Comparison of the age at cancer diagnosis of mutation-positive and -negative patients showed that mutation-positive individuals are prone to get cancer at younger age (p=0.009) (Figure 1), although this difference did not remain significant after exclusion of the probands. In *FH* mutation-positive patients, the most prominent cancer types were RCC (n=12) and uterine ULMS (n=5), affecting 22% of all mutation-positive individuals and 15% of mutation-positive females, respectively. In addition, four patients had atypical leiomyomas, a variant of leiomyoma sometimes difficult to discern from leiomyosarcoma. Several cases of breast carcinoma and hematopoietic or lymphoid malignancies were observed. Cancer cases detected in *FH* mutation-positive individuals are summarized in Table 3.

Table 3. Cancer cases in *FH* mutation-positive individuals.

Tumor	*n*	*Age at diagnosis (median)*	*Somatic second hit detected/analyzed (%)*
RCC	12	26, 32, 33, 35, 36, 39, 42, 48, 49, 68, 71, 90 (40.5)	10/12 (83)
ULMS	5	27, 30, 32, 35, 39 (32)	3/3 (100)
Breast cancer	4	50, 53, 55, 61 (52.5)	3/3 (100)
Bladder	1	71	1/1 (100)
Non-Hodgkin's lymphoma	1	63	0/1
Hodgkin's lymphoma	1	24	0/1
Chronic lymphatic leukemia	1	48	0/1
Esophagus cancer	1	53	0/1
Basal cell cancer	2	70, 83	0/1
Multiple myeloma	1	61	Not analyzed
Prostate cancer	1	63	Not analyzed
Liver/bile duct cancer (obligatory carrier)	1	82	Not analyzed
Unknown origin	1	42	Not analyzed

RCC, renal cell cancer; ULMS, uterine leiomyosarcoma;

To get insight into which tumors might have arisen due to the *FH* germline mutation, we examined somatic (biallelic) inactivation of the gene in 22 tumors (Table 3). Almost all analyzed RCCs and ULMSs as well as the one bladder and all the three studied breast carcinomas had lost the wild-type *FH* allele. One of the breast carcinomas and the bladder carcinoma were detected in the FH deficiency family. These results suggest possible association of these two tumor types with *FH* germline mutations. Two of the breast cancer patients had been diagnosed also with ULMS, supporting this concept. Strikingly, one patient with ULMS and breast cancer was affected also with multiple myeloma and non-Hodgkin's lymphoma.

WP3 Natural history of SDH associated tumors

Nationwide efforts have been undertaken in Poland and Germany and have been initiated in The Netherlands, represented by the centers in Warsaw, Freiburg and Leiden. We established compatible and coordinated databases to detect germline mutations in patients with pheochromocytomas and paragangliomas and to use as the basis to set risk profiles and as a source for adequate counseling of the patients. Three databases with patient data were created in Poland, Germany and The Neatherlands. We built up a network for patients, surgeons and physicians in Poland, Germany and The Netherlands. Special questionnaires have been elaborated in order to collect all relevant data for all registered subjects. A total of 2535 subjects were registered in the period of 2002, 2003, 2004, 2005, 2006, 2007, 2008 up to January 28, 2010. The input according to centre was Warsaw, 550 patients, Leiden 608 patients, and Freiburg 1377 patients.

Conclusions / Germany

Some of the performed in Germany under this WP extended those described in the original contract, and was funded partially the German Cancer Foundation and the German Research Foundation. Hereditary pheochromocytoma was present in 41% and hereditary HNP in 29% of patients in the Pheochromocytoma-Paraganglioma Registry, in total 37%. Patients with pheochromocytoma have mutations in the VHL, RET, NF1, SDHD or SDHB genes, whereas patients with paraganglioma predominantly have mutations in the genes SDHB, SDHD, or SDHC. Patients with hereditary syndromes were younger. Multiple and extraadrenal pheochromocytoma were more frequent in syndromic than in sporadic pheochromocytoma. Predictors of hereditary syndromes at younger age are diagnosis, multiple, malignant, bilateral and extraadrenal tumors. In particular, a founder effact for the mutation SDHD p.C11X was found in analyses performed with samples from patients of Germany and Poland in Freiburg. The data from reinvitations and complete clinical investigations of about 200 carriers of subjects with mutations of one of the genes SDHB, SDHC, and SDHD is almost finished and penetrance data will be available within the next weeks. Preliminary data show evidence that relatives of patients with SDHB mutations who were subsequently identified as mutation carriers is very low. We analyzed our data regarding economics of testing patients with paraganglial tumors. These studies have been separately published for patients with head and neck paragangliomas (HNP) in Cancer Research 2009. Of 598 patients, 30.6% had *SDHx* germline mutations: 34.4% in *SDHB*, 14.2% *SDHC*, and 51.4% *SDHD*. Predictors for an *SDHx* mutation are family history (OR 37.9), previous pheochromocytoma (OR 10.9), multiple HNP (OR 10.6), age≤40 (OR 4.0), and male gender (OR 3.5). By screening only pre-selected cases and a stepwise approach, 60% cost reduction can be achieved, with 91.8% sensitivity and 94.5% negative predictive value. Our data give evidence that clinical parameters can predict for mutation and help prioritize gene testing to reduce costs in HNP. For all other paraganglial tumors which are summarized as pheochromocytomas we published our data in Clinical Cancer Research 2009. Of 989 apparently non-syndromic pheochromocytoma cases, 187 (19%) harbored germline-mutations. Predictors for presence of mutation are age≤45, multiple pheochromocytoma, extra-adrenal location, and previous head and neck paraganglioma (HNP). If we used the presence of any one predictor as indicative of proceeding with gene testing, then 342 (34.6%) patients would be excluded, and only eight (0.8%) carriers would be missed. We were also able to statistically model the priority of genes to be tested given certain clinical features. E.g., for patients with prior HNP, the priority

would be *SDHD>SDHB>RET>VHL*. Using the clinical predictor algorithm to prioritize gene testing and order, a 44.4% cost reduction in diagnostic process can be achieved. In conclusion, clinical parameters can predict for mutation carriers and help prioritize gene testing to reduce costs in non-syndromic pheochromocytoma

Conclusions / Poland

Hereditary pheochromocytoma or paraganglioma were present in 24% of patients participating in an on-going Polish Pheochromocytoma Registry. Patients with hereditary syndromes were younger and multiple, recurrent and extraadrenal pheo were more frequent than in sporadic pheochromocytoma. Independent predictors of hereditary syndromes may be young age at the onset and multiple tumors. Till now the genetic diagnosis of polish patients identified that PPS/PGL syndrome resulting from SDHB, SDHC and SDHD genes mutations is the most common genetic syndrome associated with pheochromocytoma and paraganglioma in Poland (almost 50% of hereditary cases). It can be associated with SDHD p.C11X mutation which has been found to be due to a founder effect. We observed that there was no significant difference between SDHB and SDHD mutation carriers in the time to first diagnosis. In relation to tumor location, SDHD mutation carriers were more likely than SDHB mutation carriers to develop head and neck tumors and have a multifocal presentation. Conversely, SDHB mutation carriers were more likely than SDHD mutation carriers to present with extraadrenal abdominal tumors and with increased risk of malignancy. Penetrance analysis showed that PGL 1 syndrome can be fully penetrant whereas PGL4 and PGL3 syndromes are characterized by incomplete disease penetrance. Patients with SDHC mutations in spite of previously described solitary head and neck paraganglioma may also develop adrenal and extraadrenal metachronous pheochromocytoma. Since the tumors in PGLs syndromes can be found in head and neck, chest and abdomen or pelvis the routine diagnostic procedure in each SDHx gene mutation carrier should include three-body-area imaging examinations (CT/MRI) as well as measurement of daily urinary catecholamines excretion. SDHD mutations have infrequently been associated with malignant disease. This finding should alert clinicians to the necessity of careful monitoring of these patients for the development of tumors at new locations and also for the presence of metastases. Renal clear cell carcinoma can develop as a result of SDHB gene mutation.

Conclusions / the Netherlands

The Leiden Paraganglioma Registry now contains 448 patients with head-neck paraganglioma and 160 additional patients with pheochromocytoma. In addition to the 884 *SDHD* and *SDHB* mutation carriers previously identified, the registry has expanded slightly with now a total of 918 mutation carriers known. Once fully integrated the Leiden registry will solicit input from all academic centers in The Netherlands and will be in a strong position to graduate to a national registry. A working visit to Leiden by Prof. Neumann initiated an active collaboration in the co-ordination of these databases, with the aim of creating a database system with identical criteria, allowing seamless integration of data. The integration of the Leiden, Freiburg, and Warsaw registries and related tissue banks will represent a resource of a scale and composition unique in the world, combining the data from populations with relatively diverse mutation profiles, Freiburg and Warsaw, with those of Leiden, characterised by a relatively homogenous group defined by several founder mutations and including a significant number of very large families.

WP4 Prospective collection of 360 fresh-frozen myometrium-myoma sample pairs

The scientific reason for collecting the specimens was to feed samples to WP7, aiming at characterization of a gene expression pattern signature for TCAC deficient lesions. The idea was to examine the collected lesions for LOH at 1q and in positive cases do FH mutation analysis, to add to the numbers of FH deficient lesions available. As reported under WP7 below, the work aiming at TCAC classifier has, in the case of FH deficient lesions led to the following conclusions:
1) From large numbers of sporadic leiomyomas very few additional FH deficient samples can be anticipated.
2) When the UK and Finnish leiomyoma expression data was combined, the early TCAC classifier designed based on the Finnish samples could identify with 100% accuracy the FH deficient samples in the UK series. Thus if a smallish number of FH deficient samples were added to this analysis one would anticipate very little change in the structure of the classifier. For these reasons we decided to tone down the sample collection effort. Nevertheless the number of specimens collected in the project totals 224 normal myometrium and approximately double leiomyomas.

WP5 Collection and analysis of population based series of uterine leiomyosarcomas diagnosed under 45 years of age

The occurrence of Uterine leiomyosarcoma (ULMS) in patients with *FH* mutation led us to hypothesize that *FH* mutations could also play a role in the pathogenesis of non-syndromic early onset ULMS. This hypothesis was strengthened by findings in a small pilot series; one out of eighteen unselected ULMSs displayed a germline mutation. For this WP we collected a population-based material of all early onset (≤ 45 years) ULMS diagnosed in Finland between years 1981 and 2003 through a search from the Finnish Cancer Registry. Eighty-one patients fulfilled the criteria, and 67 tumors (83%) were available for *FH* mutation analysis. The average age at diagnosis was 39.8 years (varying between 16 and 45 years, median 41).
The mutation analysis was performed from patients' tumor tissues and presence of putative mutations was examined in the respective normal tissue. DNA extraction from paraffin blocks was performed according to standard procedures. The coding exons were amplified by PCR and analyzed by genomic sequencing.
In the mutation analysis, a total number of 653 fragments out of 670 (97.5 %) were analyzed successfully. A novel missense change K424R (1432A>G) was detected (1/67, 1.5%). The change was found in both tumor and normal tissues, and LOGH was not observed in the tumor (Figure 1). The alteration was not detected in 280 healthy controls and it occurred at a conserved residue.

To study the influence of the change to the protein function, FH enzyme activity was measured. The activity of the mutated protein was significantly reduced ($p= 0.009$) being approximately 43% of the activity of wild type protein in the model system used (Figure 2). This result indicates that K424R is a pathogenic mutation.

Because only one mutation was identified in the study, it was an obvious choice not to extend the study to other populations, because very few positive findings were anticipated.

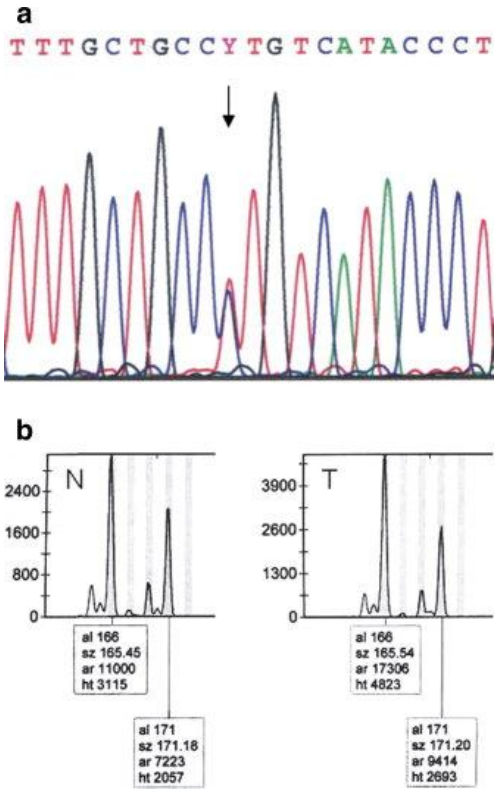

Fig1.

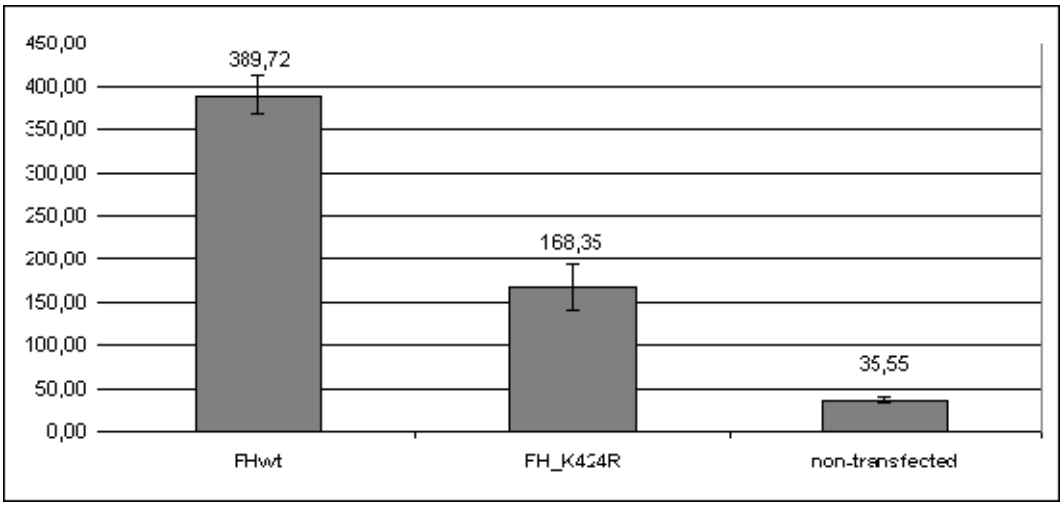

Fig 2.

WP6 Basic molecular characterization of collected sample materials

The molecular characterization of population based registries provides a unique tool for an overview of existing germline mutations and clinical relevance. The TCAC in Cancer project focused on mutations of the genes SDHB, SDHC, SDHD and FH.

Nationwide efforts were undertaken in Poland and Germany and in The Netherlands, represented by the centers in Warsaw, Freiburg and Leiden. This network is based on all University Medical Centers and many large city hospitals in Germany and Poland, and was extended in The Netherlands. For mutation screening PCR was conducted for all exons of the genes SDHB (8), SDHC (6), SDHD (4), VHL (3) and for 3 exons (exons 10, 11 and 16) of the RET gene.
Epidemiological studies were performed for Germany. Some of the work conducted under this WP extends that described in the original contract, and was funded partially by the German Cancer Foundation and the German Research Foundation.

For FH mutation screen the efforts focused on Finnish specimens, which were scrutinized by genomic sequencing. During the project also an MLPA assay for detection of FH mutations was developed. The sample sources were the ones shown in WPs 2, 4 and 5, as well as other occasional cases suspect of HLRCC

A total of 1377 SDHx and 85 FH mutation carriers were identified during this 4-year effort.

<u>The SDH mutation database</u>
The SDH mutation database was launched in 2005 (1) and is now part of the TCA Cycle Gene Mutation Database. This database has now firmly established itself as an indispensible tool for all with interest in paraganglioma/pheochromocytoma. The database attracted 15,000 page reads in 2006, 37,515 page in 2007, 76079 in 2008, and 193,544 in 2009. The database attracted an average monthly usage of 456 unique IP addresses in 2009.
The database now plays a central role in the analysis of genetic aspects of SDH tumor syndromes and is an indispensible resource for clinicians, clinical geneticists, and researchers interested in paraganglioma and pheochromocytoma. The database currently contains 353 unique variants in the SDH genes.

<u>The FH mutation database</u>
A database of FH mutations, hosted in Leiden on the LOVD database platform, has been established (2). This database includes all published FH mutations, and has become the central registry for FH mutations. The database includes an initiative to unify and standardize FH mutation nomenclature with HGVS standard mutation nomenclature. In addition contact has been initiated with the national FH testing centre in The Netherlands and input is expected. The FH mutation database currently describes 111 unique mutations.
In addition to these genes, we have plans to expand the database to describe mutations in other, recently identified TCAC-related tumor suppressor genes, including isocitrate dehydrogenase and SDHAF2.

WP7 Expression profiling of SDH and FH lesions. Creation of a classifier for TCAC defect, to examine other tumor types.

SDH-classifier

We have performed a gene expression study evaluating gene expression in sporadic, SDHD-linked and PGL2-linked head and neck paragangliomas with a focus on analysis of hypoxia, glycolysis, tricarboxylic acid cycle and oxidoreductase related pathways. We found that there is great similarity between overall gene expression profiles of sporadic, SDHD-linked and PGL2-linked head and neck paragangliomas. Furthermore, we were not able to find significant differences in gene expression between sporadic, SDHD and PGL2-linked samples in hypoxia, glycolysis, tricarboxylic acid cycle and oxidoreductase related pathways.

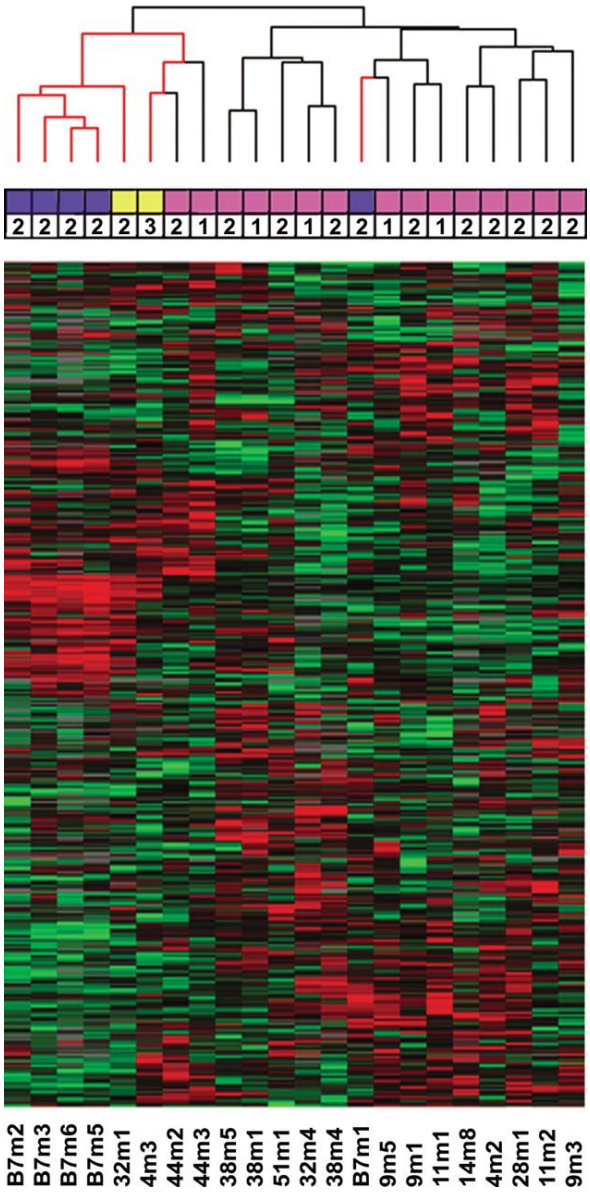

Figure 3: A dendrogram showing the unsupervised clustering of the initial set of Finnish samples. Red branches show FH-mutant fibroids. Blue, yellow and purple boxes indicate HLRCC, non-syndromic FH-mutant and FH wild-type fibroids, respectively. The numbers on the top refer to the semi-quantitative assessment of fibroid cellularity. In the expression matrix, green indicates low and red high relative expression; gray represents missing data.

FH classifier

We have used an expression microarray approach for studying differences in global gene expression pattern caused by mutations in FH. Seven uterine fibroids carrying FH mutations were compared with 15 fibroids with wild-type FH. The two groups showed markedly different expression profiles, and multiple differentially expressed genes were detected (Fig 3). The most significant increase in FH mutants was seen in the expression of carbohydrate metabolism- and glycolysis-related genes. Other significantly up-regulated gene categories in FH mutants were, for example, iron ion homeostasis and oxidoreduction. Genes with lower expression in FH-mutant fibroids belonged to groups such as extracellular matrix, cell adhesion, muscle development and cell contraction. We show that FH mutations alter significantly the expression profiles of fibroids, most strikingly increasing the expression of genes involved in glycolysis.

We then wanted to validate the previously created classifier as a predictor of the FH mutation status also in this sample set. Probe sets for six of the seven genes in the initial classifier analysis (NQO1, BNIP3, LAMA2, COL6A2, MYO15B, and CDKN1C; Vanharanta et al., 2006) passed the filtering and were present in the custom annotation files. When all 33 samples were analyzed five of the six genes (all but CDKN1C) were differently expressed between

the groups, and all tumors except one HLRCC fibroid (B7m2 in Fig 4) were correctly clustered. The classifier also correctly predicted tumors from an individual with both wild-type and FH deficient tumors (M32m1 and M32m8). When only the 15 new samples were analyzed, five of the genes were differentially expressed (all but LAMA2) and all tumors clustered correctly according to the FH mutation status (Fig 4). The classifier thus correctly predicted the FH mutation status also in this independent tumor set.

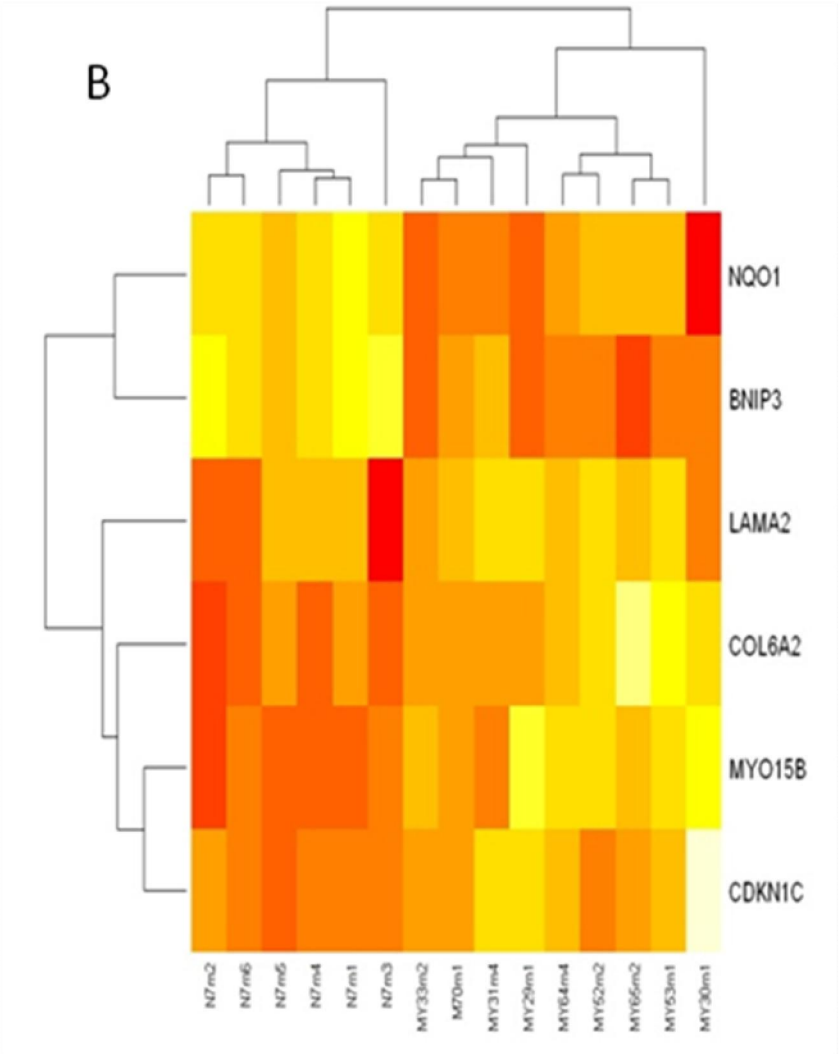

Fig 4. The classifier created by Vanharanta et al., (2006) efficiently predicts the FH mutation status in the sample set including previously analyzed and novel samples or only novel samples. B7 and N7 are HLRCC patients and M and MY refer to individuals with sporadic tumors.

To analyze whether the genes in the classifier could be used in identifying other tumor types with possible defects in TCAC, we used GeneSapiens database, which contains expression data of 17 330 genes from nearly 10 000 samples belonging to 175 healthy and pathological tissues (Kilpinen et al, Genome Biol, 2008). Maximum number of genes that can be analyzed simultaneously in the database is three, so we chose three most significant genes in the classifier, NQO1, BNIP3 and MYO15B, and evaluated the correlation of these genes and tumor types. Interestingly, tumors of the kidney and uterus that are known to associate with FH defects stood out in the correlation plot (Fig 5). Of note is also one distinguishable glioma sample as mutations

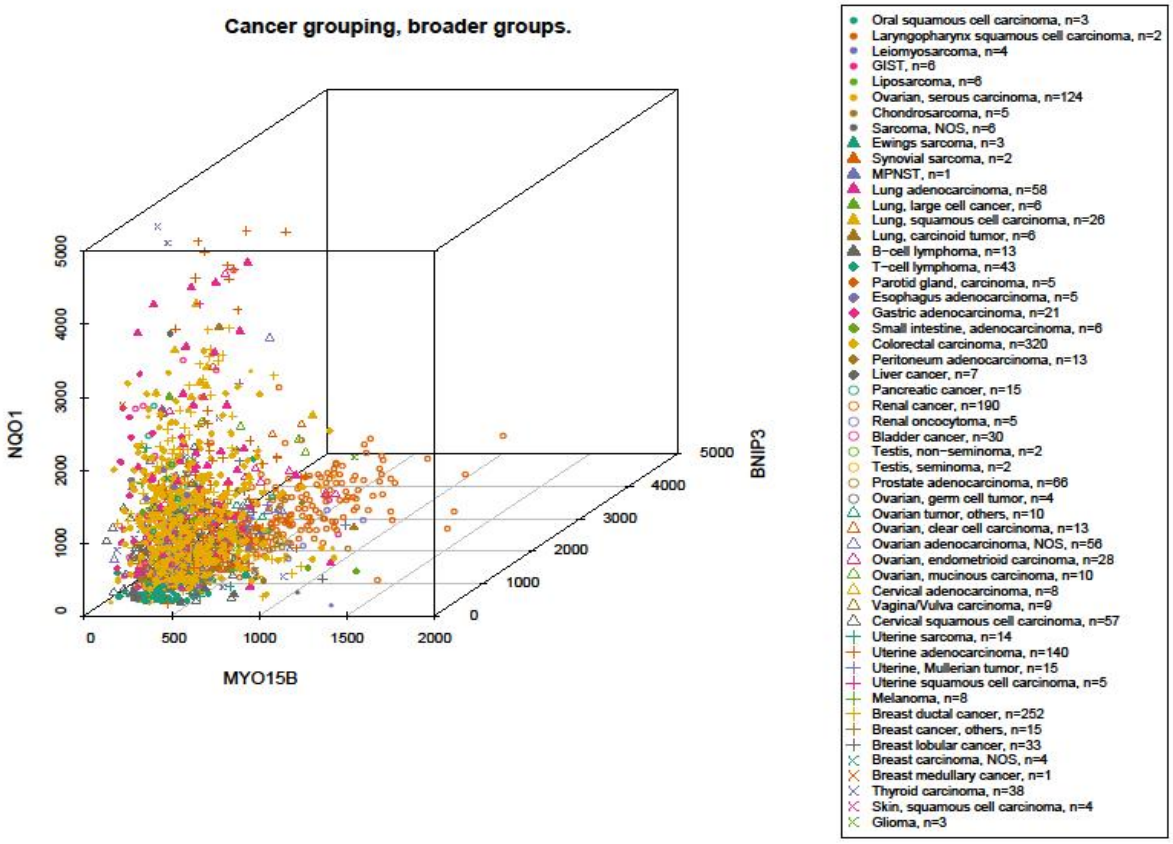

Fig 5. Correlation plot of the three most significant FH classifier genes, NQO1, BNIP3, and MYO15B, and the tumor samples in the GeneSapiens database.

WP8 Identification of the FH modifier gene

To detect putative modifier loci we first re-analyzed the Finnish HLRCC genome wide-linkage search data (Launonen *et al.* 2001) including two families with malignancies. This re-analysis of data gave 6 interesting loci. In order to gain coverage of data and to get information from an additional HLRCC family, genotyping with 2000 microsatellite markers was scrutinized. Haplotypes on linkage-positive regions were examined and loci with shared haplotypes within affected individuals in each family were further analyzed with denser marker sets. Fine-mapping on 20 loci with more than 600 markers were scrutinized in families with the malignant phenotypes. Genotype data from up to 70 individuals, including 11 RCC cases and 4 suspected uterine leiomyosarcomas, was produced for further haplotype and linkage analyses. The most promising loci were on chromosomes 6, 12, and 22. During the project one of the leiomyomatosis patients was diagnosed with RCC (patient B3). We succeeded to exclude all regions except chromosome 22 q-telomere, where all the renal cell cases shared same haplotypes within all linkage informative families. Altogether, 19 microsatellite markers and 12 SNPs were

used to fine-map the chromosome 22 shared region down to 2.3Mb in q13.31-q13.33. The added maximum LOD score for the six families included in the analyses was 2.71. LOH was examined on 18 loci. Consistent LOH shared with malignant tumors was not detected in any other than FH locus.
Because a modifier allele can be predicted to be common we postulated that a limited number of founder mutations (and identical by decent haplotypes, IBD) could be the most logical explanation for the observed segregation of malignancies in HLRCC families. In addition to fine-mapping data we performed genome-wide SNP array experiment (Affymetrix 50K and 100K SNP arrays) on individuals with RCC (4 individuals) and their healthy 1st degree relatives (7 individuals) in order to define IBD regions between families. All possible combinations were inspected but no logical overlap with interesting regions from linkage analysis was discovered.

Gene expression analysis
The rationale for expression analysis is that the modifier might affect expression level rather than coding sequence of the gene. Peripheral blood samples from 12 obligate carriers of the modifier, including two Finnish and seven foreign RCC cases, were collected for RNA extraction and expression experiments. Comparison with a set of control specimens did not reveal decisive clues to identify the FHM.

Mutation analysis of candidate genes
An affected representative (or an obligatory carrier) from each family was submitted to mutation analyses. Genomic sequencing was performed for most of the candidate genes, altogether 42 during the whole duration of the project, on 10 RCC cases and two ULMS samples and the RCC cell line. Data from linkage/ haplotype analysis, expression arrays, aCGH, LOH, IBD analysis, and in addition from Y2H experiment and information from publications and public databases were merged in order to select the most promising genes for mutation analysis.
Although the coding exons of all chromosome 22 target region genes were sequenced (except exon 1 in *FAM19A* and exon 11 in *BRD1* which were unsuccessful) no pathogenic mutations were found.
After screening the genes at the chromosome 22 locus with negative results, a fifth RCC case in FAM-1 was diagnosed. This individual was found not to share the chromosome 22 region. Thus, the only remaining region compatible with linkage was the *FH* locus itself. To study the possibility of a *FH*-linked modifier, we further examined the chromosome 1q43 region; in particular *CHRM3*, *FMN2*, and *GREM2*. All coding regions of these three genes were sequenced but no mutations were observed.

Thus despite these efforts, we have not found evidence of a genetic modifier for RCC in the context of HLRCC syndrome. Even though results obtained so far do not exclude the possible existence of a genetic modifier, *FH* inactivation alone may be the key event in malignant kidney tumor formation.

To conclude, despite vigorous efforts identification of the FHM was not successful. The possible reasons for this failure include:

1) The hypothesis, while scientifically sound and interesting, may have been incorrect. Perhaps issues such as environmental factors and coincidence play a part in HLRCC related malignancy.

2) The hypothesis is correct but the genetic modifiers are many and the sample material thus would be too limited to identify these.
3) The hypothesis is correct but the nature of the responsible genetic variant did not allow its detection. Eg it is not trivial to cover all possible forms of variation, and changes occurring in poorly characterized regulatory regions add to the challenge.

While it is unfortunate that this WP was not successful in its final scientific goal we need to conclude that the possibility of such an outcome inherently belongs to science, and the efforts described in the contract to reach the goal were fulfilled and exceeded.

WP9 Identification of the SDHD modifier gene

We approached the question through an animal model. *Sdhd* knock-out mouse was crossed to *H19* knockout mice to examine whether the *H19-IGF2* axis is capable of generating a tumor phenotype in a $Sdhd^{+/-}$ background.

The *Sdhd* KO mouse was crossed to the well-known del13 *H19* KO. The tumor cohort was established and the mean age of the mice was 22.3 months at death. There were no outward signs of tumor development and on necropsy no genotype specific pathology was found. *H19* cannot be definitively excluded as a modifier of PGL in the mouse, because no PGL tumor has yet been reported in any mouse. However, several other imprinted genes are still good candidates as the modifier of SDHD tumorigenesis.

The definitive identification of the *SDHD* modifier gene will require specific models of PGL tumorigenesis. While mouse models and specific cell lines are being developed, other avenues of investigation can still be explored including the role of loss of chromosome 11 in paraganglioma. The striking parent-of-origin inheritance pattern of *SDHD & PGL* linked tumors in man strongly suggests the involvement of an imprinted locus in paragangliomas. The most parsimonious hypothesis suggests that the maternal alleles of *SDHD & PGL2* are imprinted, negating the effect of any mutation of these alleles. While this initially seems rather unlikely because paternal mutation carriers would lack a functional gene and would be expected to die *in utero*, loss of imprinting or tissue/cell-specific imprinting remain possibilities. However, all evidence indicates that *SDHD* itself is not imprinted and paragangliomas often show loss of the maternal copy of chromosome 11, counterintuitive if the maternal *SDHD* allele is imprinted and thus non-functional. Together these data indicate that a modifier on chromosome 11 is important in at least *SDHD*-related paragangliom and perhaps more widely. The presence of the major imprinted locus of the human genome on chromosome 11p15.5 may not be coincidental to this story, and we would argue that a maternally expressed, imprinted gene on chromosome 11p15.5 is a compelling candidate as a tumor modifier, which in addition to loss of the *SDHD* maternal allele, must also be lost prior to initiation of tumorigenesis. *SDHD* & *PGL2*, together with the modifier could be targeted in a single genetic event (whole chromosome loss) in man. *SDHB* and *VHL* would require two genetic events.

To conclude, despite strong evidence to support the existence of SDHD modifier the final scientific goal, identification of the gene, was not achieved. Probably the gene is not H19. Only recently other possible approaches/candidates have been described, including imprinting of the gene for tyrosine hydroxylase, the rate limiting step of catecholamine synthesis, an essential player in the clinical manifestations of pheochromocytoma. Additional studies have also become feasible due to development of new mouse models.

WP10 Identification of novel genes predisposing to paragangliomas/pheochromocytomas

Haplotyping of the 104 available *PGL2* family members was performed first to refine the linked genetic region on Ch11q13. We eliminated 65 genes in the PGL2 region as candidates for PGL2. However, due to contact with the group of Jared Rutter at The University of Utah, we were able to identify PGL2 without further gene characterization. Together with Rutter and long-time collaborators in Nijmegen, we were able to show that PGL2 is a previously uncharacterized gene, originally designated as SDH5, but now assigned the official gene name, SDHAF2 (succinate dehydrogenase complex assembly factor 2). SDHAF2 is a soluble mitochondrial matrix protein that interacts with the catalytic flavoprotein subunit of the SDH complex (Sdh1/SDHA) and is necessary and sufficient for the incorporation of the flavin group (FAD) cofactor in this protein. We identified a mutation in a highly conserved region of human SDHAF2 gene in the Dutch SDHAF2 (PGL2) kindred, showed co-segregation with disease and described the striking parent-of-origin mode of inheritance. This mutation causes a complete loss of SDH flavination and activity.
In a follow-up study, we sought to identify further SDHAF2 mutation carriers, assess the clinical genetic significance of SDHAF2, and describe the associated clinical phenotype. We assembled a multi-centre cohort including a total of 443 non-SDHx paraganglioma/pheochromocytoma patients, who were analysed for mutations of SDHAF2, including gene deletion and somatic mutation analysis. In addition, we analysed an additional Spanish paraganglioma family for SDHAF2 mutations, and carried out haplotype analysis.
Surprisingly no germline or somatic mutations of SDHAF were identified in the 443 multi-centre cases.
Analysis of the Spanish family identified a pathogenic mutation of SDHAF2, c.232G>A (p.Gly78Arg), identical to that found in the Dutch SDHAF2 (PGL2) kindred. Haplotype analysis of the two families provided no evidence of relatedness, suggesting that this is a re-current mutation, and possibly explaining the rarity of these mutations in paraganglioma/pheochromocytoma cases. Three of the Spanish patients had head and neck paraganglioma with a strikingly young age-of-onset.
We were able to conclude that SDHAF2 mutations do not play a significant role in pheochromocytoma, and are also remarkably rare in head and neck paraganglioma. The identification of a second family with the p.Gly78Arg mutation suggested that this is a critical residue for the function of SDHAF2.

WP11 FH knockout mouse model

The plan to create of this model of the human disease aimed to provide a resource to enable the mechanisms of tumorigenesis in human HLRCC to be worked out.
We created a conditional $Fh^{fl/fl}$ knockout animal in which exons 2 and 3 of Fh were flanked by loxP sites (Fig 6).

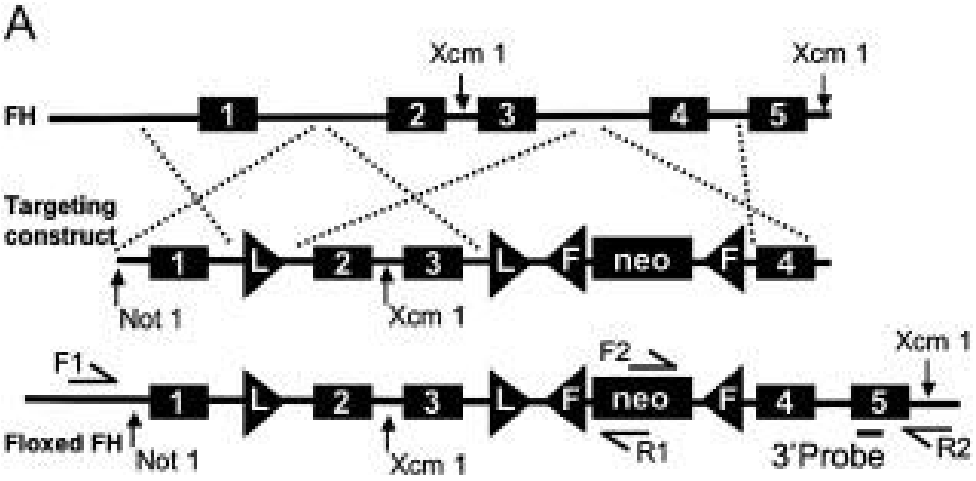

Fig 6. $Fh^{fl/fl}$ mouse construct

Cre-mediated excision of these exons creates a null allele that corresponds to the loss of function found in human HLRCC. We initially crossed these mice to Pgk-Cre expressing animals to make a simple knockout allele. The $Fh^{-/-}$ animals died in early embryogenesis of gross developmental defects, and the $Fh^{+/-}$ animals have not yet developed any specific phenotype.

We crossed the $Fh^{fl/fl}$ animals with Sm-MHC-cre transgenics that were known to express Cre specifically in smooth muscle. The mice develop hypertrophy of the circular and longitudinal muscle of the small bowel.

We then aimed to make a model of HLRCC renal disease (cysts and carcinomas). We therefore crossed our $Fh^{fl/fl}$ animals with Ksp-cadherin-Cre animals. The Ksp-Cdh promoter drives gene expression primarily in collecting ducts and developing tubules (but not glomeruli or mesenchymal cells) of newborn animals, and in renal tubules, especially collecting ducts and thick ascending limb of loop of Henle), of adults. $Fh^{fl/fl}$ Ksp1.3/Cre animals appeared healthy until 8 months of age, when polyuric renal failure became apparent in some animals. All other animals of this genotype eventually became ill, the oldest presenting at 15 months of age. Postmortem examination showed macroscopic renal cysts in all $Fh1^{fl/fl}$ Ksp1.3/Cre animals. The kidneys showed extensive cystic change in the cortico-medullary junction with irregular cysts lined by cuboidal epithelium, consistent with a derivation from the loop of Henle and subsequently confirmed by immunohistochemistry (Fig 7).

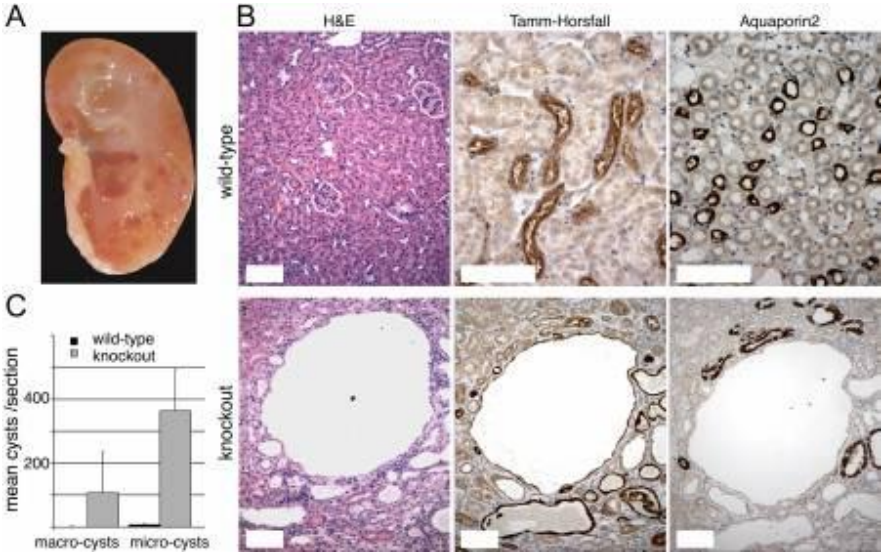

Fig 7. Mice with Renal-Specific Fh1 Knockout Develop Multiple Tubule-Derived Cysts

(A) The figure shows gross features of a bisected kidney from an *Fh1*$^{fl/fl}$ Ksp1.3/Cre mouse aged 12 months.

(B) The figure (scale bars, 250 μm) shows H&E staining of typical wild-type and cystic *Fh1* knockout kidneys. Thp immunostaining of wild-type and knockout kidneys indicates origin of this cyst from the thick ascending limb of the loop of Henle. Aquaporin2 immunostaining of wild-type and knockout kidneys indicates that the cyst is not collecting-duct derived.

(C) A cut-off of >0.1 mm diameter was used to classify a lesion as a cyst, and >0.5 mm diameter was used to classify a cyst as macroscopic. The median numbers of macroscopic and microscopic cysts detected in hemisections of kidneys of eight knockout (*Fh1*$^{fl/fl}$ Ksp1.3/Cre) and eight control mice are shown (for the microscopic cysts, p = 0.0006; for the macroscopic cysts, p = 0.0008, Mann-Whitney test). Kidney size did not differ significantly between the two groups of mice (details not shown). y error bars indicate ±SEM.

We confirmed loss of Fh1 in the cyst epithelium and showed that both complex and simple cysts were of clonal origin and expressed markers of proliferation. Renal cysts from ten animals (five knockouts and five *Fh1* wild-type littermate controls) were analyzed for expression of hypoxia pathway genes/proteins (Hif1α, Hif2α, Glut1, Caix, Vegf and Tgfa) (Fig 8). Compared with controls, Hif1α was over-expressed in the epithelia of all renal cysts from knockout animals, compared with noncystic kidney from the same animals. Nuclear Hif2α overexpression was also found consistently in cysts from the knockouts, although in a lower proportion of cyst epithelial cells. There was no association (positive or negative) between Hif1α and Hif2α expression. Glut1 and *Vegf* overexpression were found wherever there was Hif1α over-expression. Interestingly, however, expression of the HIF1α target Caix was almost undetectable in the cysts. Moreover, the Hif2α-target *Tgfa* was not expressed in any cyst, even those with positive Hif2α staining. Human HLRCC tumours showed similar patterns of expression.

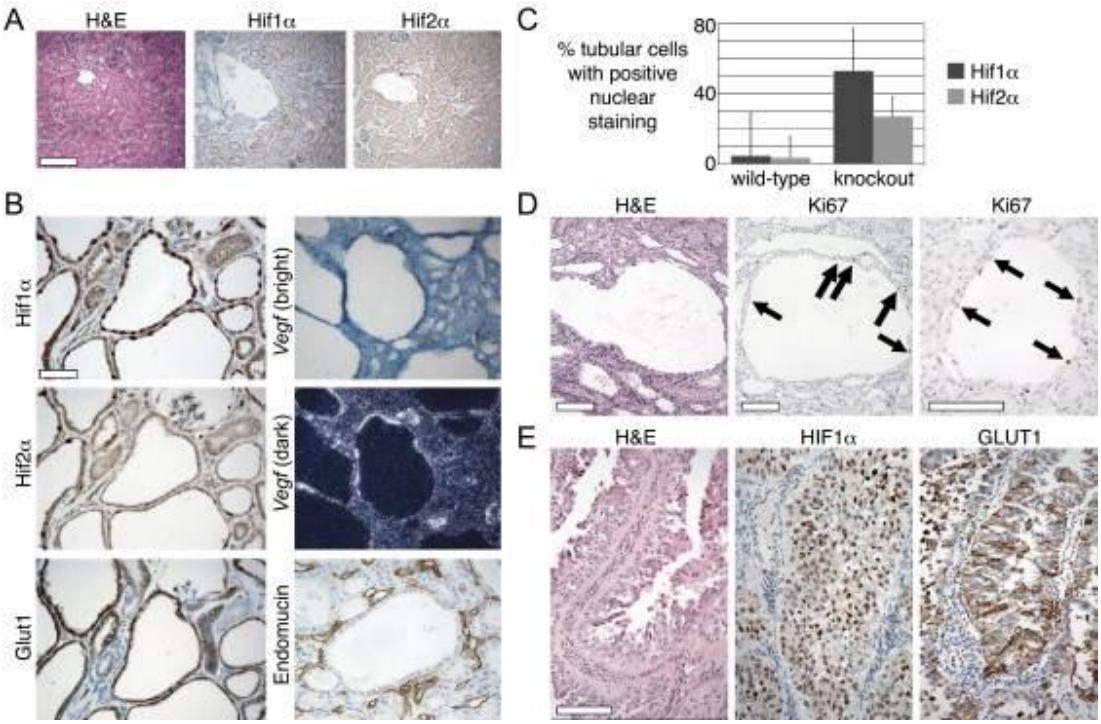

Fig 8. Analysis of Proliferation and Hypoxia-Related Genes in Fh1 Null Renal Cysts and Human Type II Papillary Carcinomas

(A) *Fh1* wild-type kidney shows weak cytoplasmic staining of Hif1α and Hif2α, and absence of nuclear positivity. Scale bar, 250 μm for all panels.

(B) *Fh1* knockout renal cysts show strong nuclear expression of Hif1α in the epithelial layer. Hif2α nuclear staining occurs at a lower frequency than that of Hif1α in cyst epithelium. The protein product of the Hif1-target gene Glut1 is expressed in the epithelial layer, as is *Vegf* mRNA (β-actin control not shown). High vascularity around the lesion is indicated by the endomucin immunostaining. Scale bar, 125 μm for all panels.

(C) The proportions of cyst cells expressing nuclear Hif1α and Hif2α in ten random high-power fields from kidneys derived from each of three control and four knockout mice are shown. The differences between the knockouts and controls were highly statistically significant ($p < 0.0001$ for both Hif1α and Hif2α, Mann-Whitney test). y error bars indicate ± SEM.

(D) A representative renal cyst from an *Fh1* knockout animal shows cells (arrows) with Ki67 immunostaining. Wild-type kidney (not shown) has very little Ki67 expression. Scale bar, 250 μm for all panels.

(E) Human HLRCC type II papillary renal carcinoma shows strong nuclear expression of HIF1α and overexpression of GLUT1. Scale bar, 250 μm for all panels.

We went on to confirm our findings of Hif1α over-expression in includicble Fh knockout and wildtype ES cells from our mice. The phenotype of the mice resembles the cystic – and presumably pre-malignant - lesions seen in HLRCC patients. The hypoxia pathway is activated in response to Fh loss. A cross with Hif1α knockouts is in progress. The phenotype of our mouse is similar to that of $Vhl^{-/-}$ animals, although there are small differences in hypoxia target expression. The mice show that hypoxia pathway activation is an early, and probably direct, event in HLRCC tumorigenesis, and that this is highly likely to eb an important cause of HLRCC renal cancers.

WP12 SDH knockout mouse models

An *Sdhd* targeting construct was designed (Fig 9), ES cells derived and an *Sdhd* KO mouse generated on the 129Ola inbred mouse background. Chimeric founder mice were crossed to wildtype 129Ola mice and to C57BL/6J mice. A tumor cohort was generated on the 129Ola background and followed for approx. 30 months or sacrificed when moribund.

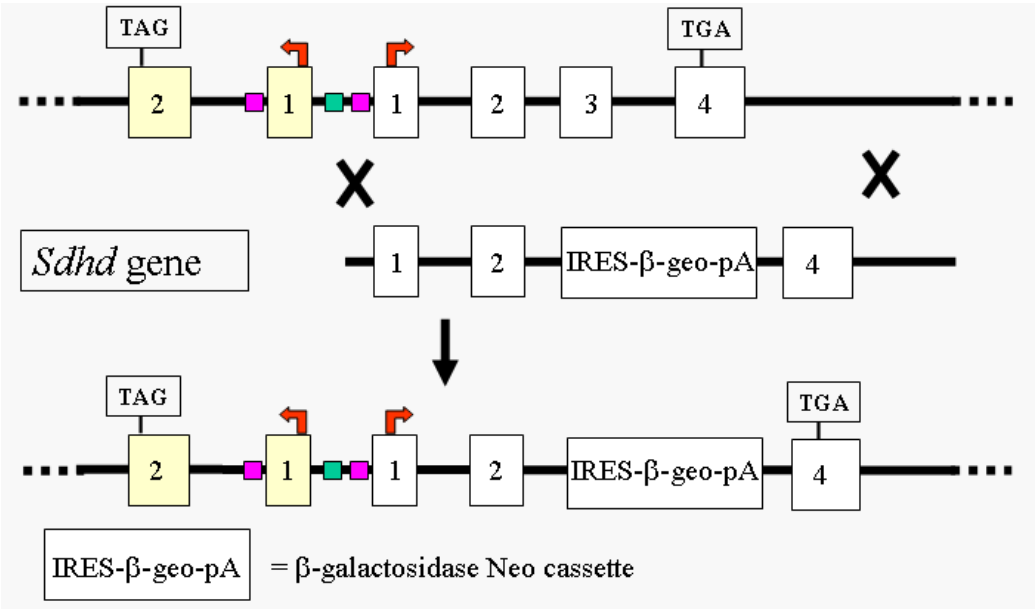

Fig 9. *Sdhd* targeting construct

Heterozygote viability was tested in cohorts on both the 129Ola and C57BL/6J backgrounds. Approximately equal numbers of wt and heterozygote mice were obtained, demonstrating that mice carrying a single allele of *Sdhd* are fully viable. Homozygote viability was tested in a cohort of 129Ola mice. Approximately equal numbers of wt (+/+) and homozygote mice (-/-) were expected, in a ration of 1:2:1. Homozygote mice (-/-) were entirely absent demonstrating that mice with a complete loss of *Sdhd* are not viable.

Quantitative RT-PCR analysis of *Sdhd* expression in several tissues revealed that expression is reduced by approximately 50%, as expected (Fig 10).

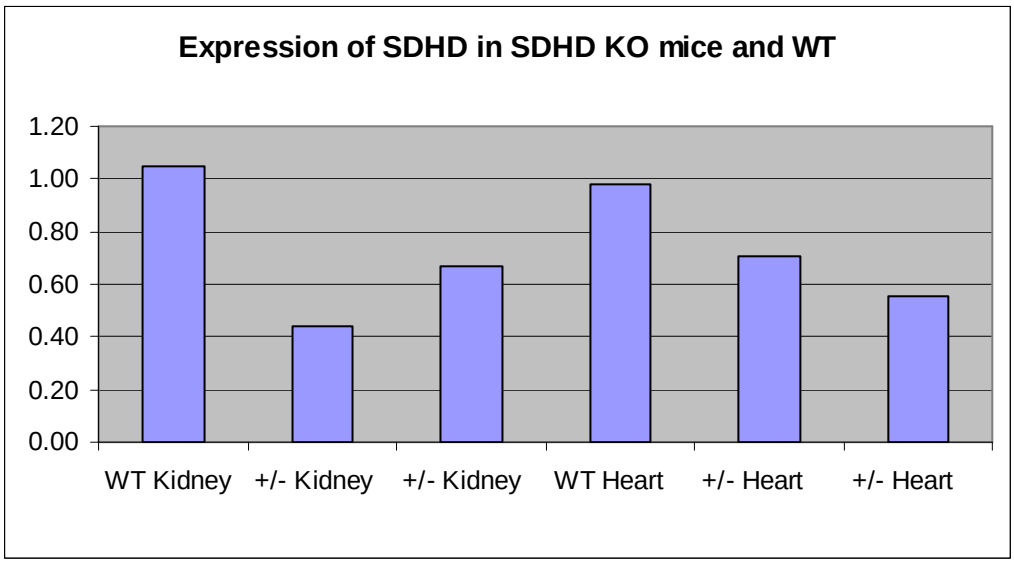

Fig 10. Quantitative RT-PCR of *Sdhd* expression in tissues of *Sdhd* KO and wt mice.

Pathology: All mice (*Sdhd*+/- and wt littermates) were examined for gross signs of neoplasia at sacrifice, and all tissues showing abnormalities and all PGL related tissues (carotid body and adrenal glands) were fixed, embedded in paraffin and HE stained slides prepared. These were examined by both the investigator and an experienced pathologist and the diagnosis noted. The mean age at death of all mice was 19 months (range 4 -32 months). Survival curves (data not shown) revealed no difference in survival between *Sdhd*+/- and wt littermates. No paragangliomas or pheochromocytomas were detected, nor any othert of the noted pathology occurred significantly more frequently in the *Sdhd*+/- mice. We conclude that there was no genotype-specific pathology occurring in this cohort.

While the above results clearly show that no tumor phenotype is present in *Sdhd*+/- mice, it is possible that loss of one allele of *Sdhd* could initiate hyperplasia in the carotid body or adrenal medulla while not leading to full blown neoplasia. To assess this possibility we removed these tissues from all sacrificed mice and prepared HE stained histological slides from these tissues. Serial sections were prepared from all carotid bodies (the leading site of HN paraganglioma), stained, photographed and quantified for gross increase in tissue size (Fig.11).

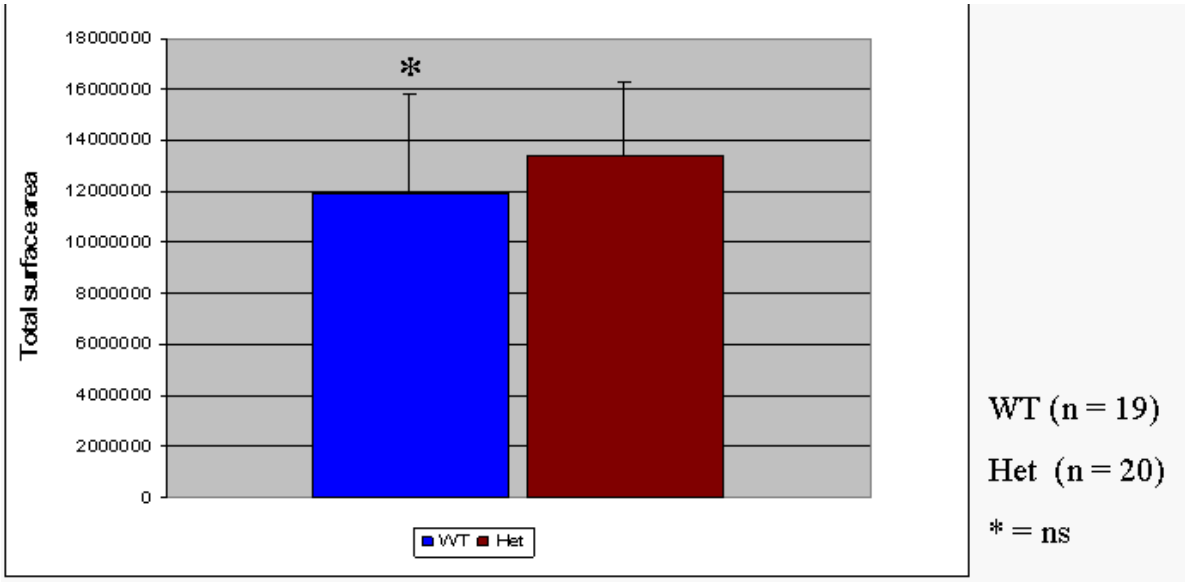

Fig 11. Relative carotid body size (females, age 20 months).

This analysis revealed a subtle but non-significant increase in carotid body size in *Sdhd*+/- mice. The carotid body consists of several cell types while only the chief cell component in thought to be directly involved in neoplasia. To examine the possibility that this cell component is increased without leading to an absolute increase in CB size, we quantified the chief cells of carotid bodies using the specific marker, tyrosine hydroxylase (Fig. 12). This analysis gave similar results to those in Fig. 5, indicating that hyperplasia, if any is actually present, is affecting the chief cell element of the carotid body in a ratio similar to that seen for the entire tissue.

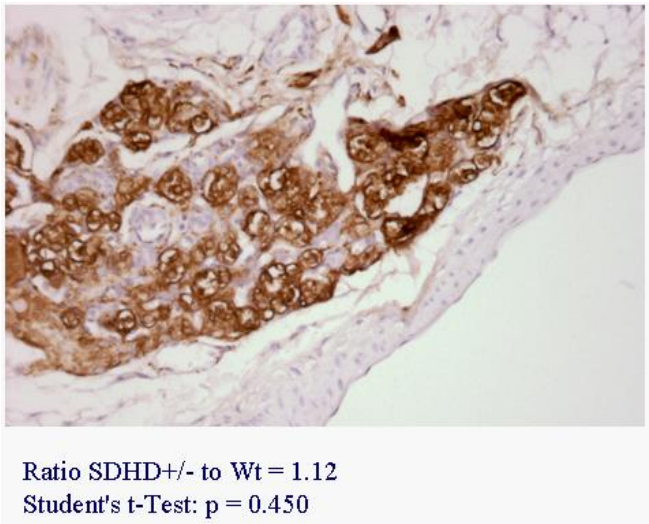

Fig 12.

In addition, we considered the possibility that the loss of *Sdhd* could lead to a metabolic defect affecting gross body size or the weights of specific organs. Neither body weight nor weights of particular tissues were affected by *Sdhd* genotype status (data not shown).

We find no paragangliomas or pheochromocytoma in the *Sdhd*+/- mice. In contrast to the situation in humans where the postulated *SDHD* modifier is located on the same chromosome as *SDHD* and is thought to be targeted in a single genetic event (whole chromosome loss), in the mouse these genetic elements are located on Ch7 and Ch9 respectively. In such a situation, loss of two separate chromosomes (or regions) would be required and could be additionally complicated by the presence of other nearby genes intolerant of allelic loss.

Generation of an *Sdhb* knockout and *Sdhb* conditional knockout.
The conventional *Sdhb* knockout mouse produces no phenotype during its lifespan. One plausible explanation is that 'second hits' at *Sdhb* are not occurring sufficiently often or at the right time during growth and development.
A new approach to create a conditional Sdhb knockout mouse has recently been initiated. The conditional Sdhb construct will be made in collaboration with Eyal Gottlieb of the Beatson Institute, Glasgow. The construct design is a simple replacement one (Fig 13). After successful targeting, the NeoR cassette will be removed by breeding with eFlp animals. Gene knockout will be achieved by Cre-mediated excision of exons 7 and 8; this knocks in expression of a beta-galactosidase marker. Cre expression may be undertaken in vitro (to make simple knockouts), by crossing with animals that express Cre in tissue-specific fashion or by lentiviral-mediated Cre expression. Xenogen Inc. will make the construct and this will be returned to the UK for ES cell targeting and all future steps in mouse production.

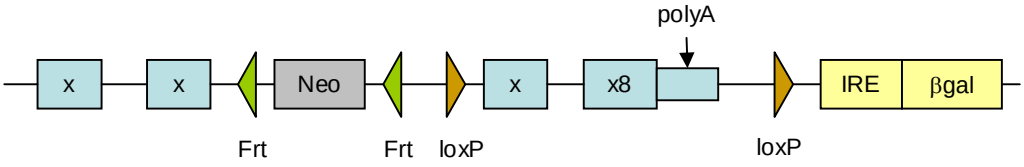

Fig 13.

WP13 Functional studies on TCAC deficiency

We have analyzed twenty-four commercial tumor cell lines and identified two cell lines with *FH* defects. A truncating mutation E181X was found in the renal cancer cell line (CRL7629) and a missense change A32T in the prostate carcinoma cell line (DU145). We have also created pEGFP- (fluorescent protein tagged expression) and pCi-Neo- (mammalian expression) based constructs expressing WT and mutant FH. Two different mutant constructs (the missense mutation A32T and the Finnish founder mutation H153R) were created to the basic FH-pEGFP and FH-pCI-neo vectors with a site-directed mutagenesis. In addition, IRES-EGFP (retroviral expression with additional GFP expression) constructs were produced to attain efficient transduction and stably restored expression of FH in the FH deficient CRL7629 cells. However, the retroviral gene delivery seemed not to be suitable for these cells; morphology of the cells changed and the proliferation slowed down (with vector control also).

Several RNAi systems were created. Using stable RNAi based on pSUPER vector we were able to decrease the FH expression up to 70%. In HEK293 cells, when using transient RNAi oligos we were able to detect over 90% decrease in mRNA expression. Moreover, we have designed vectors used to generate a stable transfectants with inducible FH knockdown.

Experiments:

Cellular distribution and characterization of WT and mutant FH. The subcellular localization of WT and mutant forms of FH was studied by overexpressing the created constructs in the FH proficient renal cancer cells (CRL1933). No striking differences in localization of the WT and mutants (FH_{H153R} and FH_{A32T}) were observed. We next measured the FH enzyme activity of the mutant cell lines (DU145 and CRL7629). In addition, the enzyme activity was measured from the FH deficient myoma cell line and from the FH deficient fibroblasts. The FH proficient CRL1933 cells were used as a control. Reduced FH enzyme activity was found in mutant cell lines as expected (Fig 14). We have also measured the FH enzyme activity of FH_{H153R} mutant in the cell model. The wild type *FH* (FH_{WT}) was used as a positive control and non-transfected cells as a negative control. The enzyme activity of cells with FH_{WT} increased 10-fold compared to non-transfected cells. In FH_{H153R} transfected cells activity was that of endogenous cells indicating FH_{H153R} to lack FH activity.

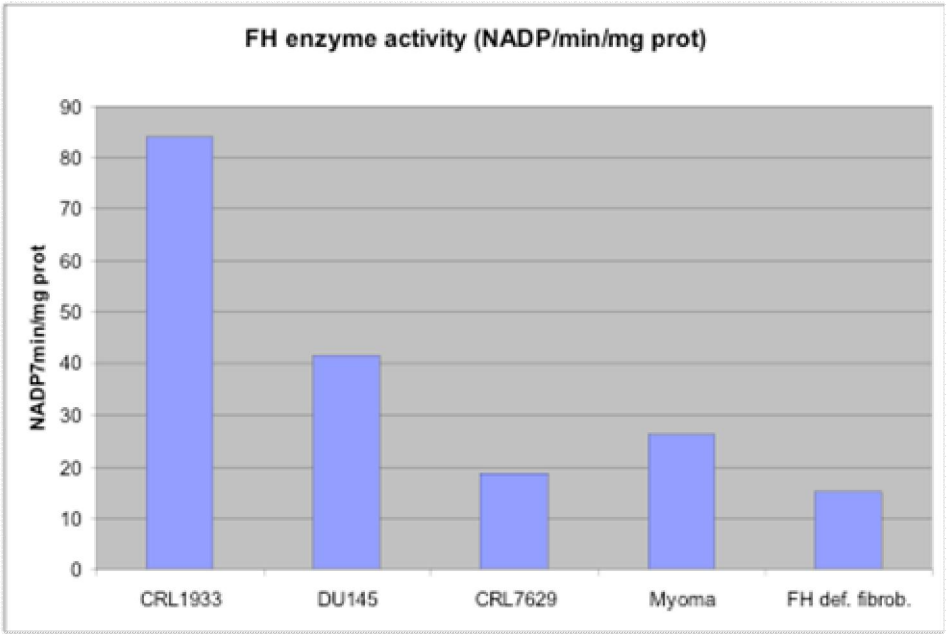

Fig 14. The results of FH enzyme activity assay. The activity of the mutant cells is reduced when compared to the FH proficient cells (CRL1933).

Functions of FH in tumorigenesis.

A set of three stable FH RNAi cell clones (established from RCC line CRL1933) were analyzed for cell proliferation rate utilizing two colorimetric assays based on MTT (diphenyltetrazolium bromide) and SRB (sulforhodamine-B). MTT is in living cells enzymatically cleaved from a pale yellow form to blue, whereas SRB dye is bound to proteins and thus correlates with protein synthesis and cell proliferation. The number of viable cells in both assays can be quantified by a spectrophotometric analysis. At least with this RNAi tool, no clear difference in proliferation rate between cells carrying FH RNAi and the controls (transfected with pSUPER vector only or siRNA construct against GFP) was seen. In conclusion, the primary effect of FH knock-down seems not to be directly related to cell division

As another approach to study functional consequences of FH and SDH defects, we conducted a study evaluating possible link between hypoxia inducible factor 1α (HIF1α) and microsatellite instability (MSI) in FH and SDH related tumorigenesis. According to previous reports, HIF1α is stabilized resulting from defect in FH and SDH function. Interestingly, in other context (in cell models and colorectal cancer in vivo) HIF1α has been suggested to cause down-regulation of the mismatch repair gene MSH2 and subsequently induce MSI. To study stabilization of HIF1α and expression of MSH2 in TCAC deficient tumors, tissue microarray containing SDHB/C/D paragangliomas/pheochromocytomas (described in WP7) and 24 samples set of FH associated tumors (RCCs, ULMs, and ULMS) were analyzed. In addition, an extensive number of tumor DNAs was examined for the microsatellite status. In agreement with previous reports, HIF1α was found to be stabilized in 77% and 67% of SDHB/C/D and FH tumors, respectively. However, no repression of MSH2 or induction of MSI was observed. In conclusion, our results do not provide supporting evidence in vivo on HIF1α driven MSI. Possible tissue specificity and additional components of the pathway, therefore, remains to be elucidated.

Effects of hypoxia, serum and glucose deprivation
Two FH proficient cell lines (HEK263 and Hela) were cultured in different stress conditions including glucose or serum deprivation, hydrogen peroxide exposure, hypoxia, and heat shock. After the treatments total RNA was extracted from the cells and relative mRNA expression of FH was analyzed by quantitative PCR. However, no changes were seen in the mRNA level of FH after treatments. We have also identified and characterized a novel *FH* variant (FHv) that contains an alternative exon 1b, thus lacking the mitochondrial signal sequence. Distinct from the mitochondrial FH, FHv localized into cytosol and nucleus and lacked the FH enzyme activity. FHv was expressed ubiquitously in human fetal and adult tissues. Heat shock and prolonged hypoxia was observed to induce FHv expression in a cell line (HTB115) to nine and four-fold, respectively. These results suggest that FH might have an alternative function outside TCAC related to cellular stress response.

Search for interaction partners of FH.
Five FH interaction partners identified by yeast two-hybrid approach have been confirmed by the mating experiment. Whether these interactions are relevant in human cells is under study.

2. Dissemination and use

The results shed new light on TCAC related tumors; mechanisms of disease and details of the clinical features. A large number of at risk individuals have been identified during the four years, and this will directly result in early diagnosis of neoplasia in this population group. The results have been published without delay in international, peer-reviewed journals. FH and SDH mutation databases have been created (http://chromium.liacs.nl/lovd_sdh/home.php). Research has also been highlighted in scientific meetings, and in journals aimed at medical professionals in different European languages. The many publications and presentations are listed in the enclosed table.

Planned / actual Dates	Type	Type of audience	Countries addressed	Size of audience	Partner responsible/ involved
2006	*Publication* Kokko A, Ylisaukko-oja SSK, Kiuru M, Takatalo MS, Salmikangas P, Tuimala J, Arango D, Karhu A, Aaltonen LA & Jäntti J. Modeling tumor predisposing *FH* mutations in yeast; effects on fumarase activity, growth phenotype and gene expression profile. *International Journal of Cancer* 118, 1340-1345, 2006	*Research*	*worldwide*		*Partner 1*
2006	*Publication* Lehtonen HJ, Kiuru M, Ylisaukko-oja SK, Salovaara R, Herva R, Koivisto PA, Vierimaa O, Aittomäki K, Pukkala E, Launonen V & Aaltonen LA. Increased risk of cancer in patients with fumarate hydratase germline mutation. *Journal of Medical Genetics* 43, 523-526, 2006.	*Research*	*worldwide*		*Partner 1*
2006	*Publication* Vanharanta S, Pollard PJ, Lehtonen HJ, Laiho P, Sjöberg J, Leminen A, Aittomäki K, Arola J, Kruhoffer M, Orntoft TF, Tomlinson IP, Kiuru M, Arango D & Aaltonen LA. Distinct expression profile in fumarate hydratase-deficient uterine fibroids. *Human Molecular Genetics* 15, 97-103, 2006	*Research*	*worldwide*		*Partner 1, 6*
2006	*Publication* Ylisaukko-oja S, Kiuru M, Lehtonen HJ, Lehtonen R, Pukkala E, Arola J, Launonen V & Aaltonen LA. Analysis of *Fumarate Hydratase* Mutations in a Population Based Series of Early Onset Uterine	*Research*	*worldwide*		*Partner 1*

	Leiomyosarcoma Patients. *International Journal of Cancer* 119, 283-287, 2006.				
2006	*Publication* Ylisaukko-oja SK, Cybulski C, Lehtonen R, Kiuru M, Matyjasik J, Szymańska A, Szymańska-Pasternak J, Dyrskjot L, Butzow R, Orntoft TF, Launonen V, Lubinski J & Aaltonen LA. Germline *fumarate hydratase* mutations in patients with ovarian mucinous cystadenoma. *European Journal of Human Genetics* 14, 880-883, 2006.	*Research*	*worldwide*		*Partner 1*
5/2007	*Publication* Lehtonen HJ, Blanco I, Piulats JM, Herva R, Launonen V & Aaltonen LA. Conventional renal cancer in a patient with fumarate hydratase mutation. *Human Pathology* 2007 vol:38 iss:5 page:793	*Research*	*worldwide*		*Partner 1*
5/2007	*Publication* Vanharanta S, Wortham NC, Langdorf C, El-Bahwary M, van der Spuy Z, Sjöberg J, Lehtinen R, Karhu A, Tomlinson IPM, Kiuru M, Arango D & Aaltonen LA. Definition of a minimal region of deletion of chromosome 7 in uterine leiomyomas by tiling-path microarray CGH and mutation analysis of known genes in this region. *Genes, Chromosomes and Cancer* 46, 451-458, 2007	*Research*	*worldwide*		*Partner 1*
9/2007	*Publication* Lehtonen HJ, Mäkinen MJ, Kiuru M, Laiho P, Herva R, van Minderhout I, Hogendoom PCW, Cornelisse C, Devilee P, Launonen V & Aaltonen LA. Increased HIF1 in SDH and FH deficient	*Research*	*worldwide*		*Partner 1, 3*

	tumors does not cause microsatellite instability. *International Journal of Cancer* 2007 Sep15;121(6):1386-9				
2007	*Publication* Lehtonen HJ, Ylisaukko-Oja SK, Kiuru M, Karhu A, Lehtonen R, Vanharanta S, Jalanko A, Aaltonen LA & Launonen V. Stress-induced expression of a novel variant of human fumarate hydratase (FH). *Gene Expression* 14, 59-69, 2007.	*Research*	*Worldwide*		*Partner 1*
2008	*Publication* Ahvenainen T, Lehtonen HJ, Lehtonen R, Vahteristo P, Aittomäki K, Baynam G, Dommering C, Eng C, Gruber S, Grönberg H, Harvima R, Herva R, Hietala M, Kujala M, Kääriäinen H, Sunde L, Vierimaa O, Pollard PJ, Tomlinson IPM, Björck E, Aaltonen LA & Launonen V Mutation screening of *fumarate hydratase* by MLPA: detection of exonic deletion in a patient with leiomyomatosis and renal cell cancer *Cancer Genetics and Cytogenetics* 183, 83-88, 2008.	*Research*	*Worldwide*		*Partner 1*
2008	*Publication* Thesis book: Lehtonen H. Molecular and Clinical characteristics of tricarboxylic acid cycle-associated tumors 2008.	*Research*	*Worldwide*		*Partner 1*
2009	*Publication* Koski TA, Lehtonen HJ, Jee KJ, Ninomiya S, Joosse SA, Vahteristo P, Kiuru M, Karhu A, Sammalkorpi H, Vanharanta S, Lehtonen R, Edgren H, Nederlof PM, Hietala M, Aittomäki K, Herva R, Knuutila S, Aaltonen LA, Launonen V. Array	*Research*	*Worldwide*		*Partner 1*

	comparative genomic hybridization identifies a distinct DNA copy number profile in renal cell cancer associated with hereditary leiomyomatosis and renal cell cancer. Genes Chromosomes Cancer. 2009 Jul;48(7):544-51.				
2010	*Publication* Vahteristo P, Koski TA, Näätsaari L, Kiuru M, Karhu A, Herva R, Sallinen SL, Vierimaa O, Björck E, Richard S, Gardie B, Bessis D, Van Glabeke E, Blanco I, Houlston R, Senter L, Hietala M, Aittomäki K, Aaltonen LA, Launonen V, Lehtonen R. No evidence for a genetic modifier for renal cell cancer risk in HLRCC syndrome. Fam Cancer. 2009 Dec 20.	*Research*	*Worldwide*		*Partner 1*
8/2006	*Conference* Launonen 8/2006 Workshop: Hereditary Leiomyomatosis and Renal Cell Carcinoma. Bethesda, USA: Finnish HLRCC families	*Research*	*worldwide*		*Partner 1*
4/2006	*Conference/poster* HJ Lehtonen et al. Cancer risk and tumor spectrum in Finnish FH mutation carriers. The 97th Annual Meeting of American Association of Cancer Reseach, Washington DC, USA				
3/2007	*Conference* Launonen 3/2007 Gordon Research Conferences: Oxidative Stress and Disease, Ventura, CA, USA: Fumarate hydratase: Linking mitochondrial dysfunction and cancer	*Research*	*worldwide*		*Partner 1*
4/2007	*Conference/poster* HJ Lehtonen et al. Increased HIF1α in SDH and FH deficient tumors does not				

10/2007	cause microsatellite instability. The 98th Annual Meeting of American Association of Cancer Reseach, Los Angeles, USA Nature Conferences, Oncogenes and Human Cancer: The Next 25 Years, Madrid, Spain 2007				
6/2006	*Publication* Barker KT, Spendlove HE, Banu NS, Bridge JA, Fisher C, Shipley J, Garrett M, Manyonda I, Houlston RS. No evidence for epigenetic inactivation of fumarate hydratase in leiomyomas and leiomyosarcomas. Cancer Lett. 2006 Apr 8;235(1):136-40.	*Research*	*worldwide*		*Partner 2*
	Lecture Cancer Research UK talk at Beatson Institute (Richard S Houlston)	*Research*	*UK*		*Partner 2*
2006 *2005*	SDH Database *Publication* The SDH mutation database: an online resource for succinate dehydrogenase sequence variants involved in pheochromocytoma, paraganglioma and mitochondrial complex II deficiency Bayley JP, Devilee P, Taschner PEM. BMC MEDICAL GENETICS 6:39 NOV 16 2005, 12 citations (9-5-2007) Website: http://chromium.liacs.nl/lovd_sdh/	*Professional medical and research*	*worldwide*	The database attracted an average monthly usage of 456 unique IP addresses in 2009.	*Partner 3*
2008	FH database *Publication* Bayley JP, Launonen V, Tomlinson IP. The FH	*Research*	*worldwide*		*Partner 1, 3, 6*

	mutation database: an online database of fumarate hydratase mutations involved in the MCUL (HLRCC) tumor syndrome and congenital fumarase deficiency. BMC Med Genet. 2008 Mar 25;9:20 Website: http://chromium.liacs.nl/LOVD2/SDH/home.php?select_db=FH				
4/2009	*Publication* Bayley JP, Grimbergen AE, van Bunderen PA, van der Wielen M, Kunst HP, Lenders JW, Jansen JC, Dullaart RP, Devilee P, Corssmit EP, Vriends AH, Losekoot M, Weiss MM. The first Dutch SDHB founder deletion in paraganglioma-pheochromocytoma patients. BMC Med Genet. 2009 Apr 15;10:34	*Research*	*worldwide*		*Partner 3*
5/2009	*Publication* Hensen EF, Goeman JJ, Oosting J, Van der Mey AG, Hogendoorn PC, Cremers CW, Devilee P, Cornelisse CJ. Similar gene expression profiles of sporadic, PGL2-, and SDHD-linked paragangliomas suggest a common pathway to tumorigenesis. BMC Med Genomics. 2009 May 11;2:25	*Research*	*worldwide*		*Partner 3*
8/2009	*Publication* Hao HX, Khalimonchuk O, Schraders M, Dephoure N, Bayley JP, Kunst H, Devilee P, Cremers CW, Schiffman JD, Bentz BG, Gygi SP, Winge DR, Kremer H, Rutter J. SDH5, a gene required for flavination of succinate dehydrogenase, is mutated in paraganglioma. Science. 2009 Aug	*Research*	*worldwide*		*Partner 3*

	28;325(5944):1139-42.				
9/2009	*Publication* Bayley JP, Weiss MM, Grimbergen A, van Brussel BT, Hes FJ, Jansen JC, Verhoef S, Devilee P, Corssmit EP, Vriends AH. Molecular characterization of novel germline deletions affecting SDHD and SDHC in pheochromocytoma and paraganglioma patients. Endocr Relat Cancer. 2009 Sep;16(3):929-37	*Research*	*worldwide*		*Partner 3*
10/2009	*Publication* Cervera AM, Bayley JP, Devilee P, McCreath KJ. Inhibition of succinate dehydrogenase dysregulates histone modification in mammalian cells. Mol Cancer. 2009 Oct 22;8:89	*Research*	*worldwide*		*Partner 3*
11/2009	*Publication* Bayley JP, van Minderhout I, Hogendoorn PC, Cornelisse CJ, van der Wal A, Prins FA, Teppema L, Dahan A, Devilee P, Taschner PE. Sdhd and SDHD/H19 knockout mice do not develop paraganglioma or pheochromocytoma. PLoS One. 2009 Nov 24;4(11).	*Research*	*worldwide*		*Partner 3*
1/2010	*Publication* Hensen EF, Jansen JC, Siemers MD, Oosterwijk JC, Vriends AH, Corssmit EP, Bayley JP, van der Mey AG, Cornelisse CJ, Devilee P. The Dutch founder mutation SDHD.D92Y shows a reduced penetrance for the development of paragangliomas in a large multigenerational family. Eur J Hum Genet. 2010 Jan;18(1):62-6	*Research*	*worldwide*		*Partner 3*

1/2010	*Publication* SDHAF2 (PGL2-SDH5) mutations in paraganglioma and pheochromocytoma Jean Pierre Bayley, Henricus P.M. Kunst, Alberto Cascon, Maria Lourdes Sampietro, José Gaal, Esther Korpershoek, Adolfo Hinojar-Gutierrez, Henri J.L.M. Timmers, Lies H. Hoefsloot, Mario A. Hermsen, Carlos Suárez, A. Karim Hussain, Annette H.J.T. Vriends, Frederik J. Hes, Jeroen C. Jansen, Carli M. Tops, Eleonora P. Corssmit, Peter de Knijff, Jacques W.M. Lenders, Cor W.R.J. Cremers, Peter Devilee, Winand N.M. Dinjens, Ronald R. de Krijger, Mercedes Robledo. Lancet Oncol. 2010 Jan 11.	*Research*	*worldwide*		*Partner 3*
2010	*Accepted* Warburg tumours and the mechanisms of mitochondrial tumour suppressor genes. Barking up the right tree? Jean Pierre Bayley, Peter Devilee. Current Opinion in Genetics and Development	*Research*	*Worldwide*		*Partner 3*
2010	*Submitted* Limited penetrance of a *SDHB* mutation in a large family. FJ Hes, MM Weiss, NF de Miranda, SA Woortman, PA van Bunderen, BA Bonsing, MPM Stokkel, H Morreau, JA Romijn, JC Jansen, AHJT Vriends, *JP Bayley*, EPM Corssmit. Head and neck paraganglioma in a large *SDHAF2* (PGL2) family. H.P.M. Kunst, M.H. Rutten, J.P. de Mönnik, H.J.L.M. Timmers, L.H. Hoefsloot, J.C. Jansen, H. Kremer, JP Bayley,	*Research*	*worldwide*		*Partner 3*

	C.W. Cremers. High-Altitude Paraganglioma and Mutation of *SDHB* Nidia Y. Cerecer-Gil, Luis Figuera, Francisco J. Llamas, Mauricio Lara, José G. Escamilla, A. Karim Hussain, Jose Gaal, Ronald R. de Krijger, Winand Dinjens, Peter Devilee, Jean Pierre Bayley Hereditary leiomyomatosis and renal cell cancer: A nationwide study of 32 families referred for *Fumarate Hydratase* germline mutation analysis. Diederik L. Smit, Arjen R. Mensenkamp, Sadhanna Badeloe, Martijn H. Breuning, Marleen E.H. Simon, Karin IJ. van Spaendonck, Cora M. Aalfs, Lies H. Hoefsloot, R. Jeroen A. van Moorselaar, Theo M. Starink, Jean-Pierre Bayley, Jorge Frank, Maurice A.M. van Steensel, Fred H. Menko. Erfelijke leiomyomatose: Kliniek (in Dutch) D.L. Smit, S. Badeloe, A. Mensenkamp JP. Bayley, Th.M. Starink, R.J.A. van Moorselaar, L.H. Hoefsloot, J. Frank, M.A.M. van Steensel F.H. Menko. Erfelijke leiomyomatose: Behandeling (in Dutch) D.L. Smit, S. Badeloe, A. Mensenkamp JP. Bayley, Th.M. Starink, R.J.A. van Moorselaar, L.H. Hoefsloot, J. Frank, M.A.M. van Steensel F.H. Menko.	*Research*	*The Netherlands*		*Partner 3*

2010	*In preparation* Mutation of SDHD in man and mouse leads to peripheral ventilatory insufficiency. Luc Teppema, Jean-Pierre Bayley, Ivonne van Minderhout, Peter Devilee, Peter E.M. Taschner, Albert Dahan.	*Research*	*Worldwide*		*Partner 3*
2006	*Brochure* For mutation VHL 505 C>T (the Blackforest) Mutation predisposing for pheo in about 50% of the carriers.	*General public/ patients*	*Germany*		*Partner 4*
2006	Summary of the findings in 10 different VHL mutations as a basis for counseling. These are done for mutations of which at least 10 carriers are included in the Freiburg VHL registry.	*General public/ patients*	*Germany*		*Partner 4*
2006	*Publication* Bausch B, Borozdin W, Neumann HP and the European-American Pheochromocytoma Study working Group. Clinical and genetic characteristics of patients with neurofibromatosis type 1 and pheochromocytoma. New England Journal of Medicine 2006;354(25): 2729-31	*Research*	*worldwide*		*Partner 4,5*
2006	*Publication* Brink et al. Fluorine-18 DOPA PET imaging of paraganglioma syndrome. Clinical Nuclear Medicine 2006;31(1): 39-41.	*Research*	*worldwide*		*Partner 4*
2006	*Publication* Walz et al. Posterior retroperitoneoscopic adrenalectomy--results of 560 procedures in 520 patients. Surgery 2006;140:943-8	*Research*	*worldwide*		*Partner 4*
	Publication Walz et al. Laparoscopic and	*Research*	*worldwide*		*Partner 4*

	Retroperitoneoscopic Treatment of Pheochromocytomas and Retroperitoneal Paragangliomas: Results of 161 Tumors in 126 Patients. World Journal of Surgery 2006;30: 1-10.				
2006	*Publication* Bausch B, Koschker AC, Fassnacht M, Stoevesandt J, Hoffmann MM, Eng C, Allolio B and Neumann HP Comprehensive mutation scanning of NF1 in apparently sporadic cases of pheochromocytoma. Journal of Clinical Endocrinology & Metabolism 2006;91(9): 3478-81.	*Research*	*worldwide*		*Partner 4*
2006	*Publication* Reisch N, Peczkowska M, Januszewicz A, Neumann HP Pheochromocytoma: Presentation, diagnosis and treatment Journal of Hypertension 2006;24(12): 2331-2339	*Research*	*worldwide*		*Partner 4, 5*
2006	*Publication* Hering A, Guratowska M, Bucsky P, Claussen U, Decker J, Ernst G, Hoeppner W, Michel S, Neumann HP, Parlowsky T and Loncarevic I. Characteristic genomic imbalances in pediatric pheochromocytoma. Genes, Chromosomes & Cancer 2006;45(6): 602-7	*Research*	*worldwide*		*Partner 4*
2006	*Publication* Gimenez-Roqueplo AP, Lehnert H, Mannelli M, Neumann HP, Opocher G, Maher ER, Plouin PF Phaeochromocytoma, new genes and screening strategies. Clinical Endocrinology	*Research*	*worldwide*		*Partner 4*

	2006;65(6):699-705				
2006	*Publication* Kreusel KM, Bechrakis NE, Neumann HP and Foerster MH Pars plana vitrectomy for juxtapapillary capillary retinal angioma. American Journal of Ophthalmology 2006;141(3): 587-9	*Research*	*worldwide*		*Partner 4*
2006	*Publication* Bausch B, Boedeker CC, Berlis A, Brink I, Cybulla M, Walz MK, Januszewicz A, Opocher G, Eng C and Neumann HP Genetic and Clinical Investigation of Pheochromocytoma: A 22-year experience, from Freiburg, Germany to International Effort. Annals of the New York Academy of Sciences 2006;1073: 112-121	*Research*	*worldwide*		*Partner 4, 5*
2006	*Publication* Fuentes C, Menendez E, Pineda J, Martinez De Esteban JP, Anda E, Goni MJ, Bausch B and Neumann HP The malignant potential of a succinate dehydrogenase subunit B germline mutation. Journal of Endocrinological Investigation 2006;29(4): 350-2.	*Research*	*worldwide*		*Partner 4*
2007	*Publication* Neumann HP, Vortmeyer A, Schmidt D, Werner M, Erlic Z, Cascon A, Bausch B, Januszewicz A, Eng C. Evidence of MEN-2 in the original description of classic pheochromocytoma. N Engl J Med. 2007;357:1311-5	*Research*	*Worldwide*		*Partner 4, 5*
2007	*Publication* Bausch B, Borozdin W, Mautner VF, Hoffmann MM,	*Research*	*Worldwide*		*Partner 4, 5*

	Boehm D, Robledo M, Cascon A, Harenberg T, Schiavi F, Pawlu C, Peczkowska M, Letizia C, Calvieri S, Arnaldi G, Klingenberg-Noftz RD, Reisch N, Fassina A, Brunaud L, Walter MA, Mannelli M, MacGregor G, Palazzo FF, Barontini M, Walz MK, Kremens B, Brabant G, Pfäffle R, Koschker AC, Lohoefner F, Mohaupt M, Gimm O, Jarzab B, McWhinney SR, Opocher G, Januszewicz A, Kohlhase J, Eng C, Neumann HP; European-American Phaeochromocytoma Registry Study Group. Germline NF1 mutational spectra and loss-of-heterozygosity analyses in patients with pheochromocytoma and neurofibromatosis type 1. J Clin Endocrinol Metab. 2007;92:2784-92				
2007	*Publication* Boedeker CC, Neumann HP, Maier W, Bausch B, Schipper J, Ridder GJ. Malignant head and neck paragangliomas in SDHB mutation carriers. Otolaryngol Head Neck Surg. 2007;137:126-29	*Research*	*Worldwide*		*Partner 4*
2007	*Publication* Neumann HP, Cybulla M, Gläsker S, Coulin C, Van Velthoven V, Berlis A, Hader C, Schäfer O, Treier M, Brink I, Schultze-Seemann W, Leiber C, Rückauer K, Junker B, Agostini FJ, Hetzel A, Boedecker CC. Von Hippel-Lindau Erkrankung. Interdisziplinäre Patientenversorgung Ophthalmologe. 2007;104:119-	*Research*	*Worldwide*		*Partner 4*

	26				
2007	*Publication* Kreusel KM, Bechrakis NE, Neumann HP, Foerster MH Juxtapapillary capillary retinal angioma with epiretinal membrane of the macula in familial Von-Hippel-Lindau-Syndrome. Ophthalmologe 2007:317-20.	*Research*	*Worldwide*		*Partner 4*
2007	*Publication* Kreusel KM, Bechrakis NE, Neumann HP, Schmidt D, Foerster MH. Solitary juxtapapillary capillary retinal angioma and von Hippel-Lindau disease. Can J Ophthalmol. 2007;42:251-5.	*Research*	*Worldwide*		*Partner 4*
2007	*Publication* Langrehr JM, Bahra M, Kristiansen G, Neumann HP, Neumann LM, Plöckinger U, Lopez-Hänninen E. Neuroendocrine tumor of the pancreas and bilateral adrenal pheochromocytomas. A rare manifestation of von Hippel-Lindau disease in childhood. J Pediatr Surg. 2007;42:1291-4	*Research*	*Worldwide*		*Partner 4*
2007	*Publication* Plate KH, Vortmeyer AO, Zagzag D, Neumann HP, WHO Classification of CNS tumors: Von Hippel-Lindau disease and haemangioblastoma in: WHO Classification of Tumours of the Central Nervous System, Eds Louis DN, Ohgaki H, Wiestler OD, Cavenee WK International Agency for Research on Cancer, Lyon 2007	*Research*	*Worldwide*		*Partner 4*
2008	*Publication* Peczkowska M, Januszewicz A, Jarzab B, Neumann HP,	*Research*	*Worldwide*		*Partner 4, 5*

	Kubaszek A, Janaszek-Sitkowetzka H, Litwin M, Antoniewicz J, Aksamit-Bialoszewska E, Roslonowska E, Prejbisz A, Januszewicz M, Michalowska I, Ciwla J, Furmanek M, Walecki. J Pheochromocytroma in children and adolescents based on the Polish Pheochromocytoma registry Ann Diagn Paed Pathol 2007;11:15-20				
2008	*Publication* Brink I, Hentschel M, Neumann HPH, Schäfer O, Moser E FDOPA-PET als Paradigma molekularer Bildgebung in der Onkologie Der Nuklearmediziner 2007;30:70-82	*Research*	*Germany*		*Partner 4*
2008	*Publication* Cascon A, Landa Í, López-Jiménez E, Díez-Hernández, A, Buchta M, Montero-Conde C, Leskelä S, Leandro-Garciá LJ, Letón R, Rodríguez-Antona C, Eng C, Neumann HP, Robledo M. Molecular characterisation of a common SDHB deletion in paraganglioma patients. J. Med. Genet. 2008;45;233-238.	*Research*	*Worldwide*		*Partner 4*
2008	*Publication* Neumann HP, Erlic ZMaternal transmission of symptomatic disease with *SDHD* mutation: fact or fiction? J Clin Endocrinol Metab 2008;93:1573-5	*Research*	*Worldwide*		*Partner 4*
2008	*Publication* Neumann HP Pheochromocytoma Harrison´s Principles of Internal Medicine Chapter 337	*Research*	*Worldwide*		*Partner 4*

	Mc Graw Hill 2008				
2008	*Publication* Margetts CD, Morris M, Astuti D, Gentle DC, Cascon A, McRonald FE, Catchpoole D, Robledo M, Neumann HP, Latif F, Maher ER. Evaluation of a functional epigenetic approach to identify promoter region methylation in phaeochromocy-toma and neuroblastoma. Endocr Relat Cancer. 2008 Sep;15(3):777-786. Epub 2008 May 22.	*Research*	*Worldwide*		*Partner 4*
2008	*Publication* Pęczkowska M, Erlic Z, Hoffmann MM, Furmanek M, Ćwikła J, Kubaszek A, Prejbisz A, Szutkowski Z, Kawecki A, Chojnowski K, Lewczuk A, Litwin M, Szyfter W, Walter M, Sullivan M, Eng C, Januszewicz A, Neumann HPH Impact of Screening Kindreds for *SDHD* p.Cys11X as a Common Mutation Associated with Paraganglioma Syndrome Type 1 J Clin Endocrinol Metab 2008 Dec;93(12):4818-25.	*Research*	*Worldwide*		*Partner 4*
2008	*Publication* Ioana Milos I, Frank-Raue K, Wohllk N, Maia AL, Pusiol E, Patocs, Robledo M, Biarnes J, Barontini M, Links TP, de Groot JW[11], Sarka Dvorakova S[12], Mariola Peczkowska M, Rybicki LA, Sullivan M, Raue F, Zosin I, Eng C, Neumann HPH. Age-related neoplastic risk profiles and penetrance estimations in multiple endocrine neoplasia type 2A caused by germline *RET* Cys634Trp (TGC>TGG)	*Research*	*Worldwide*		*Partner 4, 5*

	mutation Endocrine-Related Cancer 2008: 15(4); 1035-41.				
1/2009	*Publication* Boedeker CC, Neumann HP, Offergeld C, Maier W, Falcioni M, Berlis A, Schipper J. Clinical features of paraganglioma syndromes. Skull Base. 2009 Jan;19(1):17-25.	*Research*	*worldwide*		*Partner 4*
1/2009	*Publication* Schipper J, Spetzger U, Tatagiba M, Rosahl S, Neumann HP, Boedeker CC, Maier W. Juxtacondylar approach in temporal paraganglioma surgery: when and why? Skull Base. 2009 Jan;19(1):43-7.	*Research*	*worldwide*		*Partner 4*
1/2009	*Publication* Reisch N, Walz MK, Erlic Z, Neumann HP. Pheochromocytoma – still a challenge. Internist (Berl). 2009 Jan;50(1):27-35.	*Research*	*worldwide*		*Partner 4*
3/2009	*Publication* Erlic Z, Neumann HP. When should genetic testing be obtained in a patient with phaeochromocytoma or paraganglioma? Clin Endocrinol (Oxf). 2009 Mar;70(3):354-7.	*Research*	*worldwide*		*Partner 4*
4/2009	*Publication* Neumann HP, Erlic Z, Boedeker CC, Rybicki LA, Robledo M, Hermsen M, Schiavi F, Falcioni M, Kwok P, Bauters C, Lampe K, Fischer M, Edelman E, Benn DE, Robinson BG, Wiegand S, Rasp G, Stuck BA, Hoffmann MM, Sullivan M, Sevilla MA, Weiss MM, Peczkowska M, Kubaszek A, Pigny P, Ward RL, Learoyd D, Croxson M,	*Research*	*worldwide*		*Partner 4, 5*

	Zabolotny D, Yaremchuk S, Draf W, Muresan M, Lorenz RR, Knipping S, Strohm M, Dyckhoff G, Matthias C, Reisch N, Preuss SF, Esser D, Walter MA, Kaftan H, Stöver T, Fottner C, Gorgulla H, Malekpour M, Zarandy MM, Schipper J, Brase C, Glien A, Kühnemund M, Koscielny S, Schwerdtfeger P, Välimäki M, Szyfter W, Finckh U, Zerres K, Cascon A, Opocher G, Ridder GJ, Januszewicz A, Suarez C, Eng C. Clinical predictors for germline mutations in head and neck paraganglioma patients: cost reduction strategy in genetic diagnostic process as fall-out. Cancer Res. 2009 Apr 15;69(8):3650-6.				
8/2009	*Publication* Neumann HP, Eng C. The approach to the patient with paraganglioma. J Clin Endocrinol Metab. 2009 Aug;94(8):2677-83.	*Research*	*worldwide*		*Partner 4*
8/2009	*Publication* Erlic Z, Neumann HP. Diagnosing patients with hereditary paraganglial tumours. Lancet Oncol. 2009 Aug;10(8):741.	*Research*	*worldwide*		*Partner 4*
10/2009	*Publication* Erlic Z, Rybicki L, Peczkowska M, Golcher H, Kann PH, Brauckhoff M, Müssig K, Muresan M, Schäffler A, Reisch N, Schott M, Fassnacht M, Opocher G, Klose S, Fottner C, Forrer F, Plöckinger U, Petersenn S, Zabolotny D, Kollukch O, Yaremchuk S, Januszewicz A, Walz MK, Eng C, Neumann HPH for the European-American Pheochromocytoma Study	*Research*	*worldwide*		*Partner 4, 5*

	Group. Clinical Predictors and Algorithm for the Genetic Diagnosis of Pheochromocytoma Patients Clin Cancer Res. 2009 Oct 15;15(20):6378-85.				
11/2009	*Publication* Erlic Z, Hoffmann MM, Sullivan M, Franke G, Peczkowska M, Harsch I, Schott M, Gabbert HE, Valimäki M, Preuss SF, Hasse-Lazar K, Waligorski D, Robledo M, Januszewicz A, Eng C, Neumann HP. Pathogenicity of DNA Variants and Double Mutations in Multiple Endocrine Neoplasia Type 2 and Von Hippel-Lindau Syndrome. J Clin Endocrinol Metab. 2009 Nov 11, Epub ahead to print.	*Research*	*worldwide*		*Partner 4, 5*
2008	*Publication* Harrison's Principles of Internal Medicine* Pheochromocytoma, Chapter 343/Hartmut PH Neumann Eds: Dan L. Longo, Anthony S. Fauci, Dennis L. Kasper, Stephen L. Hauser, J. Larry Jameson, & Joseph Loscalzo McGraw-Hill Companies New York 2008.	*Higher education*	*worldwide*		*Partner 4*
2.4.06	*Meeting* Leiden, The EU consortium pheochromocytoma and paraganglioma registry	*Research*	*worldwide*		*Partner 4, 3, 5*
11/2006	*Meeting* Freiburg, Laboratory day for SDHx Genes	*Research*	*Germany*		*Partner 4*
3/2006	*Lecture* Berlin, 22.-26. März 27. Deutscher Krebskongreß Hypoxie in Tumoren: Grundlagen und klinische	*Research*	*Germany*		*Partner 4*

	Implikationen				
4/2006	*Lecture* Wiesbaden 23.04.2006 112. Kongreß Deutsche Gesellschaft für Innere Medizin Vorsymposium des BDI Sektion Endokrinologie Genetische Diagnostik und Konsequenzen bei chromaffinen Tumoren	*Research*	*Germany*		*Partner 4*
5/2006	*Lecture* Regensburg, 13.05.2006 28.Regensburger Endokrinologen-Gespräch Diagnostik und Therapie des Phäochromozytoms	*Research*	*Germany*		*Partner 4*
5/2006	*Lecture* Warschau, 27.5.2006 Diagnosis and Treatment of Secondary Hypertension Satellite Symposium of the European Society of Hypertension, Madrid, Spain Genetics of Pheochromocytoma: A New Era	*Research*	*Europe*		*Partner 4*
7/2006	*Lecture* Marseille, 7.-10.09.2006 WorldMEN 2006 Ethical, Psychological, and Legal Aspects of Genetic Testing in Familial Endocrine Tumors: Prenatal and Preimplantatory Diagnosis	*Research*	*worldwide*		*Partner 4*
10/2006	*Lecture* London, Ontario 26.10.2006 Guest Lecture, University of Western Ontario Molecular genetics and Preventive Medicine: The Paraganglioma Syndromes	*Research*	*worldwide*		*Partner 4*
10/2006	*Lecture* London, Ontario 26./27.10.2006 International Symposium for VHL patients Clinical Characteristics of	*Research*	*worldwide*		*Partner 4*

	Pheochromocytoma – associated Syndromes				
10/2006	*Lecture* Kingston, Ontario 30.10.2006 Department of Pathology and Molecular Medicine, Queens University Special Seminar "Paraganglioma and Pheochromocytoma-associated Syndromes	*Research*	*worldwide*		*Partner 4*
4/2007	Lecture Presentation, Diagnosis and Treatment of pheochromocytomas mainly by SDHD mutations in Rome April 28	*Research*	*worldwide*		*Partner 4*
7/2007	Lecture Clinical perspectives of Molecular Classification of Head and Neck Paragangliomas in Timisoara, July 3.	*Research*	*worldwide*		*Partner 4*
5/2007	*Lecture* Invited speaker on the Congress of the European Skull Base Society in Prague May 3	*Research*	*Europe*		*Partner 4*
6/2007	*Lecture* Invited speaker on the 6th European Congress of Oto-Rhino-Laryngeology, Head and Neck Surgery in Vienna June 30	*Research*	*Europe*		*Partner 4*
9/2007	*Lecture* Invited speaker on the Congress of Laboratory Medicine in Vienna September 21.	*Research*	*Europe*		*Partner 4*
9.12.07	*Meeting* Patients meeting The Blackforest Mutation of the VHLK gene	*General public/ patients*	*Germany*		*Partner 4*
30.1.07	*Meeting* International Collaborator Meeting on Pheochromocytoma	*Research*	*worldwide*		*Partner 4, 5*

	in Freiburg				
2007	*Lectures* June 30, 2007 6th European Congress of Oto-Rhino-Laryngeology and Head and Neck Surgery Invited Special Lecture for the Opening Ceremony The European Paraganglioma Network: Moleculargenetics of Paragangliomas Vienna July 02, 2007 6th European Congress of Oto-Rhino-Laryngeology and Head and Neck Surgery Clinical Perspectives of Molecular Classification of Head and Neck Paraganglioma Vienna September 21, 2007 Congress of Laboratory Medicine (Gemeinsamer Kongress der ÖGLMKC und der GGLK) Invited Organizer of the Symposium and Key Lecture Hereditary Pheochromocytoma and Paraganglioma Vienna Oktober 27, 2007 German Self Support Group Von Hippel-Lindau Krankheit Annual Meeting The Importance of control Investigations for clinical follow up in Von Hippel-Lindau disease (in German) München Neufahrn November 9 and 10, 2007 Konsensus Symposium	*Research*	*Germany/ Worldwide*		*Partner 4*

	Phäochromozytome, Paragangliome und Phäochromozytom-Paragangliom-assoziierte Syndrome Freiburg: Organizer: HPH Neumann				
2008	*Lectures* January 18, 2008; DORTMUND, Nordrhein-Westfälische Gesellschaft für Endokrinologie und Diabetologie Invited Lecture Bedeutung der Molekulargenetischen Diagnostik für die endokrinologische Praxis March 5, 2008; FRANKFURT, Gemeinsame Veranstaltung der Rhein-Main-AG für Endokrinologie, Diabetes und Stoffwechsel zum Thema: Elektrolyt-, Blutdruck-, und Stoffwechselstörungen Invited Lecture Phäochromozytome und Paragangliome: Klinische und molekulargenetische Diagnostik April 4, 2008; FREIBURG, EU Consortium Meeting Defects in the Tricarboxylic Acid (Krebs) Cycle Genes in Tumourigenesis Basic molecular characterization of collected sample materials June 5 and 6, 2008; MADRID, 3rd Familial Cancer Conference Invited Speaker Familial Pheochromocytomas and Paragangliomas	*Research*	*Germany/ Worldwide*		*Partner 4*

	June 13-18, 2008, San Francisco, USA The Endocrinology Society; A cost reduction Strategy in Genetic Medicine Practice:Predictors for Germline Mutations in Patients with Head and Neck Paragangliomas Zoran Erlic June 20 and 21, 2008; TRENTO, First Trento Paraganglioma Meeting Invited Speaker The Three Natures of Pheochromocytoma September 5, 2008; ROSKILDE, DENMARK, VHL Symposium 2008 Invited Speaker Familial Pheochromocytoma: Genetic Susceptibility, Clinical Diagnosis and Treatment Options September 17-19, 2008; CAMBRIDGE, UK Is it cost effective for every patient to have genetic screening? Invited Speaker, Lecture delivered by Dr Zoran Erlic September 29, 2008, Delphi, Greece International Cancer Conference Invited Lecture Familial Pheochromocytomas and Paragangliomas September 2008, Chicago, Congress for otolaryngeology				

	Head and neck paragangliomas and von Hippel-Lindau disease Presented by Boedeker C. C., Ridder G.J., Maier W., Neumann H.P.H.				
2009	Key Lectures: The 3rd Familial Cancer Conference in Madrid May 6 The First Trento Paraganglioma Meeting in Trento June 20 The 11th International Workshop on Multiple Endocrine Neoplasia in Delphi September 28 The 34th Symposium of Polish Association of Pediatric Surgeons in Bydgoszcz October 17, the TEMESVAR, RUMÄNIEN The 6th Congress of the Romanian Society of Endocrinology in Timisoara October 24 The 13th International Congress of Endocrinology in Rio de Janeiro November 10 The First Serbian Congress of Endocrinology in Belgrad December 5 The First Central European Conference on Pheochromocytoma in Warsaw December 13 Other Lectures: Dortmund January 18 Frankfurt March 5	*Research*	*worldwide*		*Partner 4*

	Buenos Aires, November 13.				
2006	*Publication* „Chory z obustronnym guzem chromochłonnym i nerwiakowłókniakowatością typu 1 - opis przypadku" Mariola Pęczkowska, Agata Kubaszek, Hanna Janaszek-Sitkowska, Elżbieta Florczak, Marek Kabat, Maciej Otto, Anna Okruszko, Jacek Szmidt, Andrzej Januszewicz. *Nadciśnienie tętnicze,* t 10 nr 4 2006.	*Research*	*Poland*		*Partner 5*
2006	*Publication* Book chapter „Guz Chromochłonny – Diagnostyka i Leczenie w 2006 Roku" (Pheochromocytoma-diagnosis and treatment in 2006) in „Postępy w nefrologii i nadciśnieniu tętniczym, tom V" pod red. Andrzej Więcek, Franciszek Kokot, 2006r.	*Research*	*Poland*		*Partner 5*
2008	*Publication* „Pheochromocytoma in children and adolescents based on Polish Pheochromocytoma Registry" Mariola Pęczkowska, Andrzej Januszewicz, Barbara Jarząb, Hartmut P.H. Neumann, Agata Kubaszek, Hanna Janaszek-Sitkowska, Mieczysław Litwin, Joanna Antoniewicz, Elżbieta Białoszewska – Aksamit, Elżbieta Rosłonowska, Aleksander Prejbisz, Magdalena Januszewicz, Ilona Michałowska, Jarosław Ćwikła, Mariusz Furmanek, Jerzy Walecki. Ann. Diagn. Paediatr.	*Research*	*Worldwide*		*Partner 5, 4*

	Pathol, 2007, 11; 15-19				
2008	*Publication* "Extra-adrenal and adrenal pheochromocytomas associated with a germline SDHC mutation" Peczkowska M, Cascon A, Prejbisz A, Kubaszek A, Cwikła BJ, Furmanek M, Erlic Z, Eng C, Januszewicz A, Neumann HP. Nat Clin Pract Endocrinol Metab. 2008 Feb;4(2):111-5.	*Research*	*Worldwide*		*Partner 5, 4*
2008	*Publication* Book „Nadciśnienie tętnicze w przypadkach klinicznych" (Hypertension in clinical cases) Medycyna Praktyczna, 2008. Authors: Andrzej Januszewicz, Mariola Pęczkowska, Aleksander Prejbisz, Agata Kubaszek. Book chapter:" Guz chromochłonny – choroba o wielu obliczach" (Pheochromocytoma – the disease with many faces) Mariola Pęczkowska, Agata Kubaszek, Hanna Janaszek – Sitkowska, Andrzej Januszewicz. In „ Wybrane zagadnienia z Kardiologii, diabetologii i nadciśnienia tętniczego". Medycyna Praktyczna, 2007 617-639.	*Research*	*Poland*		*Partner 5*
2009	*Publication* Diagnostyka guza chromochłonnego z perspektywy 2008 roku. Pęczkowska M, Januszewicz W, Januszewicz A, Prejbisz A. Postępy Nauk Medycznych 2009; 22:22-27.	*Research*	*Poland*		*Partner 5*
2009	*Publication (Book chapter)* Januszewicz A, Pęczkowska M, Prejbisz A, Januszewicz M. Nadciśnienie hormonozależne.	*Research*	*Poland*		*Partner 5*

	W: Syrenicz A. Endokrynologia w codziennej praktyce lekarskiej. Wydawnictwo Pomorskiej Akademii Medycznej w Szczecinie, Szczecin, 2009, 607-646.				
2010	*Publication* Pheochromocytoma-related 'classic' takotsubo cardiomyopathy P Zielen, A Klisiewicz, A Januszewicz, A Prejbisz, M Kabat, M Peczkowska, J Stepinska, P Hoffman – Journal of Human Hypertension – in press.	*Research*	*Worldwide*		*Partner 5, 4*
10/2006	*Conference* X Jubileuszowy Zjazd Polskiego Towarzystwa Nadciśnienia Tętniczego, Kraków, 5-7.10.2006 *Posters* 'Kliniczna charakterystyka pacjentów z dziedzicznymi zespołami guza chromochłonnego w Polskim Rejestrze Guza Chromochłonnego' Andrzej Januszewicz, Barbara Jarząb, Mariola Pęczkowska, Hartmut Neumann, Aleksandra Krawczyk, Kornelia Hasse-Lazar, Birke Bausch, Agata Kubaszek, Hanna Janaszek-Sitkowska, Marek Kabat, Aleksander Prejbisz 'Polski Rejestr Pheochromocytoma: występowanie dziedzicznych zespołów związanych z guzem chromochłonnym' Mariola Pęczkowska, Barbara Jarząb, Andrzej Januszewicz, Hartmut Neumann, Aleksandra Krawczyk, Kornelia Hasse-	*Research*	*Poland*		*Partner 5, 4*

	Lazar, Agata Kubaszek, Birke Bausch, Hanna Janaszek-Sitkowska, Marek Kabat, Aleksander Prejbisz 'Porównanie klinicznej charakterystyki pacjentów ze sporadycznym i dziedzicznym guzem chromochłonnym w Krajowym Rejestrze Guza Chromochłonnego' Andrzej Januszewicz, Barbara Jarząb, Mariola Pęczkowska, Hartmut Neumann, Aleksandra Krawczyk, Kornelia Hasse-Lazar, Birke Bausch, Agata Kubaszek, Hanna Janaszek-Sitkowska, Marek Kabat, Aleksander Prejbisz				
6/2006	*Conference* The 16th Scientific Meeting of the European Society of Hypertension (ESH), Madrid, Spain, 12-15.06.2006 *Posters* "Incidence and clinical characteristics of hereditary pheochromocytoma syndromes in polish pheochromocytoma registry" Andrzej Januszewicz, Barbara Jarząb, Mariola Pęczkowska, Hartmut Neumann, Aleksandra Krawczyk, Kornelia Hasse-Lazar, Birke Bausch, Agata Kubaszek1, Hanna Janaszek-Sitkowska, Marek Kabat, Aleksander Prejbisz "Prevalence of hereditary pheochromocytoma syndromes in polish pheochromocytoma registry" Mariola Pęczkowska, Barbara Jarząb, Andrzej Januszewicz, Hartmut Neumann,	*Research*	*worldwide*		*Partner 5, 4*

	Aleksandra Krawczyk, Kornelia Hasse-Lazar, Agata Kubaszek, Birke Bausch, Hanna Janaszek-Sitkowska, Marek Kabat, Aleksander Prejbisz "Incidence and predictors of hereditary pheochromocytoma syndromes in polish pheochromocytoma registry" Andrzej Januszewicz, Barbara Jarząb, Mariola Pęczkowska, Hartmut Neumann, Aleksandra Krawczyk, Kornelia Hasse-Lazar, Birke Bausch, Agata Kubaszek, Hanna Janaszek-Sitkowska, Marek Kabat, Aleksander Prejbisz				
2007	*Lectures* Central European Meeting on Hypertension and Cardiovascular Disease Prevention, Kraków, Poland, 11-13.10.2007 " Pheochromocytoma – progress in diagnostic and treatment over last decades" A. Januszewicz – lecture. The 17th European Meeting on Hypertension (ESH), Milano, Italy, 15-19.06.2007 "Clinical characteristics of sporadic and hereditary pheochromocytoma in Polish Pheochromocytoma Registry " Andrzej Januszewicz, Barbara Jarząb, Mariola Pęczkowska, Agata Kubaszek, Hartmut PH Neumann, Kornelia Hasse-Lazar, Aleksandra Krawczyk, , Hanna Janaszek-Sitkowska, Aleksander Prejbisz, Maciej Otto, Zbigniew Szutkowski,	*Research*	*Worldwide*		*Partner 5, 4*

	Andrzej Kawecki. "Prevalence and predictors of hereditary pheochromocytoma syndromes in polish pheochromocytoma registry" Mariola Pęczkowska, Agata Kubaszek, Andrzej Januszewicz, Barbara Jarząb, , Hartmut PH Neumann, Hanna Janaszek-Sitkowska, Kornelia Hasse-Lazar, Aleksandra Krawczyk, Aleksander Prejbisz, Maciej Otto, Zbigniew Szutkowski, Andrzej Kawecki.				
6/2009	*Presentation* Anatomical and functional imaging approach for initial evaluation and follow-up screening in patients with pheochromocytoma – paraganglioma syndromes. Cwikla JB, Peczkowska M, Prejbisz A, Michalowska I, Furmanek MI, Kwiatek P, Kubaszek – Kornatowska A, Walecki J, Neumann HP, Januszewicz A. 19th European Meeting on Hypertension, Milan June 12-16, 2009.	*Research*	*worldwide*		*Partner 5, 4*
6/2009	*Presentation* Cardiovascular manifestations of pheochromocytoma in patients of the Polish Pheochromocytoma Registry Peczkowska M, Januszewicz A, Jarzab B, Prejbisz A, Kubaszek-Kornatowska A, Neumann HP, Szperl M, Hasse-Lazar K, Krawczyk A, Janaszek – Sitkowska H, Otto M. 19th European Meeting on Hypertension, Milan June 12-16, 2009.	*Research*	*worldwide*		*Partner 5, 4*

6/2009	*Presentation* Blood pressure level and QT dispersion in patients after surgical treatment of pheochromocytoma. Michel - Rowicka K, Peczkowska M, Zielen P, Prejbisz A, Janaszek-Sitkowska H, Kubaszek – Kornatowska A, Janas J, Kabat M, Januszewicz A. 19th European Meeting on Hypertension, Milan June 12- 16, 2009.	*Research*	*worldwide*		*Partner 5*
6/2009	*Presentation* Early target organ damage in patients with pheochromocytoma Zielen P, Peczkowska M, Prejbisz A, Michel – Rowicka K, Pregowska-Chwala B, Kubaszek – Kornatowska A, Kuśmierczyk- Droszcz B, Januszewicz M, Michalowska I, Kabat M, Januszewicz A, Zieliński T. 19th European Meeting on Hypertension, Milan June 12-16, 2009.	*Research*	*worldwide*		*Partner 5*
12/2009	*Conference* II CENTRAL EUROPEAN CONFERENCE ON PHEOCHROMO-CYTOMA, 14 December 2009 , Warsaw, Poland organized by A. Januszewicz	*Research*	*worldwide*		*Partner 5*
4/2007	*Publication* Pollard PJ, Spencer-Dene B, Shukla D, Howarth K, Nye E, El-Bahrawy M, Deheragoda M, Joannou M, McDonald S, Martin A, Igarashi P, Varsani-Brown S, 7Rosewell I, Poulsom R, Maxwell P, Stamp GW, Tomlinson IP.Targeted inactivation of fh1 causes proliferative renal cyst	*Research*	*Worldwide*		*Partner 6*

	development and activation of the hypoxia pathway. Cancer Cell. 2007 Apr;11(4):311-9				