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Von Hippel-Lindau disease

Diagnosis and factors influencing disease outcome

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THE FOUR ORIGINAL PAPERS ARE:

I: Binderup ML, Budtz-Jørgensen E, Bisgaard ML. Risk of new tumors in von Hippel-Lindau patients depends on age and genotype. Genet Med. 2016;18:89-97.

II: Binderup ML, Budtz-Jørgensen E, Bisgaard ML. New von Hippel-Lindau manifestations develop at the same or decreased rates in pregnancy. Neurology. 2015;85:1500-3.

III: Binderup ML, Budtz-Jørgensen E, Møller Jensen A, Bisgaard ML . Survival and causes of death in von Hippel-Lindau patients. J Med Genet. 2017;54:11-18.

IV: Binderup ML, Galanakis M, Budtz-Jørgensen E, Kosteljanetz M, Bisgaard ML. Prevalence, birth incidence, and penetrance of von Hippel-Lindau disease (vHL) in Denmark. Eur J Hum Genet. 2017;25:301-307.

BACKGROUND

History and epidemiology

von Hippel-Lindau disease (vHL) (OMIM number 193300) is a rare hereditary disease with an autosomal dominant mode of inheritance. Predisposed individuals are at lifelong risk of developing benign and malignant tumors. The most common manifestations of vHL are hemangioblastomas (hbs) of the central nervous system (CNS), kidney cysts and renal cell carcinoma (RCC), pheochromocytoma (pheo), and pancreatic cysts and neuroendocrine tumors (PNETs). The characteristic vascular tumors, of the retina were first described in the 1870-90's and were named "angiomatosis retinae" by the German ophthalmologist Eugene von Hippel

in 1911 [1]. The Swedish pathologist, Arvid Lindau discovered the association between von Hippel's retinal lesions and hbs of the cerebellum, but also RCC, pancreatic, and epididymal cysts, which he published in 1926 [1]. Dr. Lindau further described the disease to be familial, and the Danish ophthalmologist Hans Ulrik Møller suggested the autosomal dominant mode of inheritance in 1929 [2]. The first clinical diagnostic criteria were suggested by Dr. Melmon and Dr. Rosen in 1964 [3], and form the basis of currently used international criteria [4-6]. The prevalence of vHL is estimated to be between 1 in 39,000 to 1 in 91,000 individuals and the birth incidence to be between 1 in 36,000 and 1 in 45,500 live births in different populations [7-10].

Pathogenesis

The VHL tumor suppressor gene

The *VHL* tumor suppressor gene was mapped to chromosome 3p25-p26 through linkage-studies in 1988 [11], and identified by positional cloning in 1993 [12], which made molecular genetic diagnosis possible. The *VHL* gene consists of three exons, and encodes two isoforms of the VHL protein (pVHL) due to alternative initiation of translation at codon 54 [6, 13]. The longest isoform p30 is 213 amino acids in length, while p19 is 160 amino acids [6, 12, 13]. Both isoforms have tumor suppression activity [14] and are expressed in adult human tissues at different tissue-specific levels [13]. The pVHL has a comprehensive range of functions in essential cellular pathways. The most important is its down-regulation of the expression of a group of transcription factors, Hypoxia Inducible Factors (HIFs), as seen in Figure 1. Without functional pVHL, the HIFs are not degraded, but are instead translocated to the nucleus where they initiate gene transcription of hundreds of target genes. Many of these promote angiogenesis, anaerobe metabolism, erythropoiesis, and cell proliferation such as Vascular endothelial growth factor (VEGF), Glucose transporter 1 (GLUT-1), Erythropoetin (EPO), Transforming growth factor α (TGFα), and Epidermal growth factor receptor (EGFR) [6, 13]. The binding of pVHL to HIF-α subunits requires oxygen, as the HIF-subunits need to be hydroxylated by prolyl hydroxylases using oxygen as a co-substrate in order to be recognized [13]. Lack of functional pVHL is equivalent to hypoxic cellular conditions, which explains the predisposition to vascular tumors.

pVHL also has several HIF-independent functions, many of which are still being discovered. The protein plays an important role in assembly of the extracellular matrix through regulation of fibronectin and collagen IV [6, 13]. Furthermore, pVHL has been shown to stabilize microtubules and modulate primary cilia formation [6, 13].

Figure 1: pVHL regulation of HIFs
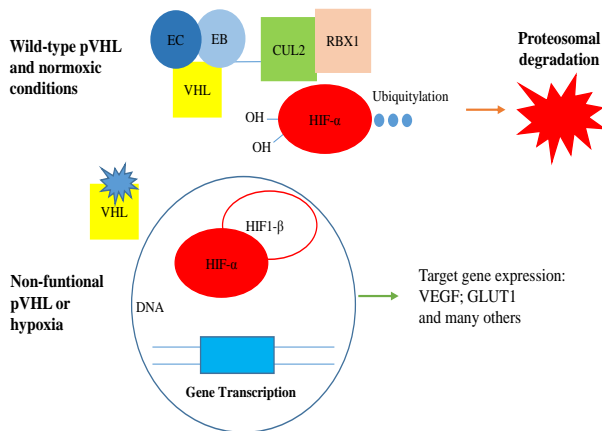
Inspired by figure 3 in [13]
Under normal circumstances, the wild type pVHL (VHL) interacts with Elongin B (EB) and Elongin C (EC) to form the VBC-complex that in conjunction with CUL2 and RBX1 functions as an E3 ubiquitin ligase. pVHL is responsible for recognizing the HIF1 and 2 α-subunits (HIF- α), which are then marked for proteosomal degradation by ubiquitination, thereby suppressing HIF downstream functions [6, 13].
VEGF= Vascular Endothelial Growth Factor, GLUT1: Glucose Transporter 1

pVHL consists of two major functional domains, the β-sheet domain which binds HIF-α subunits and the α-helix-domain which is primarily responsible for Elongin B and C binding [6]. To date more than 700 *VHL* mutations have been identified, located throughout all three exons, although no pathogenic mutations have been described corresponding to the first 54 amino acids [6, 15, 16]. Worldwide, more than half of *VHL* germline mutations are missense mutations, while large deletions, frameshift and nonsense mutations each account for more than 10% [6]. Curiously, *VHL* germline mutations are also responsible for recessively inherited polycythemias. The most well-known is Chuvash polycythemia resulting from a homozygous R200W mutation that causes only polycythemia, but no increased risk of vHL-related tumor development [6, 13].

vHL-related tumorigenesis
vHL-related tumor development is in accordance with Knudson's "two-hit-model" of tumorigenesis [17-24]: vHL patients are born with a germline mutation in one copy of their *VHL* gene in all the cells of the body – "the first hit". Somatic mutation in the other copy of the *VHL* gene – "the second hit" – initiates tumor development from that particular cell [17]. Some types of mutations result in lack of functional pVHL, while other types leave the cell with varying degrees of residual activity [6]. But, even though bi-allelic *VHL* inactivation and the resulting activation of HIF and its targets seem to be necessary for early vHL tumor development, other activating factors are also needed for initiation of tumor progression [18, 25].
The two-hit mechanism of *VHL* inactivation also applies to some of vHL-related tumors' sporadic counterparts. In sporadic RCCs, biallelic *VHL* inactivation is a key event, and has been reported in

up to 74% of tumors and in early tumor stages [26-29]. The tumorigenic pathways have not been as thoroughly investigated in sporadic CNS hbs, but some studies have suggested that the VHL-HIF pathway plays a smaller role, as only up to 50% of CNS hbs have been shown to have inactivation of both *VHL* alleles [19, 30-32]. Further, the mechanism seems to be even rarer in sporadic pheos [33, 34].

Clinical features of vHL
VHL mutation-carriers are predisposed to develop a wide variety of cysts and tumors (Table 1). Patients typically develop their first manifestation in their twenties [35-37], and the penetrance of vHL has been reported to be almost 100% at age 60 years [7, 35]. The first manifestation is most often a CNS or retinal hb (in around 40% and 25%, respectively), which are also the most common manifestation types [7, 35, 37, 38]. vHL-related tumors may remain asymptomatic for years, but can cause severe sequelae or even death. Retinal hbs entail a substantial risk of vision loss due to retinal detachment or bleeding, and are in most cases treated with photocoagulation or cryotherapy at diagnosis [39, 40]. CNS hbs can cause devastating neurological complications, as can the surgical treatment in itself [38, 41-44]. The timing and surgical approach depends on many factors, especially the location of the tumor, size, growth rate, and symptom development [45-47]. Early removal of asymptomatic hbs may be warranted in tumors with rapid growth patterns [46, 48].

vHL patients may develop clear cell RCC as well as multiple kidney cysts, which can have malignant potential, and should be frequently monitored [23, 97]. RCCs are typically asymptomatic until they have metastasized or reached a size that requires radical nephrectomy. If caught at early stages, RCCs can be monitored and removed with nephron-sparing surgery when they reach 3 cm with minor risk of metastasis [98]. Small RCCs may also be treated with ablation therapy with minimal loss of functional kidney tissue [99]. vHL patient are also at substantial risk of pancreatic cyst-development, which can be found in up to 70% of patients [100]. Other vHL-related manifestations include serous pancreatic adenomas and PNETs, which require expert care as they can become malignant [100]. Endolymphatic sac tumors (ELST) can cause sudden and permanent hearing loss at even early stages, and can be very hard to diagnose [89, 101, 102]. Cyst adenomas of the epididymis may develop in men, and cysts of the broad uterine ligament in women, although the reported incidences are low [103].

Phenotypic expression of vHL is variable and hard to predict. Several genotype-phenotype correlations have, however, been established. Clinically, vHL can be characterized based on the absence or presence of pheos in the family into type 1 or type 2, respectively [9, 60, 104-107]. Though there are exceptions to the rule, type 1 is predominantly caused by *VHL* mutations truncating the protein product, while type 2 is most often caused by missense mutations that in most cases leave an abnormal, but partly functional protein product [6, 36, 60, 104, 105, 107, 108]. Type 2 has been further subdivided into type 2A with a low risk of RCC, type 2B with a high risk of RCC, and type 2C with pheo as the sole manifestation type [60, 106]. Other genetic factors have been shown to contribute to the phenotypic variation among vHL patients. Large germline deletions also involving the *VHL*-adjacent gene *BRK1 (C3orf10/HSPC300)*, which functions as an actin regulator, are associated with significantly lower RCC and retinal hb risks [109-112].

Table 1: Frequencies of typical vHL associated manifestations and of vHL patients among patients with specific manifestations

Manifestation	Frequency of vHL pt with the manifestation type[1] (No. of vHL pt. of total no. of pt in the included cohorts, range: lowest-highest frequency in the included cohorts)	Ref	Average age of onset (N= Total no. of vHL pt reported in the included studies with the manifestation, range: lowest-highest age in the included cohorts)	Ref	Frequency of vHL pt among pt with the manifestation (N= No. of pts. with the manifestation, range lowest-highest frequency in the included cohorts)	Ref
Retinal hb	**52 %** (1,716 of 3,294, range: 15-73%)	[7, 9, 35-37, 43, 47, 49-62]	**25-37 years** (N= 980, range: 9-84)	[36, 49, 50]	**Median: 46 %** (N=145, range: 31-81 %)	[47, 63-65]
Cerebellar hb	**49 %** (786 of 1,598, range: 35-79 %)	[7, 35-37, 43, 45, 47, 52, 53, 55, 57, 58, 66, 67]	**29-30 years** (N= 484, range: 13-61)	[35, 36, 66]	**CNS hb (all locations): Median 18.5 %** (N= 563, range: 4-57 %)	[41, 63, 68-71]
Spinal hb	**27 %** (392 of 1,472, range: 7-53 %)	[7, 36, 37, 39, 43, 45, 47, 52, 53, 57, 58, 67]	**33-34 years** (N= 186, range: 8-60)	[35-37]		
Brainstem hb	**16%** (65 of 413, range 4-22 %)	[37, 47, 52, 54, 58, 67]	**25-38 years** (N= 53, range: 16-60)	[37, 72, 73]		
Cerebral hb	**4 %** (26 of 586, range: 1-7%)	[37, 43, 47, 57, 67]	**29 years** (N=1)	[37]		
RCC	**30 %** (532 of 1,784, range: 5-86%)	[7, 35, 37, 45, 53-58, 60, 62, 66, 69, 73]	**40-45 years** (N= 247, range: 20-69)	[35, 36, 66]	**< 5 %**	[58]
Renal cysts	**42 %** (99 of 231, range: 10-89 %)	[37, 45, 53-56, 58, 64, 69]	**34-39 years** (N= 51, range: 12-64)	[7, 37, 64]	-	-
Pheo	**16 %** (403 of 2,546, range: 0-32%)	[7, 35-37, 43, 45, 47, 53-55, 57-62, 74, 75]	**20-29 years** (N= 240, range: 5-62)	[35, 36, 75]	**Sporadic: 2-11 %** (N= 813) **Familial: 15-34%** (N= 236)	[76-83]
Pancreatic cysts	**21 %** (178 of 831, range: 15-35 %)	[37, 43, 45, 53-56, 58, 60, 69, 84]	**29-37 years** (N=45, range: 12-63)	[7, 37, 64]	-	-
PNET	**10 %** (170 of 1,656, range: 1-17 %)	[37, 45, 55, 59, 60, 84-86]	**32-38 years** (N=143, range: 16-68)	[85-87]	**1 %** (N= 101)	[88]
ELST	**11 %** (67 of 583, range: 3-16 %)	[45, 47, 53, 56, 89-91]	**22-40 years** (N= 69, range: 11-63)	[90-92]	**5-15 %** (N= 74)	[93-95]
Epididymal cyst adenoma	**25 %** (73 of 287, range 3-32 %)	[37, 43, 47, 53]	**24 years** (N=6, range: 10-37)	[37]	-	-

Modified from table 1 in [96] No.= Number, Pt= Patients, RCC= Renal Cell Carcinoma, Hb= Hemangioblastoma, Pheo= Pheochromocytoma, PNET= Pancreatic Neuroendocrine tumor, ELST= Endolymphatic sac tumor, Ref= references

[1] *Based on the studies of vHL cohorts (> 5 pt) reported in the literature. To avoid selection bias due to geno-phenotype correlations, cohorts selected based on a specific genotype or manifestation were excluded. It should be noted that the frequencies of specific manifestations vary with the age distributions in the individual cohorts.*

The median life expectancy for vHL patients was reported to be 49 years in 1990 [35]. Improved diagnostic techniques and treatment options, as well as the widespread use of prophylactic surveillance are expected to have improved the life expectancy, although this has not been directly assessed in an unselected vHL cohort. The main causes of death among vHL patients have been reported to be CNS hb (41-60%) and RCC (27-47%) [7, 35, 38].

Surveillance guidelines

The mainstay of vHL management is close surveillance from early childhood and surgical treatment of vHL related tumors. Currently, no preventive systemic therapies exists, and chemotherapy is used only as a second line treatment in cases of inoperable tumors, often only with moderate drug responses [113-116]. Table 2 gives an overview of the Danish vHL surveillance recommendations [96]. It is important to stress, that surveillance recommendations predominantly are meant as guidelines and are especially applicable for asymptomatic *VHL* mutation-carriers. Once patients have symptoms or diagnosed manifestations, prophylactic screening should be adjusted accordingly. The Danish surveillance recommendations correspond to international guidelines [4, 41, 71, 102, 117, 118].

Surveillance can cause considerable psychological strain on the involved vHL families [119, 120], as well as large expenses for the health care system [71]. It is widely accepted that surveillance improves morbidity and mortality [41, 43, 57, 70, 118, 121, 122], but definite evidence has yet to be reported. Current recommendations are based on best clinical judgment and experience. Besides the age-differentiated initiations, the recommendations are identical for all patients regardless of individual factors. Better knowledge about the natural course of the disease and well as influencing factors could help individualize surveillance.

Table 2: Danish vHL surveillance recommendations

From 0 to 4 years
• Annual clinical examination by a paediatrician
• Annual ophthalmoscopy in dilation
From 5 to 14 years of age
• Annual clinical examination by a paediatrician
• Annual ophthalmoscopy in dilation
• Annual plasma-metanephrine and plasma-nor-metanephrine
• Annual hearing examination in a department of audiology
• 1 x Magnetic Resonance Imaging (MRI) scan of the CNS and
• 1 x Ultra Sound (US) of the abdomen (Imaging optimally in the age interval 8-14 years of age)
From 15 years of age
• Every second year: MRI scan of the CNS including the inner ear
• Annual ophthalmoscopy in dilation
• Annual neurological examination (by a neurologist or a neurosurgeon)
• Annual US/MRI of the abdomen (kidneys, adrenal glands, pancreas, liver)
• Annual plasma-metanephrine, plasma-normetanephrine, and plasma-chromogranin A
• Annual hearing examination in a department of audiology

Based on the surveillance recommendations given in [96]. The guidelines are written by the national vHL coordination group (See Appendix 1 for a description of the group and its functions).

vHL diagnosis: clinical criteria and genetic analysis

A clinical vHL diagnosis can be made using the diagnostic vHL criteria, which vary slightly between countries (Table 3). According to the Danish criteria, an individual with a negative family history but with any two vHL-related manifestations by definition has clinical vHL [96]. In the majority of international criteria, all based on Melmon and Rosen's original criteria, at least one of the manifestations must be a hb (CNS or retinal) [3, 4, 6, 123].

Genetic testing is of great importance, especially for pre-symptomatic detection of mutation-carriers in affected families [119, 124]. The detection rate of a *VHL* germline mutation in patients with clinical vHL is up to 95-100% when a combination of direct sequencing, Southern blotting, Fluorescence In Situ Hybridization (FISH), and/or Multiplex Ligation-dependent Probe Amplification (MLPA)is used [74, 124-126]. The frequency of *VHL* mutations among clinically affected patients depends on the phenotypic spectrums in the tested cohorts [126].

Table 3: vHL clinical diagnostic criteria

	Danish criteria [96]	International criteria [3, 4, 6, 123]
Positive family history/ *VHL* mutation	1 vHL-related manifestation	1 vHL-related manifestation
Negative family history	At least 2 vHL-related manifestations	2 hb (CNS or retinal) **or** At least one hb (retinal and/or CNS) and a visceral lesion
vHL-related manifestations included in the criteria	Retinal hb Hb in cerebellum, the medulla oblongata, or the spinal cord ELST RCC Pheo, paraganglioma, and/or glomus tumor PNET and/or multiple pancreatic cysts	Retinal hb CNS hb RCC Pheo PNET ELST Pancreatic cysts* Epididymal cystadenomas*

* These manifestations are not included in all criteria, hb= hemangioblastoma, RCC: renal cell carcinoma, PNET: Pancreatic Neuroendocrine tumor, Pheo= Pheochromocytoma

National registers in Denmark

National Health Registers

The Danish health care system is tax-funded with free access to medical care. Every contact with a health provider is systematically registered and information from different registers can be linked to an individual using a unique identification number (the Civil Registration System (CRS)). This provides exceptional opportunities for genetic epidemiology studies, as information about family relations is also available in the CRS. See Appendix 2 for detailed information.

Background of the vHL Research Database

In the 1930-1940's Dr. Kai Albrechtsen collected information about Danish vHL families, which are now part of the national archives and kept at the Department of Cellular and Molecular Medicine (ICMM). Dr. Anette Møller Jensen did a follow-up study in the 1980-1990's, where she interviewed affected families, collected hospital records, death certificates, and family information. In the same period, Dr. Thomas Rosenberg started the first national vHL Register as part of the Danish Family Archive for Genetic Eye Diseases at the National Eye Clinic (which later moved to the Kennedy Center, Rigshospitalet in Glostrup) [127] with the purpose of ensuring that Danish vHL patients were offered proper surveillance and family diagnosis. In 2008 our group started the vHL Research Database and included both information from the vHL Register and from Dr. Albrechtsen's and Dr. Møller Jensen's research. We updated the pedigree information for 31 families and added an additional 12 families. From 2008-2016, we have interviewed the affected living individuals and collected hospital records to document all vHL examinations and manifestations that each affected family member had ever had. The vHL Research Database now contains detailed well-documented clinical information and pedigrees of all vHL families ever identified in Denmark going back as far as ten generations in the largest families. In May 2012, the Danish vHL Coordination Group established the official national vHL Database as a primarily clinical tool for diagnostic and management purposes [96, 127]. This database includes basic clinical information about Danish vHL patients as well as all individuals genetically tested for *VHL* mutations. It is continuously updated when new vHL families are diagnosed at the clinical genetic departments in Denmark.

Main challenges of vHL management

- vHL displays considerable phenotypic variability, both between different families, but also within the same family [7, 35, 43, 128]. It is almost impossible to predict a patient's most likely phenotype, such as which organs will be affected, how high is the risk of development of specific tumor development at certain life phases, or how fast will the tumors grow. Not knowing what to expect from their disease can cause uncertainty and distress for vHL families [119, 129, 130], and complicates counseling and clinical management.
- We hypothesize that vHL is underdiagnosed in Denmark, and that the vHL diagnosis is often delayed or unrecognized. Compared to international prevalence estimates, the Danish prevalence of 67 living diagnosed individuals in 2008 is low. Undiagnosed vHL families are not offered genetic counselling and asymptomatic genetic testing. They do not attend prophylactic surveillance and are at risk of permanent complications or death, which might have been prevented.

AIMS

The main aim of this PhD study was to improve the understanding of factors that complicate the diagnosis, genetic counseling, and surveillance of vHL families through investigation of:

Phenotypic variability: Paper I and II

How is the development of new manifestations among *VHL* mutation-carriers influenced by:

- Age
- Genotype
- Sex
- Pregnancy
- Tumor location

vHL survival: Paper III

- How has the survival of *VHL* mutation-carriers developed over time compared to siblings without vHL and the general population?
- How is survival influenced by sex, genotype, and surveillance attendance?

vHL diagnosis: prevalence, incidence, and penetrance: Paper IV

- What is the prevalence and incidence of vHL in Denmark?
- Is vHL underdiagnosed in Denmark?
- What is the penetrance of vHL in a national cohort?

MATERIAL AND METHODS

Study designs

Papers I, II, III, and a part of *IV* are based on retrospective national cohort studies. *Paper IV* also includes a register study of Danish health registers (Table 4).

Study populations and data collection

The Danish vHL cohort comprises 165 individuals identified as having vHL in Denmark (Figure 2). We had detailed clinical information concerning 150 of them. The remaining fifteen were all living individuals diagnosed with vHL, who had declined study participation (12 individuals) or whom we had not contacted (three individuals).

Paper I and II: First round of study inclusion

The initial cohort consisted of carriers of a pathogenic *VHL* germline mutation who were alive and resident in Denmark on 1st of June 2008 and who attended surveillance. We identified 59 mutation-carriers from 25 unrelated families and contacted 54 (from 22 families). We did not contact five individuals who had previously refused research participation. All 54 contacted individuals consented to participate (90%, 54 of 59). Two individuals were excluded from the analysis, as they were newly diagnosed and did not yet started their surveillance. The parents consented on behalf of children under 18 years.

Paper III and IV: Follow-up and second round of study inclusion

From 1st of January 2014 to the 1st January 2016, the 52 already included *VHL* mutation-carriers were followed-up. We identified an additional 31 living *VHL* mutation-carriers from six included and nine new families, who had been diagnosed between 2008 and 2016. Overall, we contacted 88 living individuals including the 52 individuals from 2008 and three individuals from two families not contacted in 2008. Three individuals had, via family members, asked not to be contacted. Overall, 76 individuals consented, while 12 declined. We found 74 deceased patients from 18 included and four new families. We further contacted 11 individuals (seven families) diagnosed with clinical vHL without an identifiable *VHL* germline mutation, and nine consented. We found 21 individuals with a benign *VHL* variant (N=16) or a variant of unknown significance (VUS) (N=5) [131]. These individuals were not included, as none fulfilled the clinical criteria.

Table 4: Overview of the papers' designs, observation periods, and included subgroups

Paper	Study design	Study period/ Observation time	Exclusion criteria	Included groups of individuals			
				VHL mut-carriers	Pt. with clinical vHL without a detectable *VHL* mut/mut analysis	First-degree relatives without vHL	Potential vHL patients identified in register search
I	Cohort study	Observation time: **1st Jan 1971 – 1st June 2008** (1,719 person-years)	- Deceased *VHL* mut-carriers - *VHL* mut-carriers not attending sur-veillance - Patients with clini-cal vHL without *VHL* mut	N = 52	-	-	-
II							
III	Register based Cohort study	Observation time: **16th May 1841 - 1st Jan 2016** (6,075 person years)	*VHL* mutation-carri-ers born before 2010	N= 143	-	N = 137 Siblings of *VHL* mut-carriers (Control group)	-
IV	Register study + Cohort study	Study period: Register study: **1st Jan 1977 – 10th Dec 2015** Penetrance analysis: **1878 – 2014**	Not alive in the study period	N = 97	N = 9	N =256 All known par-ents, siblings and children alive dur-ing study period	N = 335 poten-tial vHL patients and 331 of their first-degree rel-atives

Mut-carrier= Mutation-carrier

Paper III is a register study that included all deceased and living vHL patients born before 2010 from the families in which a patho-genic *VHL* mutation had been confirmed in at least one affected family member. The 143 included individuals were either con-firmed *VHL* mutation-carriers (N=89) or assumed mutation-carri-ers: (a) had at least one vHL manifestation (N= 48) or (b) were ob-ligate mutation-carriers (had affected parents/siblings as well as affected descendants) (N=6).

For the evaluation of clinical characteristics in *paper IV*, we in-cluded 106 consenting individuals (76 living and 30 deceased, from 39 unrelated families) who had been diagnosed with vHL and had been alive at some point in the study period.
The penetrance analysis in paper IV was based on data about 150 vHL patients' first manifestation diagnosis, their first manifesta-tions had been diagnosed between 1878 and 2014.
Descriptions of subgroups of the vHL cohort have previously been published: [37, 89, 132, 133]

Data collection for the included vHL patients:
The process of data collection is illustrated in Figure 3.

All consenting individuals in *papers I, II, and IV* were interviewed (the parents on behalf of children under 18 years, and living rela-tives in the cases of deceased patients) and gave us permission to collect their hospital records concerning all vHL-related examina-tions and manifestations throughout their lives. This was initially done for the 52 included individuals in 2008. During their follow-up in 2014-2016, interviews were repeated and additional hospi-tal records collected. During the second round of inclusion/fol-low-up, the consenting individuals over 18 years gave a blood sample for genetic analysis. From the vHL Research Database we obtained hospital records and/or documented patient/family in-formation for 55 deceased individuals. We interviewed the living relatives of an additional 19 deceased individuals for whom we also collected hospital records.

First-degree relatives without vHL
We drew the families' pedigrees using the pedigree software Progeny (Progeny Genetics LLC, Florida, USA). Information about familial relations was obtained during patient interviews (includ-ing records of interviews with now deceased family members per-formed 1987-1993), the Civil Registration System (CRS)), and Church records and Population censuses in the cases of individu-als who had died or emigrated before the start of the CRS in 1968.

In *paper III* we included 137 siblings without vHL (they had either been tested negative for the family's *VHL* mutation (N=59) or did not have any vHL-related symptoms (N=78)) from all known families in which the causative *VHL* mutation had been identified.

Figure 2: Flowchart of inclusion of the Danish vHL cohort
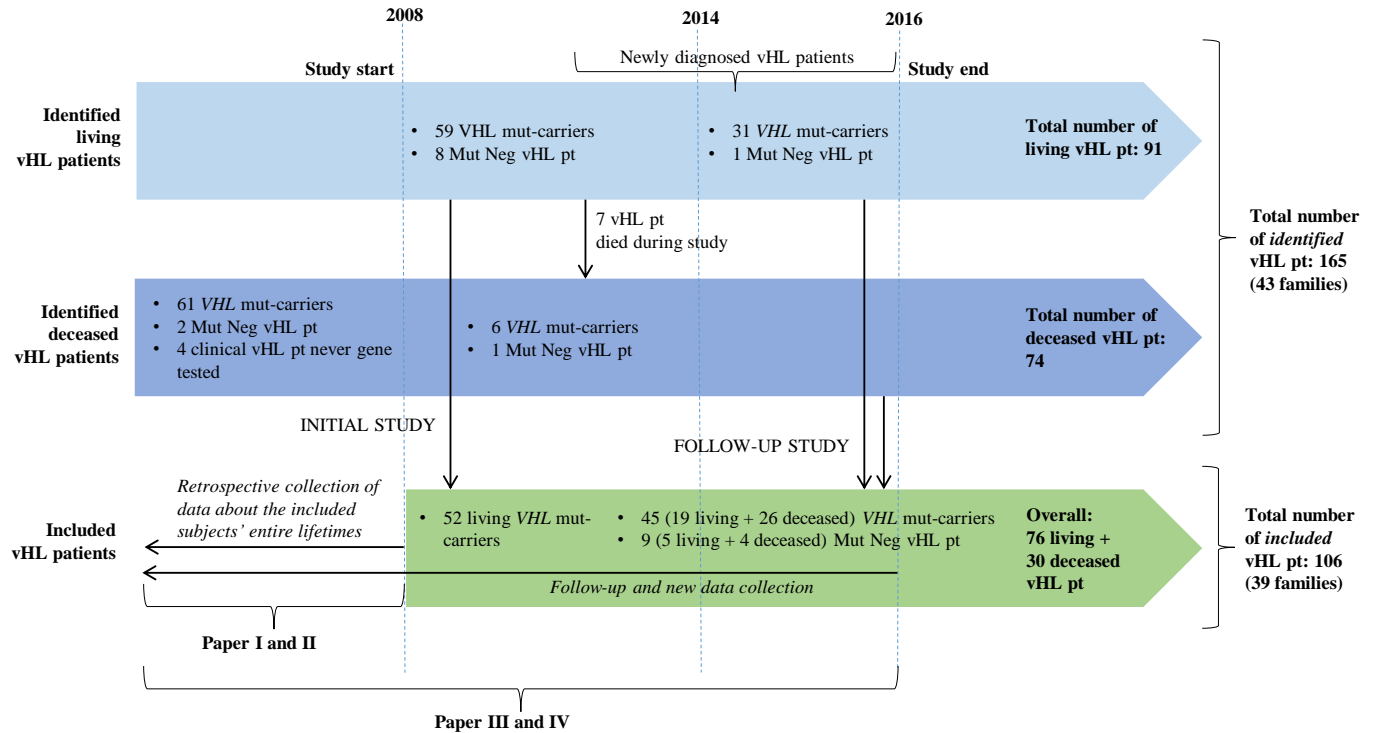
This chart shows the flow of vHL patients between different cohort subcategories: living, deceased, and included in the study. Study inclusion refers to individuals who gave their consent to active study participation: 1) Interviews, 2) permission to collect all of their hospital records regarding vHL, and 3) a blood sample for genetic testing.
VHL mut-carrier: Carrier of a pathogenic VHL mutation. Mut Neg vHL pt: Patient fulfilling the clinical diagnostic vHL criteria without an identifiable VHL mutation

Figure 3: Method of data collection
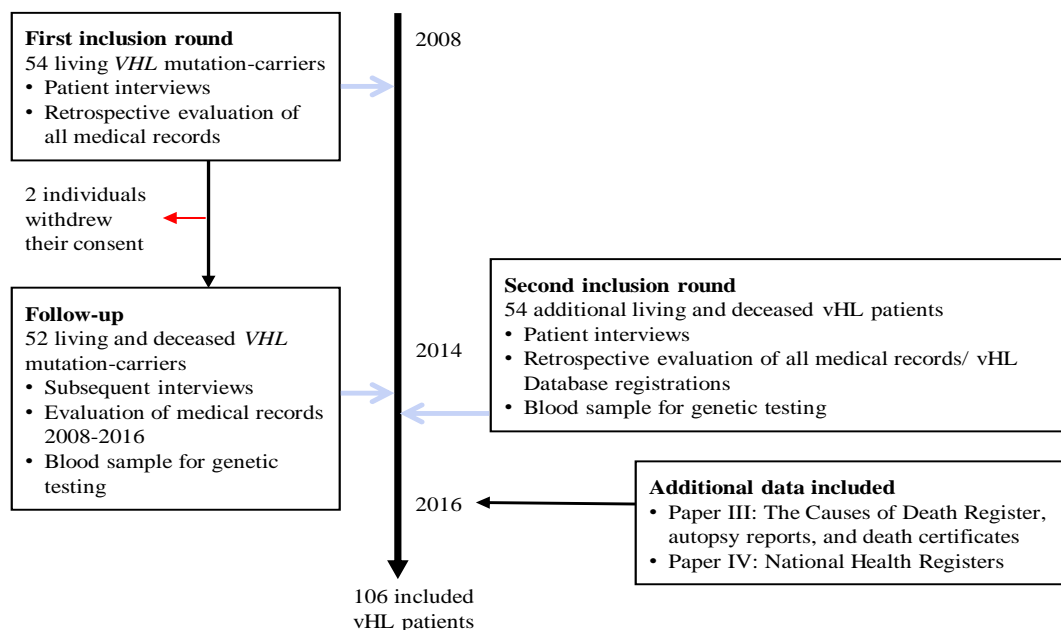
Individuals identified through Danish national health registers
We used the following national registers (see Appendix 2 for detailed descriptions): The Civil Registration System, The Patient Register, the Cancer Register, the Pathology register, and the Causes of Death Register.

In *paper III*, we utilized the Causes of Death Register to obtain information about date and specific causes of death. For patients who died before 1970, we used death certificates, medical records describing the time around the death, and autopsy reports. In the register study in *paper IV* we identified and included 335 individuals who might fulfill the clinical diagnostic vHL criteria [96] based on their registered ICD (International Classification of Disease)-codes in the Patient Register and the Cancer Register. We chose the ICD-codes used in the register search, based on a pilot study of known vHL patients whose registrations were compared to their hospital records. We reviewed the full medical histories of the 335 identified individuals (i.e. all diagnostic and pathological codes ever registered about each individual in the Patient register, the Cancer register, the Pathology register, and the Causes of Death Register) to confirm the diagnoses of their vHL-related manifestations. Using the CRS, we identified 331 first degree-relatives of 68 individuals who were assessed to fulfill the vHL clinical diagnostic criteria (assumed vHL patients). We evaluated the full registered medical histories of 205 of these relatives as well.

Information from Statistics Denmark
Statistics Denmark provides descriptive statistical information about the Danish population [134]. For *paper III* we obtained sex- and age-specific mortality rates for Danish birth cohorts born between 1900 and 2014. From here, we collected information about the size of the population and number of live births in our chosen incidence period of 1945- 1964 for *paper IV*.

Genetic analysis
We confirmed the included families' *VHL* germline mutations in at least one affected individual. A new mutation was found in a patient with clinical vHL who had not been genetically tested before. We counseled and performed genetic testing in seven first-degree relatives who had not been tested but expressed interest in such during a family interview. We found the family's *VHL* mutation in one 18-year old asymptomatic child of an affected mother, but not in the other six tested individuals (2 children and 4 siblings of affected vHL patients).

Sanger sequencing and Multiplex Ligation dependent Probe Amplification (MLPA)
Genomic DNA was purified from peripheral lymphocytes using the PureGene Blood Core Kit C (Qiagen, Hilden, Germany, cat. no. 158389) according to the manufacturer's instructions. We looked for germline *VHL* mutations through PCR-amplification of all three *VHL* exons and exon-intron boundaries (intronic primer pairs: Exon 1F: 5'-AGCGCGTTCCATCCTCTAC-3', Exon 1R: 5'-GGGCTTCAGACCGTGCTAT-3', Exon 2F: 5'- CAC-CGGTGTGGCTCTTAAC -3', Exon 2R: 5'-TGGGCTTAATTTTTCAAGTGG-3', Exon 3F 5'- GTTGGCAAA-GCCTCTTGTTC-3', Exon 3R: 5'- AAGGAAGGAACCAGTCCTGT-3'). We performed direct Sanger sequencing using the ABI BigDye® Terminator Cycle Sequencing Kit, version 1.1 on an ABI Prism® 3130XL Genetic Analyzer according to the manufacture's manual. The sequences were visualized for evaluation using the ChromasPro version 1.7.6 (Technelysium Pty Ltd, South Brisbane, Australia).

We further looked for deletions and duplications in the *VHL* gene by MLPA analysis using the MLPA-*VHL* test kit (P016-C2, MRC-Holland, Amsterdam, Netherlands), according to the manufacturer's protocol. The results were analyzed using Coffalyzer.Net (MRC-Holland, Amsterdam, Netherlands).

In silico analysis of the identified VHL mutations
Alamut Visual Software version 2.6 (Interactive Biosoftware, Rouen France) was used to confirm the pathogenicity of each identified *VHL* mutation. For further analyses, we grouped the mutations into two categories: 1) missense mutations and 2) truncating mutations (deletions, frame-shift mutations, nonsense, and splice-site mutations). Appendix 3 gives a full list of the included genotypes.

Data assessment and statistical analysis
Paper I and II:
To determine how fast new tumors developed at different stages of life, we used Poisson regression models to calculate age-related manifestation rates. That is, the average number of new manifestations diagnosed/year calculated in relation to age in predefined five-year intervals from 0-60 years. 95% Confidence intervals (CI) were based on robust standard errors (SE), which allowed for heterogeneity in the manifestation rates between the mutation-carriers. Using hazard ratios (HR), we compared the manifestation rates in groups of 1) different ages, 2) tumors in different locations (hbs in the cerebellum and retina), 3) patients with different genotypes (truncating and missense mutations), 4) men and women, and 5) women in pregnancy and in non-pregnancy intervals (the women were defined as being in a pregnancy-interval up to five years after the estimated date of conception).

Paper III:
To assess the progression in vHL survival over time, we compared Kaplan-Meier curves for vHL patients born at different times. The development was further illustrated through comparison to the survival of their siblings without vHL using a Cox regression model with birth year, sex, and vHL status (vHL patient or non-vHL sibling) as co-variates. We used similar Cox regression models to evaluate the effects of influencing factors on the vHL patients' hazard of death as well as their hazard of dying due to a vHL-related cause by a stepwise addition of multiple covariates in separate models (birth-year, sex, genotype, and surveillance attendance). The 95% CI was based on robust SE that allowed for heterogeneity between the families. Surveillance attendance was defined as regular examinations of the abdomen and/or CNS performed to screen for vHL manifestations for a period of at least three consecutive years.

We used a relative survival model to compare vHL survival to the survival of the general Danish population and estimate the mean remaining life-time at age ten for individuals born in different years. This model calculates the excess mortality due to vHL using mortality rates of the general Danish population as a baseline. Causes of death were categorized as being vHL-related or vHL-unrelated based on information given on the deceased patients' death certificates, data extracted from the Causes of Death Register, as well as autopsy records and hospital records from around the time of death in some cases. Deaths were assessed to be vHL-related if a vHL manifestation, long-term or post-operative vHL-related sequelae or surgical complications were the direct or indirect causes of death.

Paper IV:
We estimated vHL penetrance based on 150 vHL patients' ages at their first manifestation diagnosis. We used Kaplan-Meier curves to calculate cumulative age-related incidences of 1) all first manifestations (regardless of how they were diagnosed) and 2) all first manifestations that were diagnosed due to symptoms.

vHL point prevalence was calculated as the proportion of live vHL patients in the Danish population on 1st January 2014. Birth incidence was estimated as the proportion of vHL patients born between 1945 and 1964 of the total number of live births in Denmark during that period.

The level of statistical significance was set at 5% in all analyses. Statistical analyses were performed using SAS version 9.3 or version 9.4 (SAS Institute, Cary, USA) or R version 3.2.5 with the package survival version 2.28-3 [135].

Ethical considerations
The project was approved by the Danish Data Protection Agency (2009-41-3994) and the Regional Committees on Biomedical Research Ethics (H-2-2010-012), and included individuals gave their written consent for participation in *paper I, II, and IV*. The national register search and utilization of data from the national Danish vHL Research Database (*paper III* and part of *paper IV*) were conducted as register studies that did not require consent from the included individuals.

RESULTS
In total, the 150 individuals for whom we had detailed clinical information, had 5,715 vHL-related examinations and 1,209 vHL

manifestations diagnosed. Almost all manifestations (98%, 1,182 of 1,209) were verified through medical records. The rest were reported only by the patients themselves or family members. In the cases of three deceased patients, we solely had information about their manifestations through interviews. As the information was very specific, they were included in the penetrance analysis in *paper IV*. Appendix 2 and 3 show the characteristics of the included and non-included patients' genotypes and phenotypes.

Papers I, II, and III: Factors influencing the vHL phenotype and course of disease
Paper I and II: New manifestation development
We found that the number of new manifestations diagnosed per year is not constant throughout vHL patients' lives, but varies significantly with age, genotype, and the anatomical location of the tumor. New tumor development is similar for men and women, and pregnancy is associated with a decreased rate of new tumor development.

Through interviews and evaluation of medical records concerning all vHL-related hospital visits of the included 26 male and 26 female *VHL* mutation-carriers, we identified 2,583 examinations and 581 manifestations. The rate of new tumor diagnosis was highest in patients' thirties with a mean rate of almost one new manifestation diagnosed per year (Table 5). As expected, this rate was significantly higher than rates in childhood and teenage years, but also than the rates in patients' early twenties. The manifestation rate tended to decrease after patients' thirties although the difference was not significant.

Table 5: Manifestation rates (new manifestations/year) for all vHL manifestations as a function of age and hazard ratios (HR) for comparison of age-groups and sex groups

Age-intervals	Both sexes Manifestation rates (95% CI) N= Number of included pt.	Comparison of age-groups[1] HR (95% CI) p-value	Men Manifestation rates (95% CI) N= Number of included pt.	Women Manifestation rates (95% CI) N= Number of included pt.	Comparison of sex-groups HR (95% CI) p-value
0 – 14	0.040 (0.022 - 0.071) N= 52	0.045 (0.024 – 0.086) p < 0.0001	0.045 (0.020 - 0.101) N= 26	0.035 (0.015 - 0.080) N= 26	1.281 (0.401 - 4.091) p= 0.68
15 – 19	0.250 (0.126 - 0.497) N=39	0.284 (0.122 – 0.661) p= 0.004	0.232 (0.108 - 0.495) N= 19	0.269 (0.089 - 0.812) N= 20	0.862 (0.225 - 3.297) p= 0.83
20 – 24	0.386 (0.221 - 0.676) N=37	0.439 (0.225 - 0.854) p= 0.015	0.474 (0.217 - 1.032) N= 19	0.300 (0.144 - 0.626) N= 18	1.579 (0.541 - 4.607) p= 0.40
25 – 29	0.398 (0.174 - 0.911) N=35	0.452 (0.181 – 1.133) p= 0.090	0.204 (0.080 - 0.522) N= 17	0.567 (0.203 - 1.583) N= 18	0.361 (0.090 - 1.449) p= 0.15
30 – 34	0.880 (0.570 - 1.360) N=32	1 (–)	1.412 (0.841 - 2.369) N= 14	0.472 (0.278 - 0.803) N= 18	2.988 (1.424 - 6.267) p= 0.004
35 – 39	0.863 (0.562 - 1.326) N=29	0.981 (0.570 – 1.686) p= 0.944	0.858 (0.462 - 1.595) N=12	0.867 (0.483 - 1.558) N= 17	0.990 (0.422 - 2.323) p= 0.98
40 – 60	0.611 (0.441 - 0.846) N=24	0.694 (0.423 – 1.139) p= 0.148	0.572 (0.432 - 0.758) N=10	0.638 (0.383 - 1.063) N= 14	0.897 (0.501 - 1.606) p= 0.71

Based on table 2 from paper I and table 2 from paper II. N= Number of subjects included in the age-interval. We included all manifestation types (581 manifestations). Subjects were followed from birth. The age-groups 0-14 and 40-60 years were pooled due to low manifestation numbers.
[1]: In the age-group comparison: the HRs are calculated using the age-group 30-34 years as the reference interval.

When we compared the manifestation rates for patients with missense mutations (N= 22) to those with truncating mutations (N=30), the overall manifestation rate was significantly higher for truncating mutation-carriers (HR=1.85, 95% CI: 1.05-3.25, p= 0.033). This was especially due to higher rates in adulthood and more frequent cerebellar tumor development. For cerebellar tumors, the overall rate was twice as high for truncating mutation-carriers (HR=2.3, 95% CI: 1.01-5.08), while their overall rate of new retinal tumor diagnosis was less than half of the missense mutation-carriers' rate (HR=0.391, 95% CI: 0.21-0.73, p=0.003).

Seventeen of the included women had completed 30 pregnancies. The rates of new manifestation diagnosis were lower when the women were pregnant compared to when they were not pregnant (Tabel 6). Any pregnancy effect on microscopic tumor precursors was not expected to be seen immediately, as progression to a radiologically detectable tumor would take time. Therefore, we defined three pregnancy intervals of varying lengths to look for any long-term effects. Indeed, we found that the manifestation rates were significantly lower up to 5 years after conception.

Table 6: Effect of pregnancy on manifestation rates

Pregnancy interval	Hazard ratio (95% CI)	p-value
1 year	0.343 (0.084 – 1.402)	0.136
3 years	0.372 (0.194 – 0.712)	0.003
5 years	0.476 (0.239 – 0.949)	0.035

Modified from table 1 in paper II. Thirty pregnancies among 17 women were included in all three pregnancy-intervals (defined as time form estimated conception). The analysis was adjusted for number of manifestations at age 20 and genotype (truncating or missense mutation).

We assessed the rate of new manifestation development separately for only cerebellar and retinal hbs, as these were the most frequent vHL manifestation types (Figure 4). The high manifestation rate in patients' thirties seems to be primarily caused by a significant increase in the number of cerebellar tumors. From the patients' thirties to forties, new cerebellar tumor diagnosis reached a rate of more than one new tumor every second year (35-39 years: 0.57 new tumors/year, 95% CI: 0.33 – 1.00). Rates at younger ages (under 30 years) were significantly lower. In contrast, new retinal hbs were diagnosed at the highest rate in patients' teenage years, also corresponding to a rate of about one new tumor every second year (15-19 years: 0.51 new tumors/year, 95% CI: 0.22-0.75). Rates of new retinal hbs in early childhood and ages older than 25 years were significantly lower.

Paper III: vHL survival and causes of death
The survival of vHL patients has improved over time, and is significantly influenced by a patient's birth year, sex, and genotype. We estimate the mean life expectancy of *VHL* mutation-carriers born in 2000 to be 67 years for men and 60 years for women. RCC is becoming a rarer cause of death for vHL patients, although most deaths are still vHL-related.

Figure 4: Manifestation rates as a function of age: A) cerebellar and B) retinal hbs

A: Rate of new cerebellar hbs
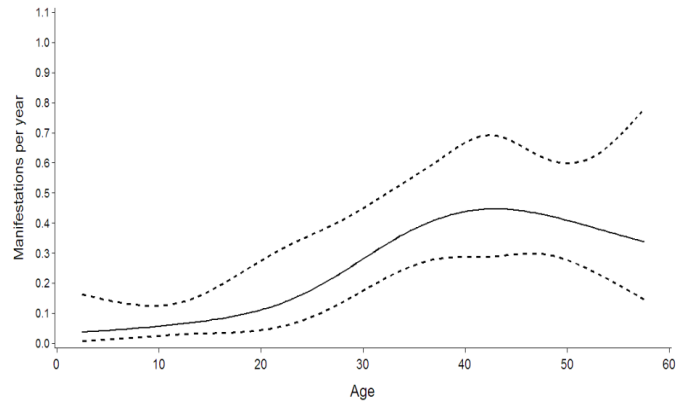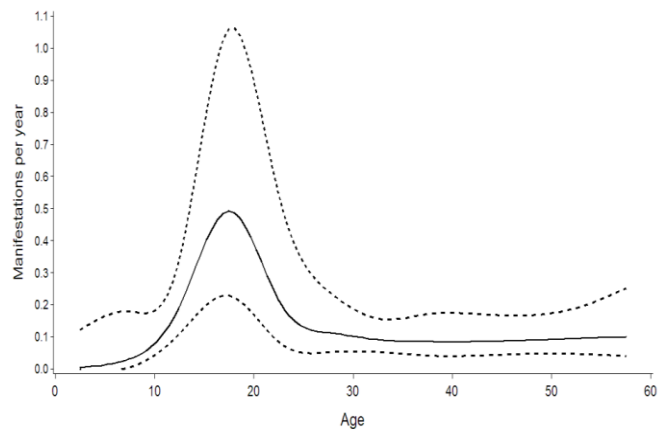
B: Rate of new retinal hbs

Figure 1 from paper I. Full-drawn line: The number of new manifestations diagnosed per year as a function of age.
Dotted line: 95% Confidence interval. Poisson regression was used to calculate age-dependent manifestation rates in pre-defined five-year age intervals. A restricted cubic spline function was used to depict the rates in smooth graphs. Subjects were included with delayed entry from the time of their first (A) MRI or CT of the CNS or (B) ophthalmoscopy. A is based on 120 cerebellar hbs. B is based on 88 retinal hbs.

We analyzed the survival and causes of death of 143 *VHL* mutation-carriers (67 deceased and 76 living). The *VHL* mutation-carriers born after 1955 had a significantly better survival compared to earlier vHL birth cohorts. Consistently, the Cox-regression model demonstrated that birth years had a significant effect on vHL survival; the later a patient was born, the better the survival. The

separate effects of birth year, sex, genotype, and surveillance attendance can be seen in Table 7.

Survival was significantly poorer for female *VHL* mutation-carriers compared to their male counterparts, as the female hazard of vHL-related death was more than twice as high (Table 7). The effect of being male or female significantly depended on the patient's genotype, with the lowest mortality rates for male missense mutation-carriers, whose hazard of vHL-related death was almost 5 times lower than for female missense mutation-carriers (p<0.001).

Overall, about half the included vHL cohort (55%, 79 of 143) had attended regular surveillance at some point in their life (median number of years with surveillance: 12 years, range: 1- 35 years). We only found a significant effect of surveillance if we took the patient's genotype in account.

Table 7: Effect of birth year, sex, and genotype on overall mortality and on vHL-related mortality rates

Covariate	Overall mortality rate			vHL-related mortality rate		
	HR	95% CI	p-value	HR	95% CI	p-value
Birth year	0.98	0.96 - 1.00	0.036	0.98	0.96 – 1.00	0.056
Sex (female vs. male)	2.25	1.16 – 4.35	0.016	2.25	1.20 – 4.20	0.011
Genotype (missense vs. truncating mutation)	0.58	0.24 – 1.40	0.224	0.54	0.24 – 1.24	0.145
Surveillance attendance (yes vs. no)	0.54	0.13 – 2.23	0.394	0.52	0.134 – 2.05	0.352

Table 4 from Paper III.
95% CI: 95% Confidence Interval. HR= Hazard ratio
Included in the analysis are 132 vHL patients born after 1900 (70 women and 62 men; 62 VHL missense mutation-carriers and 70 carriers of VHL truncating mutations).
*The separate effects of birth year, sex, genotype, and surveillance attendance on the hazards of death form any cause and death from a vHL-related cause. In this Cox regression model the four covariates are all included in the same model. In this model, we did not include interaction terms.*

Truncating mutation-carriers, who overall had the poorest survival, had the largest benefit from surveillance. Their risk of vHL-related death was significantly lower for those who had attended surveillance compared to those who had not (HR=0.22, 95% CI: 0.07-0.69, p= 0.0095).

Overall, the majority of all deaths among vHL patients are directly or indirectly related to vHL (79%, 53 of 67 deaths), Figure 5. The pattern of vHL-related causes of death has changed over time. From RCC and CNS hb being equally frequent causes of death among the older generations born between 1900-1955 (42% of deaths related to RCC and 42% to CNS hbs) to almost all

causes of death being related to CNS hbs in later years (83%, 5 of 6 deaths among patients born after 1955)

Figure 5: Causes of death among vHL patients (N= 67 deaths)
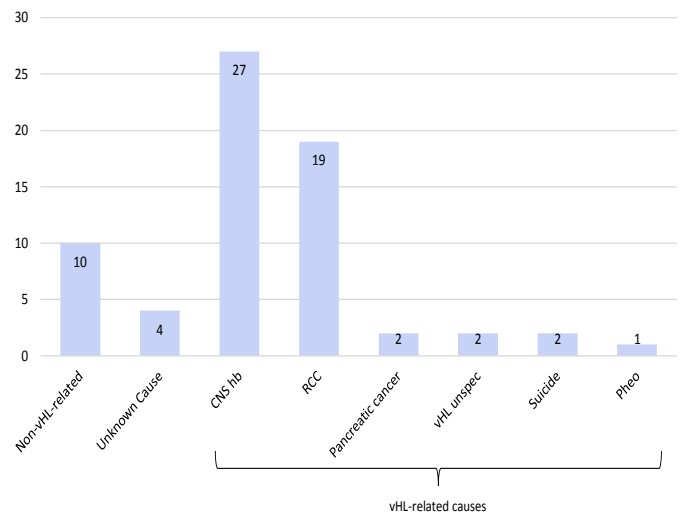
When compared to their 137 siblings without vHL, the *VHL*-mutation-carriers had a poorer survival, especially female patients who had a mortality rate that was eight times as high as their female siblings (HR=8.09, 95% CI: 4.88-13.4, p< 0.001). Comparably, the mortality rates of the male patients were about twice as high (HR=2.25, 95% CI: 1.02-4.96, p= 0.045). The same tendency was seen when vHL patients were compared to the general Danish population. The gap in the mean life expectancies of a female *VHL* mutation-carrier and a woman in the general population born in 2000 was more than twice as large as for males (22 years vs. 10 years). Nevertheless, the vHL life expectancy has moved much closer to that of the general population (Figure 6).

Figure 6: Development of mean remaining lifetime for 10-year-old individuals over time
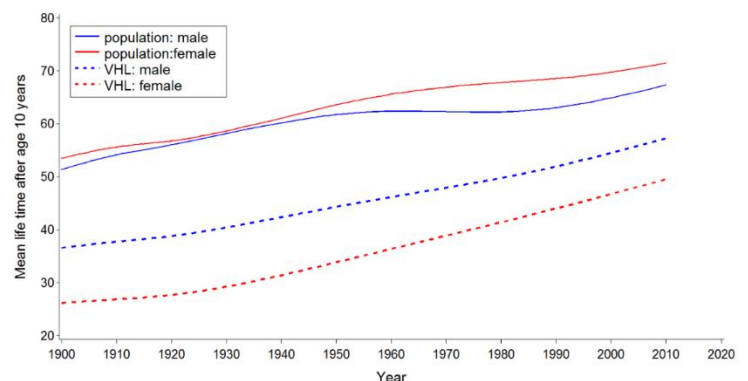
Supplementary figure 1 from Paper III. The analysis based on data from 74 female and 69 male vHL patients, as well as sex and age-specific death rates for Danish birth cohorts from 1900 to 2014. For example a male VHL-mutation-carrier, who is 10 years old in 2010 (born in 2000) has an estimated mean remaining lifetime of 57.3 years (mean total lifetime of 67.3 years); that corresponds to 10.1 years less than the estimated mean remaining lifetime of a

boy in the general population born in the same years (Mean remaining lifetime in 2010: 67.5 years/ total mean lifetime 77.5 years).

Paper IV: Penetrance, prevalence, and incidence of vHL

The penetrance of vHL was found to be up to 87% at age 60 based on the clinical information of 150 already diagnosed vHL patients. The overall penetrance, which also included manifestations diagnosed due to surveillance, was higher than the incidence of symptomatic manifestations at all ages (Table 8). The penetrance of symptomatic manifestations gives an estimate of the natural history of the vHL phenotype, if vHL patients did not attend surveillance.

vHL seems to be underdiagnosed in Denmark. We found 122 diagnosed vHL patients who had been alive at some point during the study period using the vHL Research Database. Through the search of the national health registers, we identified an additional 71 individuals who fulfilled the Danish clinical diagnostic criteria during the same period (69 by having at least two registered vHL-related manifestations, and two by having one manifestation and a first degree-relative with two manifestations) (assumed vHL patients). They were identified through evaluation of the full registered medical histories of 335 possible vHL patients (based on selected diagnostic ICD-codes) and 205 first-degree relatives.

Table 8: vHL penetrance

	A	B
	Penetrance of vHL (regardless of mode of diagnosis) **(95% CI)**	**Penetrance of symptomatic vHL** **(95% CI)**
10 years	**0.07** (0.02 - 0.11)	**0.03** (0.001-0.06)
20 years	**0.30** (0.23 -0.38)	**0.16** (0.10-0.23)
30 years	**0.57** (0.48-0.64)	**0.44** (0.34-0.52)
40 years	**0.78** (0.70-0.84)	**0.66** (0.56-0.75)
50 years	**0.86** (0.79 -0.91)	**0.78** (0.68-0.85)
60 years	**0.87** (0.80-0.92)	**0.80** (0.69-0.86)

Based on Figure 2 and Table 2 in Paper IV. N= 150. In A; all first manifestations are considered as events regardless of whether the manifestation was symptomatic or asymptomatic at diagnosis, subjects are censored at age of death or study end if no first manifestation has been diagnosed. In B; only symptomatic first manifestations are considered as events, while first manifestations that were asymptomatic at diagnosis were censored at the age at diagnosis.

We estimate the vHL point prevalence and birth incidence estimates (Table 9) based on both the international clinical diagnostic criteria (requiring that at least one manifestation must be a hb) as well as using the Danish clinical diagnostic criteria [96].

The clinical characteristics of the 68 assumed vHL patients and the 84 diagnosed patients with at least two manifestations were compared (Table 10).

Table 9: Point prevalence and birth incidence of vHL

Number of individuals alive 1st January 2014	Point prevalence	Number of individuals born between 1945-1964	Birth incidence
Estimate using international criteria			
91 (diagnosed vHL pt) + 29 (assumed vHL pt with at least 1 hb adjusted for 17% missing) = **120**	**1:46,894 individuals**	39 (diagnosed vHL pt) + 20 (assumed vHL pt with at least 1 hb adjusted for 17% missing) = **59**	**1:27,271 live births**
Estimate using Danish criteria			
91 (diagnosed vHL pt) + 52 (assumed vHLpt adjusted for 17% missing) = **143**	**1:39,351 individuals**	39 (diagnosed vHL pt) + 32 (assumed vH pt adjusted for 17% missing) = **71**	**1: 22,661 live births**

Modified from table 4 from Paper IV. Pt= Patients, Hb= Hemangioblastoma.
The size of the Danish population was 5,627,235 on the 1st of January 2014 (Statistics Denmark [134]). The number of live births from 1945-1964 was 1,608,966 (Statistics Denmark [134]).
The estimates of assumed patients were then adjusted by 17% as a measure of missed patients in the search.

Table 10: Comparison of the assumed and the diagnosed vHL patients with at least 2 vHL-related manifestations

	Assumed vHL pt with at least 2 manifestations (N=69)	Diagnosed vHL pts with at least 2 manifestations (N=84)
Frequency of pt with familial/non-familial vHL (based on family history at diagnosis)	**Familial:** 12% (8 of 69) **Non-familial:** 88% (61 of 69)	**Familial:** 89% (75 of 84) **Non-familial:** 11% (9 of 84)
Median age at first manifestation diagnosis (Range)	**50 years** (12 – 85 years)	**23 years** (6 -73 years)
Frequency of pt with at least 1 hb (CNS or retinal) **at the time of their second manifestation diagnosis**	**67%** (46 of 69)	**87%** (73 of 84)
Frequency of pt only visceral lesions (pheo, paraganglioma, pancreatic lesions, RCC) **at the time of their second manifestation diagnosis**	**33%** (23 of 69)	**13%** (11 of 84)

Modified from Table 1 from Paper IV. Pt= Patient, Hb= Hemangioblastoma, RCC= Renal Cell Carcinoma, Pheo= Pheochromocytoma/Paraganglioma

DISCUSSION

We primarily wanted to increase the understanding of why the vHL phenotype varies so much between vHL families and even members of the same family. Furthermore, we wanted to establish how often the vHL diagnosis may be missed in Denmark as well as to evaluate vHL penetrance based on a national cohort.

Key findings:

- Risk of new vHL manifestations varies with age, genotype, and tumor location.
- Men tend to have higher manifestation rates in adulthood compared to women.
- Pregnancy is associated with a lower frequency of new manifestations.
- vHL survival has improved significantly over time, and seems to be influenced by a patient's birth year, sex, genotype, and surveillance attendance.
- vHL has been underdiagnosed in Denmark; we estimate the prevalence to be about 1 in 46,900 individuals and the birth incidence to be about 1 in 27,300 live births.
- We found a penetrance at age 60 of 87%, and only 80% among patients who have not attended surveillance prior to diagnosis, accordingly vHL does not exhibit full penetrance as previously reported.

Which factors influence the phenotypic variability of vHL?

Previous studies of variation in vHL manifestation development have focused either on the patients' mean ages at first manifestation, total or cumulative manifestation incidence, or on the growth rates of individual tumors [7, 35-37, 40, 44, 45, 47, 59, 70, 136-138]. We have demonstrated how the risk of new vHL manifestations is not constant, but varies over the course of a vHL patient's lifetime.

The impact of genotype

Genotype significantly modulates a vHL patient's risk of specific manifestation types throughout life. Generally, *VHL* mutations that lead to an absent or truncated protein product are associated with the most severe phenotypes. We found that truncating mutation-carriers' risk of new manifestations was almost twice as high as for missense mutation-carriers. The two genotype groups have similar patterns of new manifestation development until the patients' twenties, when the rate of new manifestations increases more for truncating mutation-carriers, especially due to a significantly higher rate of new cerebellar hbs. In contrast, missense mutation-carriers have an overall higher rate of new retinal hbs, especially from 10-20 years of age. In line with our results, others have found truncating mutation-carriers to have a significantly higher risk of developing multiple CNS hbs and associated cysts compared to missense mutation-carriers [44, 62, 71, 139]. The association of truncating mutations with a lower risk of retinal hbs is in accordance with other studies [40, 59, 109-112]. The association is however not entirely due to concurrent loss of *BRK1* along with *VHL* deletions [109-112], as only one family of three patients had a deleted *BRK1* in our study. This points to other contributing mechanisms being behind this correlation or perhaps tissue-specific effects of different mutation types.

Consistent with our study, Maher et al. found vHL survival to be similar for the two genotype groups when no other factors are considered [107]. Nevertheless, our interaction analysis revealed that the genotype actually has a considerable effect on vHL survival, as the effects of sex and surveillance depend significantly on the patient's germline mutation. Male missense mutation-carriers had a substantially better survival than their female counterparts did. This was not true for patients with truncating mutations. Consistent with their more severe phenotype, truncating mutation-carriers had the greatest benefit of surveillance attendance in relation to vHL survival.

Correlations between germline *VHL* mutations and the resulting phenotype depend on the ability of the mutant pVHL to downregulate HIF-pathway activity [13]. Accordingly, phenotypes with high risk of CNS hb and RCC (type 1 and type 2B) are associated with *VHL* mutations that lead to either an absent or truncated pVHL that can no longer regulate the HIF pathway [140]. Missense mutations responsible for a type 2A phenotype (high CNS hb, but low RCC risk) result in a pVHL that is able to impair HIF-mechanisms to some extent [140]. Type 2C (pheo only) associated protein products have normal abilities to suppress the HIF pathway, but have instead lost their ability to regulate fibronectin matrix assembly [140, 141]. A multitude of HIF-independent pVHL functions are still being discovered. Most functions are undoubtedly also affected differently by specific mutation types, which contributes further to the complex modulation of the vHL phenotype.

Influence of pregnancy and sex

Pregnancy was associated with lower rates of new tumour development in our study. This protective effect may be long-term, as the rates were significantly lower up to five years after conception compared to when the patients were not pregnant. In line with our results, a prospective study by Ye et al. found no significant differences in the rates of new CNS hbs or in the growth of existing lesions in pregnant and non-pregnant-intervals (N= 9 women, 13 pregnancies), not even when compared to a control group without pregnancies (N=27)[142]. Contrary, a retrospective study by Frantzen et al. found that pregnancy induced cerebellar hbs growth (N= 12 pregnancies), but had no effect on other manifestation types, including hbs in other CNS locations, although the manifestation numbers were small [143]. The observation periods in both previous studies were short (mean 18.7 and 18.4 months after delivery, respectively) and any possible long-term effects may have been missed. Also, the studies did not take the women's ages into account. This may have provided a bias, as we have now shown that manifestation rates increase in patients' twenties and thirties. In our analysis, the tumor risk in pregnancy was compared with age-matched non-pregnant periods in the same female cohort. We further took the women's genotypes and manifestation burdens at age 20 into account to avoid bias in comparing women with different basis for choosing to have children. However, 46% of pregnancies (14 of 30) occurred before the women initiated surveillance, and were likely not aware of their tumor risk. The same was true for almost 60% (17 of 29) of women in the study by Frantzen et al., while all women in Ye et al's study attended vHL surveillance at the time of their pregnancies [142, 143]. Previous vigilance of adverse pregnancy-induced tumor progression may be due to the concurrence of fertile periods with symptomatic first vHL presentations in women with undiagnosed vHL, giving rise to biased reports.

Only a few studies have included sex as a variable in their analyses of vHL disease progression and survival [44, 45, 49, 138, 144]. Two studies found male and female patients' risk of new retinal and CNS hbs to be similar [45, 49], and another found no differences in men and women's tumor burdens [138]. We, on the other hand, found a tendency for men to have a more severe phenotype than women. The two sexes had similar risks of new vHL manifestations until their mid-twenties, when the male rate tended to increase compared to the female rate. This underlines that the decreased tumor risk found during and after pregnancy is not due to a concurrence of a naturally milder tumor development in women in the years when they have their children compared to their non-fertile ages. Male patients had a slightly increased risk of CNS hb, but a lower retinal hb risk, although this was not significant. A recent prospective study of CNS hbs found that male vHL patients overall had significantly more CNS hbs as well as a significantly faster tumor growth compared to women [44].

Paradoxically, we found that vHL survival seemed to be significantly poorer for women than for men despite the apparently more severe male phenotype. This was especially evident for missense mutation-carriers, where women had a mortality rate that was almost five-time as high as for men. Another smaller study likewise found a tendency for a poorer female life expectancy, but this was not significant [144]. These sex differences may be due to hormonal factors although this has not been investigated. In any case, sex differences in tumor development, tumor growth and possibly differences in vHL-related morbidity between men and women need to be further investigated.

Other influencing factors
As expected, we found that the rate of new manifestation development varies with age. This is in line with other demonstrations of age-related risks of specific manifestation types [35, 36, 40, 49, 59, 66]. In contrast to displaying cumulative manifestation risks or age-stratified prevalence [35, 36, 40, 49, 59, 66], we showed that the rate of new tumor development varies at different ages throughout life in the same vHL cohort. The risk of new manifestations does not increase steadily with age, but varies in distinct patterns according to the location of the manifestation. Consistently, biallelic *VHL* inactivation may be established as early as during embryogenesis as multiple tumor precursors, which can later be activated to grow by yet unknown factors [18, 145]. The varying patterns of two-phased growth for individual tumors as well as parallel and asymmetric growth of separate tumors in different organs or CNS locations that are described in *paper I* and reported in [44, 45, 47, 138, 139, 146], make local tissue-specific activating factors such as micro environmental stimuli likely. The patient's germline mutation may have different tissue-specific effects according to the mutational consequence, as missense mutations have a higher risk of hbs in the retina but a lower in the cerebellum, while truncating mutation-carriers have the opposite risk pattern.

Initiation of tumor development is in all likelihood a complex process requiring multiple factors. Other activating factors could be circulating endocrine stimuli, additional random genetic alterations including epigenetic changes, or external environmental factors [47, 145, 147]. External risk-modifying factors could possibly be physical activity, obesity, or smoking, although this has not been systematically studied. A recent animal study demonstrated that exercise is associated with decreased tumor burden and growth in a mouse melanoma model through up-regulation of immune responses [148]. Smoking and obesity are known risk factors for sporadic RCC development [149]. Although, curiously, in sporadic RCC tumors, smoking is associated with a lower frequency of somatic *VHL* inactivation [150].

There are wide phenotypic variations even between family members with the same *VHL* mutation suggesting additional non-allelic inherited genetic modifiers [49]. Variants in the gene for the Cyclin D1 protein, which is involved in cell cycle control, has been suggested to modulate the risk of hb development in vHL patients, although the association has only been demonstrated in a single study [151].

Also, the type of second hit, i.e. the somatic inactivation of the wildtype *VHL* allele (point mutation, deletion, epigenetic inactivation etc.) as well as additional somatic genetic alterations will contribute to the cellular phenotype of the individual precursor lesions. Recent studies using next generation sequencing (NGS) of multiple tumors from individual vHL patients have shown that distinct somatic genetic changes have occurred in the individual tumors [152, 153]. The tumors from the same individual do however also share similar patterns of somatic variation, suggesting that both tumor-specific as well as individual factors, either genetic modifiers in the germline or environmental factors, are important for tumor development [152, 153]. The sequence of vHL-related tumorigenesis is still not fully understood, which limits our ability to truly predict vHL tumor progression.

Implications for genetic counseling and surveillance
Our estimates of how frequently new vHL manifestations are diagnosed in a given age-interval can help clinicians to assess a vHL patient's probability of manifestation development at specific ages. This is useful in a genetic counseling setting, and could form the basis of future targeted surveillance strategies that better ensure efficient surveillance for the individual patient that both minimizes the risk of missing potential life-threating new manifestations, while also keeping the psychological strain at a minimum. Pre-symptomatic diagnosis of retinal hbs is especially important, as vision loss can be prevented by early treatment in most cases [39]. The risk of vision loss is reported to be significantly lower when diagnosed pre-symptomatically versus symptomatically (p < 0.001, cumulative risks of vision deficits by 40 years: 35% versus 82%, respectively) [39]. Retinal hbs are most frequent in patients' teenage years and early twenties, and regular ophthalmological surveillance is especially important in this period of their lives. After about 30 years of age the rate of new retinal tumors is persistently low corresponding to only about one new tumor every tenth year. In line with this, a large study of retinal hbs found that the risk of permanent vision loss due to retinal tumors was highest before the age of 30 [39]. We believe that retinal surveillance could be relaxed in older individuals with longer intervals between examinations, especially for truncating mutation carriers who have an overall lower risk of retinal hbs. Particular focus on screening for CNS hbs is advised from vHL patients' thirties where manifestation rates peak, mainly for truncating mutation-carriers. As CNS hbs are the major cause of vHL-related death and responsible for most long-term sequelae [138], an early diagnosis is essential to give the surgeons the most optimal conditions for planning the appropriate treatment approach [48].

Based on our results, pregnancy should not be discouraged for female vHL patients based on their predisposition alone, and we do not recommend intensified surveillance of pregnant vHL patients, which contrasts recommendations of previous studies [117, 143].

If women have already diagnosed manifestations, appropriate specialist evaluations of risk of growth or complications due to pregnancy might occur, and any additional measure can be taken based on the individual situation.

The frequency of RCC-related deaths has decreased over time. This can most likely be attributed to surveillance-facilitated early RCC diagnosis. Ong et al. compared a British vHL cohort (N=573) of which 20% had been diagnosed with vHL pre-symptomatically, and who had attended surveillance to the clinical characteristic of part of the same cohort diagnosed due to clinical vHL (N=152) when they had just initiated surveillance 17 years earlier [35, 36]. They found that the frequency of RCC diagnosis had increased in patients who had attended surveillance (35% vs. 28%). Furthermore, the RCCs were diagnosed at a significantly younger age in the surveillance group (39.7 vs. 44 years, p= 0.026) [36], suggesting diagnosis at earlier tumor stages.

We did not find any significant effects of surveillance attendance on vHL survival, when genotype was not considered. This could in part be due to the late start of surveillance for many patients (median age of surveillance initiation: 24 years, range: 0-67 years). Also, our strict definition of regular surveillance attendance may underestimate the true extent of surveillance, and our material may be too small to see any significant effect. Before official guidelines were published [154], many vHL families did attend pre-symptomatic screening, but not always in regular intervals. Many of these periods are not counted as surveillance attendance, which may underestimate the true effect of surveillance. In fact, we have previously found a surprisingly high compliance to surveillance in part of this cohort as far back as in the early 1990's [37], which contrasts other reports of low surveillance compliance among vHL patients [53, 132, 155].

A very important purpose of surveillance is to minimize vHL-related morbidity, especially long-term sequelae such as blindness, neurological deficits, loss of kidney function, thereby improving the patients' quality of life. We did not account for these factors in the present study, and the impact of surveillance on vHL-related morbidity has yet not been directly assessed. A recent prospective Italian study evaluated vHL-related disabilities among patients who had attended surveillance, but had no control group. They found a high rate of patients with neurological impairments, however, despite this the majority of patients had only minor effects on their ability to carry out normal daily activities [138]. Even though some patients in this study were followed for up to 16 years, the median follow-up time was 45 months, suggesting that many patients may not have attended long-term surveillance [138]. The effects of surveillance are expected to be even more evident in pre-symptomatically diagnosed *VHL* mutation-carriers who have attended surveillance from early childhood.

In the future, as antiangiogenic drugs or other systemic treatments may be available for pre-symptomatic treatment of especially CNS tumors, the benefit of very early manifestation diagnosis through surveillance can become even more important [124].

Challenges in vHL diagnosis
vHL is not always acknowledged on account of its rarity as well as involvement of numerous clinical specialties. Many vHL patients may not be diagnosed at the most optimal time, which is essential for establishment of surveillance and the offer of genetic counseling. We demonstrate that vHL has been underdiagnosed in Denmark. Many individuals in whom the diagnosis is delayed or missed entirely may be asymptomatic or very mildly affected *VHL* mutation-carriers, even late in life. vHL does not seem to be as highly penetrant as previously reported, and as many as 20% of *VHL* mutation-carriers may still be asymptomatic and undiagnosed at age 60, if they have never attended surveillance.

vHL prevalence and incidence in Denmark
When including both diagnosed and yet undiagnosed assumed vHL patients, we found high estimates of vHL prevalence (1 in about 46,900 individuals) and birth incidence (1: 27,300 live births) compared to most previous studies [7-10]. Our estimates are even higher, if we use the Danish diagnostic criteria, as they also include individuals without a hb, but with two other vHL-related manifestations. The estimates based on Danish criteria are however not directly comparable to previous estimates based on international diagnostic criteria. Also, our register search for assumed vHL patients is more complete according to international criteria, as we were able to find individuals with multiple hbs, but not those with multiple manifestations of most other types. Previous prevalence and incidence estimates vary widely, from 1 in 39,000 to 1 in 91,000 individuals and the birth incidence from 1 in 36,000 and 1 in 53,000 live births in different populations [7-10]. All were based on selected patient cohorts and have been regional estimates, that may be influenced by random genetic drift and founder effects [9]. Further, most previous estimates were based on cohorts of clinically affected patients, which would underestimate the number of *VHL* mutation-carriers [7-10]. Our estimates are based on a national cohort that includes all vHL patients with a genetic diagnosis as well as those clinically affected. The increase in the Danish prevalence of diagnosed vHL patients from 67 living patients in 2008 to 91 living patients in 2014 can in part be attributed to improved vHL diagnosis and survival. Also, many *VHL* mutation-carriers are in their reproductive age, and nine of their children born after 2008 were pre-symptomatically diagnosed as *VHL* mutation-carriers. In recent years, the national Danish vHL coordination group (see Appendix 1) has been formalized, and has gained many new specialists, which enables a strong multi-disciplinary effort and strengthened awareness about vHL among medical professionals throughout the country.

vHL penetrance
We found that vHL penetrance was 87% at age 60, and if we only considered patients who had not attended surveillance prior to their first manifestation diagnosis, only 80% would have had symptoms at this age. This is much lower than previous estimates, where vHL penetrance has been reported to be almost 100% at 60 years [7, 8]. Previous estimates were based on cohorts of clinically affected patients diagnosed before accurate genetic testing was available. This could have biased the estimates towards the more severe phenotypes, as asymptomatic or mildly affected *VHL* mutation-carriers were not included. In our study 14% (15 of 106) of the cohort were asymptomatic mutation-carriers, while only 3.6% (3 of 83) and less than 0.5% (1 of 236), respectively, were asymptomatic obligate mutation-carriers included in the previous cohorts [7, 8]. Since genetic testing has become the main diagnostic tool for vHL, several cases of asymptomatic mutation-carriers older than 60 years have been reported [39, 133, 156]. In our cohort, 16 mutations-carriers had lived to be more than 60 years old without any symptoms of vHL.

Implications for vHL diagnosis and genetic counseling

The notion that vHL is fully penetrant at age 60 years has probably influenced the diagnostic approach to vHL families. In the Danish families with a known pathogenic *VHL* mutation, a surprisingly high number of the living first-degree relatives (39%, 55 of 141) have never been genetically tested (median age: 62 years, range: 1 – 99 years). The finding of a lower penetrance than previously assumed could have important consequences for genetic counseling of adult asymptomatic first-degree relatives and patients with late-onset vHL-related tumors.

The lower penetrance seen in our study could also have implications for assessment of vHL as a possible differential diagnosis. Based on the observed lower penetrance, many *VHL* mutation-carriers may have milder phenotypes with only a single vHL-related manifestation and perhaps no family history of vHL. Up to 11% of patients with an apparently isolated pheo with no family history and no other vHL related symptoms, have a germline *VHL* mutation [34, 77, 83]. The same goes for up to and 18% of solitary CNS hb patients, respectively [41, 70, 71, 157]. Several groups recommend that all patients with a single retinal or CNS hb, a single pheo, or a single early-onset RCC (< 50 years) should be genetically tested for *VHL* mutations [96, 119, 156].

Through the national Danish health registers, we identified 71 individuals who we assessed to fulfill the Danish clinical diagnostic criteria based on their registered diagnostic ICD-codes (assumed vHL patients). According to the national vHL Research Database they had not been diagnosed with vHL. The majority of the assumed vHL patients were non-familial cases, which contrasts the high proportion of familial cases usually found among the diagnosed patients [7, 62, 158, 159],*paper IV*. On one hand, non-familial cases are more easily overlooked, and we would expect most undiagnosed *VHL* mutation-carriers to be *de novo* cases. On the other hand, previous estimates of *de novo* mutations are much lower, between 3 and 21% [6, 62, 64, 158]. Based on our finding that vHL is not fully penetrant, previous reported *de novo* mutation cases might in reality be cases of non-penetrance in an asymptomatic parent with a germline *VHL* mutation, who has not been genetically tested. We assessed that 22 patients had *de novo* mutations based on their family histories in *paper IV*, but this had only been genetically confirmed in one family. Apparent *de novo* cases could also be due to mosaicism in a parent, though this is reported in only up to 5% [159].

Some of the identified assumed vHL patients may have a different diagnosis. About a third of the assumed patients (20 of 69) had only visceral lesions and would not have fulfilled the international diagnostic criteria [4]. The majority (60%, 12 of 20) had a combination of a RCC and a neuroendocrine tumor (a pheo, a paraganglioma, or a PNET). Some may be phenocopies, while others may have vHL type 2C or another pheo predispositions such as Multiple Endocrine Neoplasia 2A or *SDH*-associated hereditary pheo/paraganglioma syndrome [160]. *VHL* germline mutation have been found in up to almost 40% of familial pheo cases, while the rest or caused by other genes or unknown causes [81, 161, 162]. Among the diagnosed vHL patients, as many as 13% (11 of 84) had only visceral manifestations and no hbs at the time of their second manifestation diagnosis, when they would have fulfilled the diagnostic criteria on a purely clinical basis. This underlines the usefulness of the more inclusive Danish diagnostic criteria compared to the international criteria.

In a clinical setting, multiple aspects are taken into account before a vHL diagnosis is made, including the clinical characteristics of the individual and the family. Rarer phenotypic presentations,

such as solely visceral lesions require particular attention to differential diagnoses, and often multigene testing will be considered [96]. The greatest challenge lies in assessing whether patients with only visceral lesions, in whom no *VHL* mutation has been found and all other differential diagnoses have been ruled out, should attend vHL surveillance, as they would still fulfill the Danish diagnostic criteria. Better knowledge about the risk of additional manifestations in such families is needed to facilitate appropriate surveillance measures.

STUDY STRENGTHS AND LIMITATIONS

Several elements contribute to the strength of the reported results. The included papers are all based on national studies that identified all known *VHL* mutation-carriers regardless of genotype or phenotype, which limits the risk of selection bias. Further, due to the free and equal health care system in Denmark, any bias caused by socio-economic disparities in surveillance or treatment options is negligible.

The results are based on a comprehensive, reliable, and well-documented data set that accounts for the entire lifetime of each of the included individuals. Almost all (98%, 1,182 of 1,209) of the diagnosed manifestations were documented through hospital records or register data. The approach of using a longitudinal perspective in which we included the patients' entire life spans enables us to demonstrate changes over several decades, both for the individual vHL patient, but also for the entire cohort.

Certain study limitations should, nevertheless, be taken into account:

Study design

- The included papers are all based on retrospective studies, as this approach allowed us to include long follow-up time for each individual.

Risk of selection bias

- As the study cohort has been selected based on a specific diagnosis, any yet undiagnosed families are not included. The possible vHL probands identified in *paper IV* were for example not included in the previous papers' analyses. Although most were seemingly *de novo cases*, the extent of any asymptomatic mutation-carriers in their families is unknown. Also, a large number (39%, 55 of 141) of currently living first-degree relatives in known vHL families have not been genetically tested. Although apparently asymptomatic, some may be mutation-carriers.

- In *papers I, II, and III* we solely included confirmed *VHL* mutation-carriers, as the underlying genetic cause of the vHL phenotype or the natural history of the disease is unknown in families without identifiable *VHL* mutations. The group of vHL patients who fulfill the clinical diagnostic criteria without identifiable *VHL* mutations is, however, very small, and any differences would have minimal influence on the overall estimates.

- In the clinical characteristics analysis in *paper IV*, not all known vHL patients were included. Nevertheless, when we compared the 16% (15 of 91) who were not included to the 150 included patients, their characteristics were similar, except for an overweight of asymptomatic *VHL* mutation-carriers in the non-included group compared

to the included group (40%, 6 of 15 vs. 13%, 20 of 150). (Appendix 3).

Limitation of use of national health registers

In paper *IV*, we searched directly for undiagnosed individuals who could fulfill the clinical diagnostic vHL criteria (assumed vHL patients). Nevertheless, our search strategy probably underestimated the frequency of assumed vHL patients.

- For every contact with the health care system, the treating hospital department/physician is responsible to code the procedures performed and the diagnoses suspected/given during the contact and to report these codes to the relevant registers. Therefore, different doctors may not use the diagnostic codes uniformly to code the same manifestation types. The variation is largest for manifestations that do not have their own specific ICD-code, such as retinal hb. Even though specific codes are available for hbs of the cerebellum, they are still often coded using ICD-codes for benign or malignant supratentorial tumors or tumors in unspecified locations (see Supplementary report about the pilot study in *Paper IV* for details). Nevertheless, as we included all hbs based on their morphological code, regardless of location-specific ICD-codes, which would identify all hbs that have been surgically removed. Since 2000, the government funding of hospital departments/private practitioners depends on a system in which the codes used for diagnoses and procedures lead to different payment rates, which may have biased the use of certain codes over others [163].
  To our knowledge, the ICD-codes used in our register search have not been validated on a larger scale to ensure that the specific codes are used for the intended underlying conditions that we wish to find. This has been done for a variety of other conditions (stroke, cholesteatoma, and venous thromboembolisms) with positive predictive values of the ICD-codes registered in the Patient Register of about 80-90% [164-166]. We did perform a pilot study to choose the codes to be used in the search. In the pilot study, the hospital records of diagnosed vHL patients (N=37) were compared to their diagnostic codes.
- In *paper IV* we used only the Patient Registry combined with the Cancer registry in our initial search. We subsequently used the Pathology Register for diagnosis confirmation, and discovered that this register had a more accurate and complete registration of vHL-related manifestations diagnosed among the known vHL patients. Inclusion of the Pathology Register in our initial search would have yielded a more accurate search result.
- In the register search in *paper IV*, we were only able to evaluate the full medical records for individuals who were alive in 1968 (and received a CRS identification number). Assumed vHL patients who were born in the time period used to calculate birth incidence (1945-1964), but wo had died before 1968 or who had manifestations diagnosed before the start of the study period (1977) from when we had register data, would not have been found.

- In *paper IV* we did not obtain register data for 38% (126 of 331) of the identified first-degree relatives to the assumed vHL patients. This may have underestimated the frequency of familial cases among the assumed vHL patients. We do, however, not asses this bias to be very large, as any first-degree relatives with a retinal or CNS hb or two different vHL-related manifestations would have been identified in our initial search. Among the 126 evaluated first-degree relatives, we found about 3% (6 of 205) to be affected. From this, we can extrapolate that up to four affected first-degree relatives (3% of 126) may have been missed among the non-evaluated first-degree relatives.

Genotype classification

- In our genotype analyses in *paper I and III*, we focused on two broad genotype categories (missense and truncating *VHL* mutations). *VHL* genotype-phenotype correlations have been categorized in further detail, both according to the mutation type, its intragenic location, relation to functional domains, and structural integrity of pVHL [36, 108, 167, 168]. Due to the limited number of Danish vHL families, we were not able to account for the genotype effects in greater detail.
- The Danish distribution of missense and truncating mutations (44% and 56%), Appendix 3 seems to be slightly skewed compared to distributions reported internationally, where between about 50-65% of all identified germline *VHL* mutations are missense mutations [6, 16]. As families with missense mutations are more likely to be missed than those with truncating mutation-carriers, owing to the often milder phenotypes of missense mutation-carriers, there may have been a tendency for families with missense mutations to be underdiagnosed. The higher frequency of truncating mutation-carriers in the Danish cohort might be because less Danish vHL families are overlooked.

Factors not accounted for and conservative definitions

- In *papers I and II*, we focused only on the development of new manifestations and did not take growth or associated cyst development of any already diagnosed manifestations into account. We have no evidence that the same factors are involved, however, hypothesize similar growth mechanisms for initial growth of microscopic precursor lesions and of larger detectable tumors, when their sizes increase.
- In *paper I*'s analyses of retinal or cerebellar manifestation rates, any hbs diagnosed at the patients' first ever brain scan or ophthalmoscopy were not included, as it was impossible to determine the exact age at which the manifestation could have been detected. We did this to avoid a falsely high manifestation rate at the time of their first examination. This approach may nevertheless cause a systematic bias that could generate underestimated manifestation rates in the youngest age groups. But, when we considered all manifestation types, we included all first manifestations and the manifestation rates were not markedly higher in the younger age groups.

CONCLUSION AND PERSPECTIVES

The full complexity of vHL-related tumorigenesis is unraveling, and many yet unidentified factors are likely to influence tumor development and subsequently the patients' phenotypes. The more widespread use of NGS techniques and increasing focus on epigenetics will facilitate identification of potential genetic modifiers in the germline as well as genetic drivers of tumorigenesis, which could be used in phenotype predictions, or as potential drug targets.

Even though we cannot yet accurately predict the specific phenotype of the individual patient, our results are useful when answering families' questions about what to expect from their disease, as often posed in a clinical genetic setting. We document that vHL survival has improved, and is getting closer to the survival of individuals without vHL. This could have important implications for many international patients' problems with life and health insurances. Our results form the basis of improved individualized surveillance recommendations. We provide evidence as to which factors influence new tumor development and vHL survival.

Especially the finding that pregnancy is associated with decreased tumor development is important for female patients, who can be reassured that pregnancy does not increase their risk of new tumors, and who do not need intensified surveillance during pregnancy. The finding that women have a lower life expectancy than men, even though men seem to have more severe phenotypes is surprising and needs to be further studied in other vHL cohorts. The concept of vHL as a very rare and highly penetrant disease may be changing. New genetic testing approaches has made multi-gene testing available. The indications for when to include the *VHL* gene in multigene analyses may become wider, the easier and more cost-effective analyses becomes. This could lead to a wider phenotypic spectrum with a higher frequency of mildly affected *VHL*-mutation-carriers, giving rise to even lower penetrance estimates. Many at-risk family members, especially parents or siblings to adult probands have not been genetically tested. Based on previous penetrance estimates, the probability of a *VHL* mutation-carrier having had a manifestation in their thirties or forties would be around 80% and 90%, respectively. The risk of older first-degree relatives may in some cases have been perceived as negligible, if they had reached a certain age without symptomatic manifestations. Based on our penetrance calculations, however, the age-related penetrance is much lower, which greatly influences risk assessment in a clinical genetic setting. vHL is still not recognized as the underlying cause of disease in many families, who are not offered genetic counseling or surveillance. Even though the Danish prevalence has increased over recent years, vHL still seems to be underdiagnosed, and there is further need for increased awareness about vHL for doctors who may encounter the disease.

APPENDIX 1: THE DANISH VHL COORDINATION GROUP

As vHL management is a multidisciplinary effort, it is essential that surveillance is organised in collaboration between the involved specialists. In Denmark, a national vHL coordination group has been established, which consists of medical specialists from all over the country covering all aspects of vHL-associated specialties: Clinical and molecular genetics, Ophthalmology, Neurosurgery, Urology, Endocrinology, Radiology, Otology, Gastroenterology, vHL Researchers, and The Danish vHL Patient Society.

Besides writing the national surveillance recommendations [96], the group has developed a mobile chart for patients to help them keep track of their surveillance program and the current recommendations. Also, most group members function as clinical coordinators for vHL patients in their region, and are in charge of managing the patients' referrals to their surveillance examinations.

APPENDIX 2: DESCRIPTIONS OF THE EMPLOYED NATIONAL HEALTH REGISTERS

The Civil Registration System, (CRS) [169, 170]: Contains each resident's unique CRS number, name, vital status, dates of birth and death, sex, places of birth and residence, information about migration, and the CRS numbers of parents, children, and spouses. Individuals deceased before the initiation of the CRS in 1968 are not registered. A source of uncertainty is that family registrations are based on legal and not biological relationships, so adopted and biological children cannot be differentiated. Family relationships are only registered for individuals born after 1950, and when the CRS started, parent-child relationships only if they had the same address.

The Patient Register [163]: Contains data about all somatic hospital admissions from 1977 (including all diagnoses and surgical procedures performed). From 1995, emergency room and outpatient contacts were also registered. Diagnoses are coded using ICD-codes (ICD-8 from 1977-1994 and ICD-10 from 1995-present). The data is registered electronically by the treating doctor, who is also responsible for choosing the most appropriate ICD-codes for the contact.

The Cancer Register [171]: Contains information about the incidence of different malignancies and benign CNS tumors. The register was initiated in 1943, but registration was not mandatory before 1987. ICD-7 were used before 1978, and ICD-10 codes after. Each tumor is registered separately, and morphology was registered from 1978. Registrations are received from treating hospitals. Data is validated through cross-reference with the Patient register and the Pathology Register.

The Pathology Register [172]: Contains information about all histological specimens examined at Danish Pathology departments. The register was initiated with standardized national registration in 1997, but also includes data dating back to the 1970ies from some departments (the different departments have varying start dates, so registration cannot be considered complete before 1997). The register is updated daily. Diagnoses are coded and reported electronically by the examining pathologist using SNOMED (Systematized Nomenclature of Medicine)-codes.

The Causes of Death Register [173]: Contains information about date of death, manner of death, and causes of death (direct and indirect causes as well as other conditions that may have contributed to the death) as registered on the Death Certificate from 1970. Diagnoses have been registered with ICD-10 codes since 1994. Until 2007, the ICD-codes were chosen by trained coders in the National Board of Health based on the written diagnoses on the death certificate filled out by a doctor. From 2007, doctors themselves reported the death certificate electronically and chose the ICD-codes.

APPENDIX 3: ADDITIONAL TABLES

Characteristics of the included 150 vHL patients for whom we have specific clinical information and the 15 vHL patients who have declined active study participation.

	Included vHL patients (N= 150)		Non-included vHL patients: (Total N= 15)[1] 1) have decline active study participation (N= 6), 2) not responded to our study invitation (N= 6), 3) were not contacted (N= 3)	
Vital status and age	**74 deceased** (median age at death: 45 years, range: 12 – 88 years) **76 living** (median age[2]: 39 years, range: 1-73 years)		**0 deceased** **15 living** (median age: 33 years, range: 3- 71 years)	
Genotype				
Truncating mutation-carriers	48% (72 of 150)		40% (6 of 15)	
Missense mutation-carriers	44% (66 of 150)		40% (6 of 15)	
No identifiable *VHL* mutation	5% (8 of 150)		20% (3 of 15)	
Never genetically tested	3% (4 of 150)		0 % (0 of 15)	
Clinical characteristics				
Frequency of individuals without diagnosed vHL manifestations	13% (20 of 150)		40% (6 of 15)	
Median age at first manifestation diagnosis	26 years (Range: 6 – 73 years)		21 years (Range: 16 -55 years)	
Manifestation types				
	Incidence (N= Number of affected subjects)	**Subjects with type as first manifestation** (% of all subjects with manifestations N= 130)[2]	**Incidence** (N= Number of affected subjects)	**Subjects with type as first manifestation** (% of all subjects with manifestations N=)[2]
Retinal hbs	46% (69 of 150)	32% (42 of 130)	56% (5 of 9)	33% (3 of 9)
Cerebellar hbs	61% (91 of 150)	38% (49 of 130)	44% (4 of 9)	33% (3 of 9)
Brainstem hbs	15% (22 of 150)	4% (5 of 130)	22% (2 of 9)	11% (1 of 9)
Spinal Hbs	27% (41 of 150)	3% (4 of 130)	44% (4 of 9)	11% (1 of 9)
Supratentorial hbs	3% (4 of 150)	1% (1 of 130)	11% (1 of 9)	11% (1 of 9)
ELSTs	2% (3 of 150)	0% (0 of 130)	0% (0 of 9)	0% (0 of 9)
RCC	30% (45 of 150)	11% (14 of 130)	33% (3 of 9)	0% (0 of 9)
Renal cysts	41% (62 of 150)	-	11% (1 of 9)	0% (0 of 9)
Unspecified renal tumors	5% (8 of 150)	8% (10 of 130)	0% (0 of 9)	0% (0 of 9)
Pancreatic cysts	29% (44 of 150)	8% (10 of 130)	0% (0 of 9)	0% (0 of 9)
PNETs	2% (3 of 150)	8% (10 of 130)	0% (0 of 9)	0% (0 of 9)

Other pancreatic tumors (unspecified or adenocarcinoma)	9% (14 of 150)	2% (2 of 130)	0% (0 of 9)	0% (0 of 9)
Pheos	14% (21 of 150)	8% (10 of 130)	11% (1 of 9)	0% (0 of 9)
Paragangliomas	1% (2 of 150)	0% (0 of 130)	0% (0 of 9)	0% (0 of 9)
Epididymal cystadenomas	47% (7 of 150)	4% (5 of 130)	0% (0 of 9)	0% (0 of 9)
Cysts of the broad uterine ligament	1% (2 of 150)	0% (0 of 130)	0% (0 of 9)	0% (0 of 9)

Inclusion: Here refers to active study participation: interviews, letting us use hospital records, providing a blood sample.

Out of the 165 known living and deceased vHL patients ever known to be diagnosed with vHL in Denmark. From the vHL Research Database and the current study, we have documented clinical information about 150 of them (99% (1198 of 1209) of their manifestations are documented by medical records, the rest (1%, 11 of 1209) only through family information). Of these 150 individuals, 40 (27%) had not been systematically examined for vHL manifestations or had an autopsy. Their manifestations were only diagnosed due to symptoms, and the number of manifestations that are unaccounted for is unknown.

[1]: The 3 first-degree relative with possible vHL diagnosed in the register study in paper I are not included in this group, as their diagnosis has not been confirmed. Information about the 15 non-included individuals was obtained based on information from previous research projects and registrations in the vHL Research Database and/or health registers. The information is therefore not documented through hospital records and information about manifestations may be incomplete. The 15 non-included patients came from 12 families, all but 3 families were represented in the study as other family members were included.

[2]: Current age was taken as age on the 1st January 2016

[3]: If more than 1 manifestation type was diagnosed at the same time, we counted both as first manifestations.

The genotypes of the diagnosed Danish vHL families with pathogenetic *VHL* germline mutations

Family no.	No. of *VHL* mut-carriers in the family	*VHL* mutation (c.DNA/p.Protein)	Assessed to be truncating (T) /missense (M)[1]
1	22	c.278G>A (p.Gly93Asp)	M
2	2	c.407 T>C (p.Phe136Ser)	M
3	4	c.341-? - 463+?del	T
4	34	c.433C>T (p.Gln145*)	T
5	8	c.341-?_463+?del	T
6	4	c. 499C>T (p.Arg167Trp)	M
7	3	c.319C>T (p.Arg107Cys) AND c.353T>C (p.Leu118Pro)	M
8	10	c. 194C>T (p.Ser65Leu)	M
9	3	c.337C>T (p.Arg113*)	T
10	2	c.520_521ldupAA (p.Asn174Lysfs*29)	T
11	3	c.293A>G (p.Tyr98Cys)	M
12	1	c.269A>T (p.Asn90Ile)	M
13	2	c.239G>T (p.Ser80Ile)	M
14	3	c.l-?_463+?del	T
15	1	c.194C>G (p.Ser65Trp)	M
16	1	c.341_343insGGT	T

17	2	c.481C>T (p.Arg161*)	T
18	4	c.496G>T (p.Val166Phe)	M
19	1	c.341-?_463+?del	T
20	1	c.463+1G>T	T
21	4	c.1-?_340+?del	T
22	1	c.606dupA (p.Gln203Thrfs*53)	T
23	1	c.194C>G (p.Ser65Trp)	M
24	2	c.548C>A (p.Ser183*)	T
25	1	c.464-?_642+ ?del	T
26	3	c.464-?_642+ ?del	T
27	2	c.l-?_463+?del	T

28	2	c.341-?_463+?del	T
29	1	c.1-?_340+?del	T
30	1	c.464-?_642+ ?del	T
31	1	c.257C>T (p.Pro68Leu)	M
32	6	c.500G>A (p.Arg167Gln)	M
33	6	c.191G>C (p.Arg164Pro)	M
34	1	c.388G>A (p.Val130Ile)	M
Total	**15 families with missense mutations (44%) and 19 families with truncating mutations (56%)**		

Supplementary table 1 from paper IV.
*The reference VHL sequence used is NM_000551.3, *= stop codon*
[1:] Missense mutations defined as a single nucleotide substitution leading to substitution of an amino acid. Truncating mutations defined as mutations predicted to truncate the gene product (nonsense, frame-shift, splice-site mutations, and exon deletions)

ABBREVIATIONS

CRS	Civil registration system
CT	Computed tomography
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ELST	Endolymphatic sac tumor
EPO	Erythropoietin
FISH	Fluorescence in situ hybridization
GLUT-1	Glucose transporter 1
Hb/hbs	Hemangioblastoma(s)
HR	Hazard ratio
ICD-7/8/10	International classification of disease v. 7/8/10
MLPA	Multiplex ligation-dependent probe amplification
MRI	Magnetic resonance imaging
N/ No	Number
NGS	Next generation sequencing
OMIM	Online mendelian inheritance in man
Pheo/Pheos	Pheochromocytoma(s)
PNET	Pancreatic neuroendocrine tumor
Pt	Patient
pVHL	The VHL protein
RCC	Renal cell carcinoma
SE	Standard error
SNOMED	The systematized nomenclature of medicine
TGF-α	Transforming growth factor α
US	Ultra sound
VEGF	Vascular endothelial growth factor
vHL	von Hippel-Lindau disease
VHL	The VHL gene
VUS	Variant of unknown significance
95% CI	95% Confidence interval

SUMMARY

von Hippel-Lindau disease (vHL) is a hereditary tumor predisposition caused by mutations in the VHL tumor suppressor gene. VHL mutation-carriers are at life-long risk of multi-organ tumor development. The mainstay of vHL management is close surveillance and surgical tumor removal. The disease has been reported to be fully penetrant at 60 years of age, and has a highly variable phenotype, which complicates vHL management and causes distress and uncertainty for affected families. vHL survival has historically been poorer than the survival of the general population, with a median life expectancy for vHL patients of only 49 years. vHL life expectancy is expected to be improved by better surveillance, tumor diagnosis, and treatment approaches, although this has not yet been directly demonstrated. The prevalence of vHL is between 1 in 39,000 and 1 in 91,000 individuals, and the birth incidence is between 1 in 36,000 and 1 in 45,500 live births in different populations. Based on these estimates, vHL is underdiagnosed in Denmark, and many undiagnosed families are not offered genetic counseling or prophylactic surveillance.

We aimed to assess 1) how the rate of new manifestation development is influenced by age, sex, genotype, tumor location, and pregnancy, 2) how vHL survival has developed over time, and is affected by sex, genotype, and surveillance attendance, 3) to determine the prevalence and incidence of vHL, and 4) to calculate vHL penetrance based on an unselected national cohort.

We including almost all diagnosed vHL patients in Denmark in a retrospective cohort study. We further used the national health registers to find individuals who had a missed vHL diagnosis despite fulfilling the clinical diagnostic criteria.

We found that the risk of new vHL manifestations varies with age, genotype, and tumor location. The risk of new retinal tumors is highest in the patients' teenage years, while cerebellar tumors developed at the highest rates in patients' thirties. Patients with truncating mutations had higher rates of new manifestation diagnosis than patients with missense mutations. Men tend to have higher manifestation rates in adulthood compared to women, and pregnancy was associated with a lower frequency of new manifestations. vHL survival has improved over time, and is getting closer to that of their siblings without vHL and the general population. Survival is significantly influenced by a patient's birth year, sex, and genotype. We estimate the mean life expectancy of VHL mutation-carriers born in 2000 to be 67 years for men and 60 years for women.

We estimate the vHL prevalence to be about 1 in 46,900 individuals and the birth incidence to be about 1 in 27,300 live births. We found a penetrance at age 60 of 87%, and only 80% among patients who have not attended surveillance prior to diagnosis, which is considerably lower than previous estimates.

Our findings form the basis of a more targeted vHL surveillance and counseling. The lower age-related penetrance greatly influences risk assessment in a clinical genetic setting. Even though the prevalence has increased over recent years, vHL is still underdiagnosed, and there is a need for increased awareness about the disease.

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