Mutation analysis of TGF-β signaling pathway genes among Finnish patients with primary pulmonary hypertension and hereditary hemorrhagic telangiectasia

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ACADEMIC DISSERTATION

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Original publications
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:


IV Sankelo M, Halme M, Laitinen T: Mutational analysis of ALK1, Endoglin, BMPR1A, SMAD4, and Serotonin Transporter genes among the Finnish PPH patients, manuscript

The publications are referred to in the text by their roman numerals.
ABBREVIATIONS

aa  amino acids
aaPPH  anorexigen associated primary pulmonary hypertension
ACVRL1  activin A receptor, type II-like 1
ALK1  activin like kinase type I
ALK3  activin like kinase type III
AV  arteriovenous
BMP  bone morphogenetic protein
bp  base pairs
BMPR1A  bone morphogenetic protein receptor, type IA
BMPRII  bone morphogenetic protein receptor type II
Ca\(^{2+}\)  calcium
CAVM  cerebral arteriovenous malformation
CCB  calcium channel blocker
Co-SMAD  Cooperating Smad
CT  computer tomography
eNOS  endothelial nitric oxide synthase
ET-1  endothelin-1
ETA  endothelin receptor A
ETB  endothelin receptor B
FDH  Finnish disease inheritance
FPAH  familial pulmonary artery hypertension
FPPH  familial primary pulmonary hypertension
GFP  green fluorescent protein
HAART  highly active antiretroviral therapy
HAVM  hepatic arteriovenous malformation
HHT1  hereditary hemorrhagic telangiectasia type 1
HHT2  hereditary hemorrhagic telangiectasia type 2
HIV  human immunodeficiency virus
hivPPH  human immunodeficiency virus associated pulmonary hypertension
5-HTT  5-hydroxytryptamine transporter
ICD-9  International Statistical Classification of Diseases and Related Health Problems, the Ninth Revision
ICD-10  International Statistical Classification of Diseases and Related Health Problems, the Tenth Revision
i.v.  intravenous
IPA AH  idiopathic pulmonary arterial hypertension
JPS  juvenile polyposis syndrome
K\(^{+}\)  potassium
1.5Kv  1.5 voltage-gated potassium channel
MIM  Mendelian Inheritance in Man
mPAP  mean pulmonary artery pressure
6MWT  6 minute walking test
NMuMG mouse epithelial cell line
NO  nitric oxide
NYHA New York Heart Association
PAF platelet activating factor
PAH pulmonary arterial hypertension
PAI plasminogen activator inhibitor
PAVM pulmonary arteriovenous malformation
PCR polymerase chain reaction
PDGFα platelet derived growth factor α
PPH primary pulmonary hypertension
PVR pulmonary vascular resistance
RFLP restriction fragment length polymorphism
R-SMAD receptor regulated SMAD
RT-PCR reverse transcriptase polymerase chain reaction
s.c. subcutaneous
SLC6A4 solute carrier family 6, member 4
Smad4 Sma gene and mothers against decapentaplegic homolog 4
SNP single nucleotide polymorphism
SPH secondary pulmonary hypertension
SSCP single-strand conformational polymorphism
Tx tromboxane A2
vWF-ag von Willebrand factor antigen
ABSTRACT

Pulmonary hypertension is a disorder characterized by a rise in pulmonary arterial pressure leading to right heart failure. The etiology and pathophysiology of the disease varies. Primary pulmonary hypertension (PPH), or according to the recent classification idiopathic pulmonary hypertension (IPAH), is a rare, progressive disease of pulmonary vasculature with a mean survival of four years. The main symptoms are dyspnea, fatigue and syncope or near syncope. Patients are mainly young adults with female predominance. Most of the patients are sporadic but in about 6% of cases the disease is familial (FPPH). In 2000 two different groups identified the gene predisposing to PPH. Bone morphogenetic protein receptor type 2 (BMPR2) encodes a subunit of transforming growth factor β (TGF-β) receptor complex. Mutations of BMPR2 have been found in about 55 % of the familial and 26 % of the sporadic PPH cases.

Hereditary hemorrhagic telangiectasia (HHT) or Osler-Weber-Rendu disease is a bleeding disorder characterized by local telangiectasias. The most common symptom of HHT is recurrent epistaxis, but arterio-venous (AV) malformations may also be found in internal organs like the liver or lungs. Some patients also have pulmonary hypertension clinically indistinguishable from PPH, suggesting clinical and genetic overlap between PPH and HHT. Mutations in genes encoding endoglin and activin like kinase type I (ALK1 or ACVRL1), another members of the TGF-β have been found to predispose to HHT and also to HHT-related pulmonary hypertension.

In this study we identified all of the Finnish PPH patients for the years 1986-1999 using the hospital discharge registries of Finnish university hospitals. During this period we found a total of 59 confirmed PPH patients: 55 sporadic and 4 familial representing 3 different families. In 1999 the prevalence of PPH was 5.8 per million and the annual incidence varied between 0.2-1.3 per million.

Using direct sequencing we identified four mutations of BMPR2 altogether among 28 PPH patients. Heterozygous BMPR2 mutations were found in 12% (3/26) of sporadic patients and in 33% of the PPH families (1/3). All the mutations found were different.

As a candidate gene approach we also studied ALK1, Endoglin, Bone Morphogenetic Receptor Type IA (BMPR1A or ALK3), Mothers Against Decapentaplegic Homolog 4 (SMAD4) and Serotonin Transporter Gene (SLC6A4) using single-strand conformational polymorphism (SSCP) analysis and direct sequencing. Altogether 21 patients, 9 sets of parents of PPH patients (7 sporadic, 2 familial) and a parent with HHT were studied. We found 7 different polymorphisms altogether in these genes. In addition, we found two mutations in ALK1 in two unrelated samples. The other mutation was found in a parent with HHT whose child with PPH was deceased. The same mutation was found in the child, who had been diagnosed with PPH as a young adult, without signs of HHT. The other ALK1 mutation was found in a middle-aged PPH patient who had no signs of HHT in hospital records. The family history of HHT of this patient is unknown.
We also identified all the HHT patients treated at the Department of Otorhinolaryngology at Helsinki University Central Hospital between the years of 1990-2005 and 8 of the patients were studied for Endoglin and ALK1 mutations using direct sequencing. A total of seven mutations were found and all the mutations were different. The absence of a founder mutation in the Finnish population in both PPH and HHT was somewhat surprising. This suggests that the mutations of BMPR2, ALK1 and Endoglin are quite young and the older mutations have been lost due to repetitive genetic bottlenecks and/or negative selection.

In summary, between the years of 1986-1999 we identified a total of 59 patients with a confirmed diagnosis of PPH in Finland. Four of these patients had a familial background of the disease and one patient had HHT patients in the same family. 28 patients and 2 sets of parents were screened for mutations of the BMPR2 gene. We found a mutation of this gene in 12% of the sporadic and 33 % of the familial cases. In addition a mutation in ALK1 was found in 2 patients. We also screened eight Finnish HHT patients for Endoglin and ALK1 mutations and a mutation was found in all except one patient. Both in PPH and HHT all the mutations were different suggesting that there is no founder effect in Finland. One may also assume that other genes must be involved in the pathogenesis of these diseases.
1. INTRODUCTION

Pulmonary hypertension is a syndrome characterized by an increase in pulmonary vascular resistance potentially leading to right heart failure. The etiology and pathophysiology of the disease varies. Pulmonary hypertension has traditionally been classified as primary pulmonary hypertension (PPH) or according to the recent classification idiopathic pulmonary arterial hypertension (IPAH, Mendelian Inheritance in Man, MIM 178600) when there is not an identifiable cause (eg. like pulmonary embolism) for pulmonary hypertension. The term secondary pulmonary hypertension (SPH) is used when such a cause or risk factor for the disease is found. Although both terms (PPH and SPH) were officially abandoned in the recent classification, both the terms are still widely used.

PPH is a progressive and severe disease of pulmonary vasculature with the median survival time of about 4 years from the diagnosis (Appelbaum et al 2001). PPH is usually a diagnosis of young adults with female predominance, although people of all ages may be affected (Appelbaum et al 2001, Dolara et al 1988, Rich et al 1987, Braman et al 1991, Sandoval et al 1995). The mean time from the onset of symptoms to the diagnosis is 2-3 years (Appelbaum et al 2001, Rich et al 1987). The most common initial symptoms of PPH are dyspnea, fatigue, chest pain, syncope or near syncope (Rich et al 1987). Most of the patients are sporadic but in about 6% of cases there is more than one person affected in the same family (familial PPH, FPPH) (Rich et al 1987).

An important step in understanding the pathogenesis and the genetic background of PPH was achieved in 2000 when two separate groups reported mutations in the gene encoding bone morphogenetic receptor type II (BMPRII), a member of the transforming growth factor β (TGF-β) signaling pathway, in a majority of patients with FPPH. Later on mutations in the same gene were also found in a subgroup of patients with sporadic PPH (Deng et al 2000, Lane et al 2000, Thomson et al 2000).

A rare type of pulmonary hypertension is associated with hereditary hemorrhagic telangiectasia (HHT; MIM 187300, 600376 and 601101) also known as Osler-Weber-Rendu syndrome. HHT is an autosomal dominant inherited disease where arteriovenous (AV) malformations in different organs are found. AV malformations of nasal mucous membranes lead to recurrent nose bleeds, which is the most common symptom of the disease. In more severe cases AV malformations in the liver, lung as well as in brain occur (Porteous et al 1992, Garcia-Tsao et al, 2006 Cottin et al 2007). Previously, mutations in the genes encoding activin like kinase type 1 (ALK1 or ACVRL1) and Endoglin, members of the same signaling pathway, were found among patients with hereditary hemorrhagic telangiectasia (HHT) (McAllister et al 1994, Johnson et al 1996). Pulmonary hypertension associated with HHT is clinically indistinguishable from PPH and the phenotype caused by the same mutation can vary (Trembath et al 2001). In cases with pulmonary hypertension related to HHT, mutations in the gene encoding ALK1 are found (Trembath et al 2001). Among HHT patients with pulmonary hypertension, mutations in
Endoglin are reported in HHT patients with PAVM and pulmonary hypertension associated with dexfenfluramine use (Harrison et al 2005, Chaouat et al 2004).

The population history of Finland theoretically creates an ideal playground for geneticists. Based on the unique population history of Finland consisting of resettlement of rural areas, repetitive bottlenecks, rapid growth of population in addition to geographic, cultural and linguistic barriers, the genes of the Finnish population are thought to originate from a relatively small founder population. This forms the background of the concept of Finnish disease inheritance, a spectrum of rare, mostly recessive diseases, where often one or two founder mutations are found (de la Chapelle 1993).

In previous studies in mixed populations no founder effect has been found among the PPH patients but dozens of mutations have been described. However, in some isolated populations there is some evidence of founder mutation among HHT patients (Gallione et al 2000). Based on knowledge of the the Finnish disease inheritance and Finnish population history one may assume that a founder effect may also be found in relation to these diseases. The purpose of this study was to study the genetic epidemiology of PPH in Finland, to find a potential founder effect among the Finnish PPH and HHT patients and if possible, to create new diagnostic tools for clinicians treating the patients.
2. REVIEW OF THE LITERATURE

2.1 Finns as a study population in genetic studies

Several factors make Finland and the Finns an ideal population for mapping and identifying genes predisposing to especially monogenic and, to a lesser extent, the complex trait diseases (de la Chapelle and Wright 1998, Peltonen et al 2000). First of all, the Finns are a relatively young population. Thus, fewer crossovers have influenced the chromosomal regions harboring the founder mutation and long haplotypes are divided between affected individuals with high levels of linkage disequilibrium (LD, e.g. two alleles co-segregate more often than suspected) (de la Chapelle and Wright 1998). Environmental advantages for Finland are also significant. Compared to other populations, the Finns are a culturally homogenous population. The standard of living is high and healthcare in Finland is available for everybody. The medical training for physicians is given in only five medical schools with shared traditions, which makes the diagnoses comparable. In addition, church records in Finland provide easily accessible and profound genealogical data (Peltonen et al 2000).

The low genetic diversity among Finns is due to a unique Finnish population history. Until the 16th century most Finns lived on a narrow strip of land in the coastal region. Especially during the reign of Gustavus of Vasa (1523-1560) migration began partly due to changes in taxation. The people of this early settlement, especially farmers from the Savo region, started to migrate to eastern, central and northern parts of Finland. The migrants lived in rural isolates for centuries and mating probably occurred in units consisting of only hundreds of people (Kere 2001). Most of the Finnish population lived in these units until the industrialization, and movement to southern cities started after the Second World War. During the centuries the population of Finland has gone through genetic bottlenecks because of wars and famine which has dramatically affected the genetic pool of Finns. For instance, practically all the people in Kuusamo born before 1939 were descendants of the 39 families that survived the great famine 1695-1697 (Varilo et al 2000). After that the population of Finland started to grow quickly due to improved living conditions (Norio et al 1973). In a period of one hundred years from 1750 to 1850 the population increased fourfold from 400 000 to 1.6 million and further from 2.6 million in 1900 to it’s present 5.2 million.

Because of a small number of initial founders, isolation, repetitive bottlenecks, genetic drift and a fast increase in population, several alleles are enriched in the Finnish population, whereas some disease-causing alleles are rare compared to other populations (e.g. thalassemia, phenylketouria). This creates a basis for a theory of Finnish disease inheritance (FDH) meaning that certain rare hereditary diseases are more common in Finland and in most of these diseases the founder mutation is found (Kere 2001). Since
1966 when Norio described the genetic background of congenital nephrotic syndrome more than 30 diseases belonging to FDH have been identified. Most of these diseases are inherited as autosomal recessive traits, but also two are X-chromosomal recessive (choroideremia, X-linked juvenile retinoschisis) and two dominant diseases have been identified (Finnish type amyloidosis, tibial muscular dystrophy). Many diseases of FDH cause neurologic or metabolic abnormalities, growth retardation, vision abnormalities and some are lethal intrauterally (Kere et al 2001). A typical feature of FDH diseases is that the same mutation is found in most, up to 98% of the cases (Peltonen et al 1999). The founder effect is also present in some diseases not particularly belonging to FDH. Typical examples of this phenomenon are the genes predisposing to breast cancer BRCA1 and BRCA2 where founder mutations are found in up to 55% of the mutation positive patients (Huusko et al 1998). In addition, FH-North Karelia mutation in low density lipoprotein receptor in familial hypercholesterolemia in Finnish North Karelia explains 85% of all mutations and potassium channel mutation KCNQ1-Fin in long QT-syndrome 30% (Vuorio et al 1997, Piippo et al 2001).

2.2 Primary pulmonary hypertension (PPH) and hereditary hemorrhagic telangiectasia (HHT) as clinical entities

2.2.1 Diagnosis and clinical classification of the disease

Primary pulmonary hypertension (PPH, MIM 178600) is a disease of pulmonary vasculature. The first symptom of PPH is often shortness of breath. Later on patients may have chest pain caused by right ventricular ischemia, syncope, tiredness, and peripheral edema related to right heart failure. Some patients may also have hoarseness caused by compression of nervus recurrens by an enlarged pulmonary artery, referred to as Ortner’s syndrome (Rich et al 1987, Gaine and Rubin 1998).

PPH is characterized by elevated pulmonary arterial pressure. The diagnostic criteria include mean pulmonary arterial pressure of more than 25 mmHg at rest and 30 mmHg during exercise and the exclusion of all known causes of pulmonary hypertension such as congenital pulmonary or heart disease, myocardial disease, congenital or acquired myocardial or valvular defects, pulmonary thromboembolic disease, sickle cell anemia, history of intravenous drug use, or clinically significant respiratory or connective tissue disease, pulmonary venous hypertension with pulmonary capillary wedge pressure exceeding 12 mmHg (Rich et al 1987).

In the past pulmonary hypertension was classified as secondary (SPH) when an identifiable cause or risk factor for pulmonary hypertension was present.
1. **Pulmonary arterial hypertension**

1.1. Idiopathic (IPAH)
1.2. Familial (FPAH)
1.3. Associated with
   - 1.3.1. collagen vascular disease
   - 1.3.2. congenital systemic-to-pulmonary shunts
   - 1.3.3. portal hypertension
   - 1.3.4. HIV infection
   - 1.3.5. drugs and toxins
   - 1.2.6. other (thyroid disorders, glycogen storage disease, Gaucher disease, hereditary hemorrhagic telangiectasia, hemoglobinopathies, myeloproliferative disorders, splenectomy)
1.4. Associated with significant venous or capillary involvement
   - 1.4.1. pulmonary veno-occlusive disease
   - 1.4.2. pulmonary capillary hemangiomatosis
1.5. Persistent pulmonary hypertension of the newborn

2. **Pulmonary hypertension with left heart disease**

2.1. Left-sided atrial or ventricular heart disease
2.2. Left-sided valvular heart disease

3. **Pulmonary hypertension associated with lung diseases and/or hypoxemia**

3.1. Chronic obstructive pulmonary disease
3.2. Interstitial lung disease
3.3. Sleep-disordered breathing
3.4. Alveolar hypoventilation disorders
3.5. Chronic exposure to high altitude
3.6. Developmental abnormalities

4. **Pulmonary hypertension due to chronic thrombotic and/or embolic disease**

4.1. Thromboembolic obstruction of proximal pulmonary arteries
4.2. Thromboembolic obstruction of distal pulmonary arteries
4.3. Non-thrombotic pulmonary embolism (tumor, parasites, foreign material)

5. **Miscellaneous**

   Sarcoidosis, histiocytosis X, lymphangiomatosis, compression of pulmonary vessels, (adenopathy, tumor, fibrosing mediastinitis)

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**Table 1**  
Revised clinical classification of pulmonary hypertension (Venice 2003, Simonneau et al 2004)

In 1998 a new clinical Evian classification of pulmonary hypertension, was proposed, aiming to divide the clinical entities with similar pathophysiological mechanisms, clinical presentation and treatment options (Simonneau et al 2004). This classification was further revised in Venice in 2003 (Table 1). In this new classification the category pulmonary
arterial hypertension (PAH) includes idiopathic pulmonary artery hypertension (IPAH) and familial pulmonary artery hypertension (FPAH). This category also includes PAH related to certain identifiable causes of pulmonary hypertension, PAH associated with significant venous or capillary involvement and persistent pulmonary hypertension in newborns. The other categories include PAH associated with left heart disease, lung diseases and/or hypoxemia, PAH due to chronic thrombotic and/or embolic disease and miscellaneous causes. Although in this classification IPAH and FPAH are recommended and the term secondary pulmonary hypertension abandoned, the terms PPH and SPH are still widely used.

After diagnosis the estimated median survival of PPH patients has been reported to be 2.8-4 years in adults and 4.12 years in children under 16 years of age (Sandoval et al 1995, Appelbaum et al 2001, D’Alonzo et al 1991). However, the prognosis of PPH varies and there are reports of non-progressive disease and even spontaneous regression of the disease (Halank et al 2004, Bourdillon and Oakley 1976).

Hereditary hemorrhagic telangiectasia (HHT, MIM 187300, 600376 and 601101) or Osler-Weber-Rendu disease is a bleeding disorder characterized by localized arteriovenous (AV) connections without an intervening capillary bed. Some patients also have pulmonary hypertension without pulmonary AV malformation (PAVM) suggesting clinical and genetical overlap between between PPH and HHT (Trembath et al 2001, Abdalla et al 2004). The AV malformation lesions are usually mucocutaneous, but visceral manifestations are also found in many cases. Recurrent nosebleeds are usually the first and most common symptom of HHT occurring in more than 90% of patients while teleangiectasies of skin occur in about 50-80% of patients (AAssar et al 1991, Sadick et al 2006). Pulmonary arteriovenous malformations that may lead to hemoptyysis, hypoxemia and CNS complications such as strokes and brain abscesses are found in 15-33% of HHT patients (Kjeldsen et al 2000, Cottin et al 2007, Cottin et al 2007). Central nervous system AV malformations are found in about 4-12% of patients with annual 0.4-0.7% bleeding risk (Willemse et al 2000, Maher et al 2001). Most of the neurological manifestations among HHT patients are caused by pulmonary AV malformations. Gastrointestinal bleeding as a sign of mucous teleangiectasies, is found in up to 33% of patients (Kjeldsen and Kjeldsen 2000). Using computed tomography (CT), liver AV malformations that may cause high-output heart failure, portal hypertension and biliary disease have been found in up to 74% of the patients, although only a minority of the patients are symptomatic (Ianora et al 2004).

Presently the clinical diagnosis of HHT is based on Curaçao criteria (Shovlin et al 2000). The diagnosis is definite if three of four of the criteria are present (Table 2). The diagnosis of HHT cannot be established in patients with only two criteria, but should be considered as possible or suspected. If fewer than two criteria are present, HHT is unlikely. In a study of 98 HHT patients, 62% of the patients had some symptom of HHT by the age of 16 and all the patients by the age of 40 (Portenous et al 1992). In many cases however, the symptoms may remain mild and thus the disease undiagnosed.
Table 2  Curaçao Criteria for clinical diagnosis of Hereditary Hemorrhagic Telangiectasia (Shovlin et al 2000). The diagnosis is definite, if 3 criteria are present, possible or suspected if 2 criteria are present and unlikely if fewer than 2 criteria are present

<p>|</p>
<table>
<thead>
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<th>Curaçao criteria</th>
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<td>1. Epistaxis</td>
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<td>2. Teleangiectasies</td>
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<tr>
<td>3. Visceral lesions</td>
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<td>4. Positive family history</td>
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Compared to age and gender matched controls of the Danish follow-up study, the mortality of HHT patients was slightly increased, but among the subgroup of patients under 60 years the mortality was twice that expected (Kjeldsen et al 1999). In a retrospective Italian study the affected parents (n=70) of HHT patients were compared to their spouses. 57.1 % of the parents with HHT vs. 51.4 % of the non-affected parents had died at the time of study. Median age at death was 63.2 vs 70 years, respectively. HHT was the main risk factor influencing their life expectancy and death was often related to the complications of PAVMs, CAVMs or anemia (Sabbà et al 2006).

### 2.2.2 Epidemiology of PPH and HHT

Systematic studies of the incidence and prevalence of PPH are scanty. Published estimates are based on small and not fully representative cohorts. In Israel the estimated annual incidence of PPH was 1.4 per million per year and prevalence 8 per million (Appelbaum et al 2001). The mortality trend has been found to increase in the United States especially among African-Americans, probably because of better diagnostical methods and due to anorexigen exposure (Lilienfield and Rubin 2000). PPH is considered a disease of young adults, and the mean age at the time of diagnosis varies between 36 and 48 years (Rich et al 1987, Dolara et al 1988, Appelbaum et al 2001, Thenappan et al 2007). However, people of all ages have been diagnosed (Braham et al 1991, Sandoval et al 1995). There is a female predominance among the PPH patients 1.7:1-3.4:1 (Rich et al 1987, Dolara et al 1988, Appelbaum et al 2001). In the first report on Finnish patients with pulmonary hypertension they were diagnosed as having the Ayerza's syndrome (cyanosis, hypertrophy of the right side of the heart, chronic lung infection, sclerosis of the pulmonary arteries) and the diagnosis was based on clinical picture and autopsy findings (Koulumies and Mustakallio 1952). The following year two patients with essential
pulmonary hypertension were reported and heart catheterization was used to confirm the diagnosis (Sipilä et al 1953). The first report on familial pulmonary hypertension in Finland was published in 1990 when two brothers were diagnosed with unexplained pulmonary hypertension (Puolijoki et al 1990).

The prevalence rates of HHT obtained in different studies vary from 1 in 40,000 inhabitants in northern England (Porteous et al 1992) to as high as 1 in 1,300 inhabitants in Netherlands Antilles (Westermann et al 2003). However, HHT might be underdiagnosed (Guttmacher et al 1994). There is some data about female predominance among HHT patients and among HHT patients having visceral manifestations of the disease (Letteboer et al 2006). HHT is inherited in an autosomal dominant fashion with maximum penetrance of at least one manifestation in 97 % (Plauchu H et al 1989). In the Finnish literature the first case of a patient with HHT was published in 1935 and more cases with reported family history of the disease in 1949 (Wilenius 1935, Saksela et al 1949, Saksela et al 1949).

2.2.3 Etiology of PPH

PPH is usually a sporadic disease, but in about 6 % of all cases there is more than one affected person in the same family (familial primary pulmonary hypertension, FPPH, Rich et al 1987). Profound genealogical studies have shown that sometimes the familial background is missed (Elliot et al 1995). The mode of inheritance of FPPH is thought to be autosomal-dominant with reduced penetrance meaning that not all family members with the mutation become affected (Loyd et al 1995, Nichols et al 1997). There is no difference in the clinical picture of familial and sporadic PPH, but in FPPH families genetic anticipation and abnormal gender ratio of the newborns are found (Loyd et al 1995). In successive generations the age of onset of the disease has been found to be lower, suggesting clinically more severe disease, and thus genetic anticipation. In addition one study showed that female offspring are more common than expected among the carriers of the mutation suggesting loss of male fetuses or primarily abnormal gender ratio (Loyd et al 1995).

In most reported cases the etiology of PPH has remained unknown. During the last decades, PPH has been associated with certain anorexigens, HIV infection (hivPPH), portal hypertension and collagen vascular diseases. PPH associated with these factors is clinically indistinguishable from the conventional PPH and the pathogenic mechanisms are unknown, but in recent classification they belong to another category (Simonneau et al 2004).

In the 1960s there was an epidemic of PPH in Switzerland, Austria and Germany associated with anorexigen Aminorex Fumarate (Follath et al 1971, Gurtner 1985). Another wave of anorexigen-associated PPH (aaPPH) in the1990s was related to long term use of fenfluramine derivatives (Simonneau et al 1998, Rich et al 2000, Abenhaim et
al 1996, Souza et al 2008). The clinical picture of aaPPH patients is similar to other PPH patients. In some cases however, aaPPH patients showed a clinical improvement or even remission after discontinuation of the medication (Simonneau et al 1998, Nall et al 1991).

The clinical picture of human immunodeficiency virus infection associated PPH (hivPPH) is usually less severe than in non-hivPPH at the time of the diagnosis, but the survival rates do not differ (Petiprez et al 1994). The prevalence of hivPPH among HIV-positive patients has remained the same, 0.5% of the HIV patients, as before the modern treatment opportunities and during the highly active antiretroviral treatment (HAART) era (Speich et al 1991, Sitbon et al 2008). The pathogenesis of PPH in HIV patients is unknown, but direct involvement of the HIV virus has not been found in the wall of pulmonary arteries (Humbert et al 1998). It has been suggested that the virus stimulates the release of proinflammatory cytokines, predisposing to pulmonary hypertension (Voelker and Tuder 1995). HivPPH patients have also showed a distinct expression of endothelin-1, a known vasoconstrictor, in their circulating monocytes (Ehrenreich et al 1993). Also elevated levels of platelet derived growth factor-α (PDGFα), which has an ability to stimulate smooth muscle and fibroblast proliferation, have also been reported in these patients (Humbert et al 1998).

There are also reports about familial PPH associated with hemoglobinopathies (Rich and Hart 1991, Wille et al 1996) and PPH is also related to the use of hormone replacement therapy and oral contraceptives (Morse et al 1999, Kleiger 1976).

2.2.4 Pathological findings in arterial walls and pathophysiology of PPH and HHT

The pathological findings in the pulmonary arteries of PPH patients include pulmonary arterial medial hypertrophy, plexiform lesions, concentric laminar intimal fibrosis, eccentric intimal fibrosis and in some cases also recanalized trombi (Pietra et al 1989).

The pathological mechanisms behind PPH are not fully understood, but several hypotheses have been suggested. Imbalance between vasodilative and vasoconstrictive substances is considered one possible pathogenic mechanism in PPH. Nitric oxide (NO) is a potent vasodilator of the pulmonary vascular system. The expression profiles of both NO and endothelial NO-synthase (eNOS) are widely studied and the results are conflicting. The expression has been shown to be both increased and decreased in patients with PAH suggesting a disturbed NO system (Mason et al 1998, Giaid et al 1995). Prostacyclin is an important vasodilator and inhibitor of platelet adhesion while thromboxane A2 (Tx) has completely opposite effects. Decreased expression of prostacyclin synthase, a critical enzyme in production of prostacyclin has been found in lungs of patients with PAH but not in healthy individuals (Tuder et al 1999). Also an imbalance between the urinary excretion of Tx and prostacyclin has been found in patients with PAH, suggesting platelet aggregation and abnormal vasoactivity of pulmonary
vascular endothelium (Christman et al 1992). Increased expression as well as increased plasma levels of endothelin-1 (ET-1), a potent vasoconstrictor and mitogen of pulmonary vasculature are found in patients with PAH and the severity of the disease correlates with their plasma levels (Giaid et al 1993, Stewart et al 1991, Nootens et al 1995, Rubens et al 2001). Dysfunctional voltage-gated $K^+$ channels leading to membrane depolarization and increase of cytoplasmic calcium, vasoconstriction and pulmonary artery smooth muscle cell proliferation seem also to have a role in the pathogenesis of PPH (Yuan et al 1998).

Serotonin-mediated smooth muscle hyperplasia of the arterial walls may have a role in the pathogenesis of PPH. Patients with PPH are found to have overexpression of the gene encoding the serotonin transporter (5-HTT, also known as SLC6A4, solute carrier family 6, member 4), leading to fast growth of pulmonary artery smooth muscle cells when stimulated by serotonin or serum. This overexpression is related to the long-allelic variant of the 5-HHT gene promoter and homozygosity of the long variant was also found in children with PPH. Interestingly, the growth-stimulating effects of serotonin were partly inhibited by 5-HTT blocker fluoxetine (Eddahibi et al 2001, Vachharajani and Saubders 2005).

Protrombotic aggregation mechanisms, increased coagulation and impaired fibrinolysis as well as thrombocytes are also suggested to be involved in pathogenesis and disease progression of PPH. Plasma activity of plasminogen activator inhibitor (PAI), leading to decreased fibrinolysis, is found to be increased in patients with PPH (Hoeper et al 1998). Increased levels of fibrinopeptide A reflecting increased activity of thrombin, which converts fibrinogen to fibrin, are also found in PPH patients (Eisenberg et al 1990). In addition, elevated plasma von Willebrand factor antigen levels (vWF-ag) predisposing to coagulation are found in patients with PAH and which also independently predicts long-term survival (Hoeper 1998, Kawut et al 2005). Phospholipid-dependent antibodies, a known risk factor for thrombosis, are also found in 10% of PPH patients (Wolf et al 2000).

Trombocytes have also been studied as a potential pathogenetic factor in the pathogenesis of PPH both by direct and indirect mechanisms. Increased spontaneous and platelet activating factor (PAF) induced platelet aggregation has been found in PPH patients (Nanonechnicov et al 1996). Also, increased urinary excretion of tromboxane A2, a potent stimulator of trombocyte aggregation, and decreased urinary excretion of vasodilator prostacyclin is found in these patients (Christman et al 1992). In in vitro animal models aggregating platelets are found to release serotonin and contract isolated pulmonary arteries in canines (McGoon and Vanhoutte 1984). Platelets and serotonin are also suggested to be involved in the pathogenesis of certain familial platelet and enzyme defect disorders. PPH has been reported in a patient with a familial platelet δ-storage pool disease, a disorder where platelet dense granules are rapidly released leading to platelet activation and vasoconstriction (Hervé et al 1990). In patients with type Ia glycogen storage disease, an autosomal recessive disorder caused by a deficiency of glucose-6-phosphatase, elevated plasma serotonin levels and severe pulmonary hypertension,
histologically similar to PPH, has been described (Pizzo 1980, Humbert et al 2002). Elevated levels of serotonin are found in these patients even without pulmonary hypertension (Humbert et al 2002).

In HHT, the earliest signs of pathological finding of the skin lesions are the cutaneous telangiecstatic lesions which are histologically locally dilatated postcapillary venules. When they continue to enlarge, the walls of the postcapillary venules become thicker because of an increased number of pericytes and increased intraluminal diameter. Finally, there is a direct arteriovenular connection without intervening capillary segments predisposing to bleeding. A characteristic finding in the telangiecstatic lesions are also aggregated mononuclear cells around the vessels affected (Braverman et al 1990). In liver the macroscopic lesions can be diagnosed by radiological methods (Ianora et al 2003, Buonamico et al 2008). Three-dimensional reconstruction of HHT liver shows abnormal direct communications between arterioles and ectatic sinusoids and between portal veins and ectatic sinusoids (Sawabe et al 2001). In lung, AV malformation consists of direct connections between the pulmonary artery and vein through a thin-walled aneurysm. Compared to other patients with PAVMs, in HHT patients there are often multiple lesions (Cottin et al 2007).

Pulmonary malformations may lead to serious pulmonary hemorrhage or cerebral abscesses or thromboembolic events that are common reasons for neurological symptoms among HHT patients (Cottin et al 2007). Other neurological complications of HHT are caused by cerebral arteriovenous malformations, dural arteriovenous fistulas and cavernous malformations (Maher et al 2001).

2.2.5 Treatment of PPH and HHT

The traditional medication for pulmonary hypertension includes calcium channel blockers (CCB), warfarin, diuretics, digoxin and oxygen supplementation. CCBs have long been the treatment of choice for the subgroup of PPH patients which tested responsive to vasodilators defined by a fall of mean pulmonary arterial pressure (mPAP) by at least 10 mmHg to 40 mmHg or less and maintenance of cardiac output during exposure to the drug (Galiè et al 2004). In a prospective, non-randomized study of 64 patients receiving either nifedipin or diltiazem, the 5 year survival rate was 94% in the group who respondend to CCB in the vasodilator test vs 55 % in the group that did not respond (Rich et al 1992). Unfortunately long-term responders are only 10-15 % of the patients who acutely respond to CCB (Sitbon et al 2002).

Survival benefit, although not always statistically significant, has also been reported in patients with PPH with oral anticoagulation. In a study by Rich et al (1992) the use of warfarin was associated with improved survival especially in patients who did not respond to CCB therapy. When patients with aPPH were studied, the long term prognosis improved significantly with warfarin anticoagulation. However, among patients with PPH
the survival benefit though, not statistically significant was found only after five years of treatment (Herbert et al 1997).

During the last decade the patients not responding to CCB have got new treatment options. The development of these therapies is mostly based on the increased knowledge of the pathogenesis of PPH. Prostacyclin analogues are potent vasodilators of the pulmonary vasculature and have been found to improve capacity, hemodynamics and survival in patients with PPH. In a 12 week prospective, open label study of 81 patients receiving continuous intravenous (i.v.) prostacyclin analogue, epoprostenol, 6 min walking test (6MWT) results and haemodynamics significantly improved and survival benefit was also seen when compared to patients receiving conventional therapy alone (Barst et al 1996). 3-year survival of PPH patients using continuous epoprostenol improved from 35.4 % to 62.8 % compared to historical data (McLauiglin et al 2002). The need of constant i.v. infusion has led to development of alternative administration routes. Iloprost is a well-tolerated and effective, inhaled prostacyclin analogue, which has been shown to improve the mean 6MWT distance, mean pulmonary arterial pressure (mPAP), cardiac output (CO) and pulmonary vascular resistance (PVR) (Krause and Krais 1986, Hoeper et al 2000, Olschewski et al 2000). However, iloprost has relatively short duration of action and analogues with longer half-lifes were needed (Olschewski et al 1996). Trepostinil is an inhaled prostacyclin analogue with a half-life of 3 hours and the effectiveness of the agent has been proven (Voswinckel et al 2006). Trepostinil has also been administered subcutaneously as monotherapy and the survival was 91-72% over 1-4 years compared to predicted survival of 69-38%, respectively (Barst et al 2006). Beraprost, an oral prostacyclin analogue has been shown to decrease PAP in some patients and improve exercise capacity and symptoms (Okano et al 1997, Galiè et al 2002). However, the beneficial effects were attenuated with time during the study period of 12 months (Barst et al 2003).

Endothelin-1 (ET) is a potent vasoconstrictor and smooth muscle mitogen of the pulmonary vasculature which is thought to mediate vascular hypertrophy associated with chronic pulmonary embolization related PAH (Kim et al 2000). Two different isoforms of endothelin receptors, A and B were identified. ETA receptors are mainly responsible for vasoconstriction while ETB receptors are also involved in the clearance of endothelin and release of prostacyclin and thus, vasodilation (Sauvageau et al 2007, Dupuis et al 1996, de Nucci et al 1988). Bosentan, the dual (ETA and ETB) endothelin receptor antagonist was the first oral therapy approved for the treatment of pulmonary arterial hypertension. In a study of 169 PPH patients with bosentan as their first-line therapy, the survival estimates were 96% at 12 months and 89% at 24 months while predicted survival rates were 68% and 57 %, respectively (McLaughlin et al 2005). Bosentan has also been an effective treatment for patients refractory to epoprostenol (Kataoka et al 2005). Bosentan has showed liver toxicity while this side effect was less frequent with the second generation ETA antagonist ambrisentan (Rubin et al 2002, Galiè et al 2005). A third endothelin receptor antagonist is sitaxsentan, which is highly specific for ETA while preserving the ETB activity. This may be beneficial, because production of prostacyclin and clearance of
ET-1 are thus maintained by ETB. Sitaxsentan improved functional class by 45% and also 6MWT result, mean right atrial pressure, mPAP, cardiac index and pulmonary vascular resistance were significantly improved in a 12 week randomized study (Langleben et al 2004).

Sildenafil is a potent and specific phosphodiesterase type 5 inhibitor. It metabolizes guanosine monophosphate, affects capacitative Ca$^{2+}$ entry and thus, enhances the guanosine monophosphate mediated relaxation and growth inhibition of vascular smooth muscle cells (Wharton et al 2005, Wang et al 2008). It has been shown to significantly reduce mPAP, improve WHO functional class and 6MWT distance in patients with PAH and the positive effects continued during the study period of 12 months (Galié et al 2005). Another phosphodiesterase inhibitor, vardenafil, has also been shown to improve haemodynamics in patients with PAH (Aizawa et al 2006).

Combination therapies have also been studied. Combination of epoprostenol and bosentan compared to epoprostenol alone showed improvement, though not statistically significant, in pulmonary resistance, exercise capacity and NYHA class (Humbert et al 2004). When iloprost was combined to existing bosentan 6MWT, mPAP and pulmonary vascular resistance (PVR) were significantly improved in a 12 week study of 67 patients (McLaughlin et al 2006). In the same study the combination therapy also delayed the time of deterioration. In a study of 58 PPH patients on bosentan therapy whose response declined after a few months of therapy the combination of sildenafil to the treatment significantly improved the 6MWT distance from 277+/−80 m to 392+/− 61 m (Hoepfer et al 2004). A combination of bosentan and sildenafil has also been shown to be effective in PAH although bosentan significantly decreases sildenafil plasma concentrations (Lunze et al 2006, Paul et al 2005).

When medical therapy is failed, surgical procedures are needed. The treatment procedures used are atrial septostomy, single or bilateral lung transplantation and lung-heart transplantation. Atrial septostomy is a palliative procedure to create right to left shunting and thus increase preload of the left ventricle, systemic output and oxygen transport. In a study of 15 patients with severe PPH treated with graded balloon dilation atrial septostomy, the NYHA class improved significantly, 6MWT distance was improved, and also survival benefit was seen (Sandoval et al 1998). In a study including 29 PPH patients the 1-, 3- and 5-year survival was 52, 40 and 35% after heart-lung and lung transplantation. The survival rate after heart-lung transplantation was higher than after bilateral lung transplantation although the difference was not statistically significant (Franke et al 2000). In a study by Whyte et al (1999) the percentage of survival after heart-lung transplantation was somewhat higher. When single and bilateral lung transplantation (SLT, BLT) were compared, BLT had a survival benefit in a 4-year follow-up (Conte et al 2001).

Literature on the treatment of HHT-related pulmonary hypertension is sparse, but there are case reports about successive use of bosentan, an endothelin antagonist and intravenous
prostacyclin as treatment for these patients (Bonderman *et al* 2006, Minai *et al* 2007). Pulmonary arteriovenous malformations predisposing to cerebral abscesses and ischemia are treated with embolization or in some cases, with surgical resection. The follow-up of the patients is needed because of recanalization of the occluded malformations and growth of the untreated lesions (Cottin *et al* 2007, Cottin *et al* 2007).

### Treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mechanism of action</th>
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<tr>
<td><strong>CCB</strong></td>
<td><strong>Vasodilator (patients with positive vasoreactivity test)</strong></td>
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</tbody>
</table>
| **Prostacyclin and prostacyclin analogues:** | **Multifactorial:**  
Vasodilation, 
Smooth muscle relaxation, 
Inhibition of platelet aggregation, 
Clearance of ET1 
Inotropic effect 
Cytoprotective and antiproliferative properties |
| Epoprostenol (continuous i.v) |  |
| Trepostinil (continuous s.c.) |  |
| Sodium beraprost (p.o) |  |
| Iloprost (inhaled) |  |
| **ET-antagonist:** | **Dual ETA/ETB antagonist**  
Selective ETA antagonist  
Selective ETA antagonist |
| Bosentan (oral) |  |
| Sitaxentan (oral) |  |
| Ambrisentan (oral) |  |
| **Sildenafil** | **Type 5 phosphodiesterase inhibitor, vasodilator antiproliferative effects** |
| **Oral anticoagulants** | **Prevent thrombotic events in pulmonary microcirculation and pulmonary arteries** |
| **Diuretics** | **Decrease fluid retention** |
| **Digitalis, dobutamine** | **Increase cardiac contractility** |
| **Surgical treatment:** |  |
| Balloon atrial septostomy |  |
| Single or bilateral lung transplantation |  |
| Heart-lung transplantation |  |

Table 3  
*Treatment options of PPH*

In HHT, cutaneous telangiectasies cause mostly cosmetic problems but there is also a risk of bleeding. Management of these lesions is based on laser treatments (Fernandez-Jorge *et al* 2007, Werner *et al* 2007). Recurrent epistaxis is usually the most common symptom
affecting the daily life of HHT patients. Lesions on nasal mucous membranes can be destroyed with different laser treatments, but the results are not long-lasting (Karapantzos et al 2005, Pagella et al 2006). To reduce the blood flow, embolization and in some cases ligation of the feeding arteries may sometimes be performed (Abdelkader et al 2007, Layton et al 2007, Geisthoff et al 2006). Septodermoplasty or septectomy and septal dermoplasty are also shown to reduce the need for blood transfusions and the volume and frequency of bleeds (Geisthoff et al 2006, Lesnik et al 2007). Medical treatment of epistaxis includes tranexamic acid, which is found to both reduce fibrinolysis and stimulate the expression of ALK-1 and endoglin and TGF-β-signaling (Fernandez-Lopez et al 2007). Combination therapy with estrogen and progesterone has been found to reduce bleeding tendency in some patients with HHT. In addition, antiestrogen tamoxifen has eliminated HHT-related bleeding in some postmenopausal women. Topical estriol has also reduced epistaxis of some HHT patients (Jameson and Cave 2004, Sadick et al 2005). Operative treatment of liver manifestations of HHT include liver transplantation and liver resections (Lerut et al 2006, Orlando et al 2008). Lately a new vascular endothelial growth factor inhibitor bevacizumab has been used successfully on liver lesions (Mitchell et al 2007). Cerebral arteriovenous malformations are treated with embolization and surgery. The treatment of non-hemorrhaged lesions should follow the principles of the AVMs in the general population (Maher et al 2001).

2.2.6 Structure of BMPR2, ALK1 and Endoglin genes

BMPR2-gene encoding BMPRII receptor, a type II serine/threonine kinase receptor was cloned by Rosenzweig et al (1995). They also showed that BMP7, and less efficiently BMP4, bind to BMPRII and the binding is facilitated by type I receptor for BMPs. The cDNA was cloned and genomic organization of BMPRII was studied by Beppu et al in 1997, and the cDNA was found to encode a protein of 1038 amino acids (aa) containing a single transmembrane domain, a serine-threonine kinase domain and a long carboxy-terminal tail. The cDNA is divided in 13 exons spanning over 80 kb. In 1999 this gene was located to 2q33-q34 (Åström et al 1999).

ALK1-gene encodes ALK1, a type I receptor for the ligands of TGF-β superfamily. It is highly expressed in placenta and lungs (ten Dijke et al 1993). In animal studies ALK1 was found to be predominantly expressed in arteries over veins and the expression was diminished in adult mice when their growth slowed (Seki et al 2003). The gene has 9 coding exons spanning over 15 kb of genomic DNA and encodes a protein of 503 aa (Berg et al 1997, ten Dijke et al 1993). In 1998 by Röijer et al the gene was localized to 12q13. With other type I receptors ALK1 has a high degree of similarity as to serine-threonine kinase, subdomains, a glycine- and serine-rich region preceding the kinase domain and cytoplasmic tail (ten Dijke et al 1993).
Endoglin was first mapped to human chromosome 9 and, by fluorescence in situ hybridization to 9q34q terminus, distal to the breakpoint of the Philadelphia chromosome (Fernández-Ruiz et al 1993). It encodes accessory receptor for the TGF-β superfamily of ligands. The cDNA of Endoglin was originally cloned by Gougos and Letarte in 1990 and later on an isoform with a shorter cytoplasmic tail was found (Bellon et al 1993). The gene has 14 coding exons with an 25 aa leader peptide, an 561 aa extracellular portion and a 25 aa transmembrane domain (McAllister et al 1994). The longer variant has a 47 aa cytoplasmic tail while the shorter variant has 14 aa (Gougos and Letarte 1990, Bellon et al 1993). Endoglin is expressed in endothelial cells, placenta, bone marrow stromal cells and different hematopoietic cells (Gougos and Letarte 1988, Lastres et al 1992, Gougos et al 1992, Robledo et al 1996, Lastres et al 1992, Rokhlin et al 1995).

Figure 1 Structure of BMPR2 (A), ALK1 (B) and Endoglin (C) genes

2.2.7 TGF-β-signaling pathway

TGFβ-signalling pathway is responsible for proliferation and differentiation of cells, embryonic development, wound healing, and angiogenesis. The TGF-β-superfamily consists of structurally related cytokines, TGF-βs, activins and different bone morphogenetic proteins (BMP). Their biological effects are exerted through a receptor
complex consisting of two serine-threonine kinase receptor subunits (Figure 2): type II receptor like BMPRII and type I receptor like ALK1, ALK3 and ALK6. Five type II receptors and seven type I receptors, also called activin like kinases (ALK) have been identified. In this receptor complex endoglin functions as an accessory or type III receptor. In the signaling cascade TGF-βs bind to type III receptor which presents it to type II receptor, or directly to type II receptor. When activated the type II receptor recruits, binds and phosphorylates the type I receptor and stimulates its kinase activity. Activated type I receptor in turn phosphorylates Smads (mothers against decapentaplegic homolog genes, mad and related sma-genes), transcription factors that form a heteromeric complex and move to the nucleus where this complex interacts in a cell-specific manner with various transcription factors and regulates the transcription of TGF-β responsive genes (Itoh et al 2000, Massaque and Chen 2000, Derynck et al 1996).

In addition to Smad-mediated pathway, there is an alternative pathway for TGF-β and BMP signaling. They are also found to activate MAP kinases, JNK and p38MAPK kinases (Massaque and Chen 2000). The pathway activated is found to depend on whether the ligand stimulate preformed typeI/typeII receptor complex or lead to recruitment of the receptors (Nohe et al 2001). The balance between these two alternative pathways has been found to be disturbed in PPH where expression of mutant bone morphogenetic receptor type II -receptors (BMPRII) leads to constant activation of the pathway even in the absence of the ligand (Rudarakanchana et al 2002). Several different mutations have been described in different members of the signaling pathway causing clinically distinct diseases.

Figure 2  
TGF-β signaling pathway. Endoglin(III) presents ligand to type II receptor, which recruits and phosphorylates type I receptor. Type I receptor in turn phosphorylates Smads. Smad-complex moves to nucleus to regulate gene transcription.
2.2.8 Molecular genetics of PPH and HHT

In 1997 and 1998 the gene locus of FPPH was mapped to 2q31-32 by two independent linkage studies (Nichols et al 1997, Morse et al 1997). Three years later the disease-causing mutation was found in the gene encoding bone morphogenetic protein type II (BMPRII) among patients with FPPH and later the same year among patients with sporadic PPH (Deng et al 2000, Lane et al 2000, Thomson et al 2000). BMPRII is a member in a receptor complex of the TGF-β superfamily signaling pathway (Liu et al 1995). Mutation of BMPR2 was originally found in about 55 % of familial cases and 26 % of sporadic PPH cases (Machado et al 2001, Thomson et al 2000). Mutations in BMPR2 have been found in all functional domains of the gene and the majority of the mutations lead to a premature termination codon (Trembath and Harrison 2003, Machado et al 2001). At present the different mutation types included partial missense and nonsense mutations, frameshift mutations, exonic deletions and duplications, and splice site mutations resulting in aberrant splicing, alternative splicing has not been described. Some rearrangements of BMPR2 may be missed by direct sequencing. Reverse transcriptase polymerase chain reaction (RT-PCR) combined with multiplex ligation-dependent probe amplification sometimes shows a mutation in some of the patients previously tested negative for BMPR2 mutations (Cogan et al 2005, Cogan et al 2006, Aldred et al 2006).

In addition to mutations in BMPR2 gene, overexpression of SLC6A4 is found in PPH, and long-allelic variant of the promotor region is associated with this phenomenon (Eddahibi et al 2001). There is also a report about 3 families with PPH where no mutation was found in BMPR2 but the disease locus was linked to more proximal region 2q31 (Rindermann et al 2003).

In vitro expression analysis has demonstrated loss of BMPRII function in many of the identified mutations suggesting haploinsufficiency as a molecular mechanism in both sporadic PPH and FPPH. Missense mutations also altered the charge, polarity, hydrophobicity and/or the size of the substituted amino acid residue changing the three-dimensional structure of the ligand binding domain of BMPRII (Machado et al 2001). In transfection studies transport of cysteine-substituted BMPRII was prevented and the activity of BMP/Smad-responsive reporter gene was reduced suggesting a dominant negative effect as another molecular pathogenetic mechanism in PPH (Rudarakanchana et al 2002). BMPRII with non-cysteine substitutions was transported to the cell surface but the activation of the reporter gene was suppressed. When the cytoplasmic tail was mutated BMPRII was trafficked to the cell surface and the reporter gene was activated. Interestingly, transfection with mutant receptors led to activation of an alternative, p38MAPK –pathway even in the absence of ligand leading to proliferation of cells. This suggests that Smad-dependent signaling is not the only pathway downstream to BMPRII in the pathogenesis of PPH.

Gene expression studies of the lung tissues obtained in transplantation or autopsy of the PPH patients with unknown BMPR2 mutation status have shown decreased expression of genes encoding several kinases and phosphatases, and upregulation of several oncogenes and ion channel proteins (Geraci et al 2001). Also microsatellite instability in DNA
mismatch gene, and microsatellite mutations and reduced expression of proapoptotic Bax
gene and transforming growth factor have been found in plexiform lesions of PPH patients
(Yeager et al 2001). In another study, microsatellite instability was not found in five genes
studied. In addition heterozygosity in BMPR2 locus was retained which does not support
the idea about the loss of wild-type allele, known as the second hit theory (Machado et al
2005).

There are several mouse models on the effect of BMPR2 mutations on embryogenesis and
pulmonary arterial pressure. Mice homozygous for BMPR2 gene deletion encoding the
kinase domain and the aminoterminal end of the kinase domain were arrested before
gastrulation and failed to form organized structures and lacked mesoderm. The heterozygous mice were phenotypically normal and fertile (Beppu et al 2000). When the
mutant gene was under control of tetracycline gene switch and the mutation was activated
after birth, the mice developed increased pulmonary artery pressure, right ventricle/left
ventricle plus septum weight and pulmonary artery muscularization without an increase in
systemic arterial pressure. Interestingly, the PAP was higher in mice raised in mildly
hypoxic conditions compared to those raised at sea level showing the effects of
environmental factors in addition to genetic factors on pulmonary hypertension (West et al
2004). Also decreased expression of voltage-gated potassium 1.5 (Kv) channel expression
and increased right ventricular pressure without significant modeling of the vasculature
has been found in transgenic mice with BMPR2 mutation. In vivo, the right ventricular
systolic pressure was decreased after exposure to CCB nifedipine, suggesting that
activation of L-type Ca\textsuperscript{2+} channels caused by reduced Kv1.5 mediates vasoconstriction and
increase in pulmonary pressure (Young et al 2006). Decreased expression of genes related
to smooth muscle differentiation and angiogenesis and increase of cytokines and markers
of immune response especially in female mice have also been found in lungs of transgenic
mice with BMPR2 mutation (Tada et al 2007).

In HHT, many studies have shown genetic heterogeneity and at least four different locuses
have been found. In 1994 a subgroup of HHT patients were found to have mutations in
Endoglin, known as HHT1 (McAllister 1994). This was also the first human disease where
mutations in a member of the TGF-β-receptor complex were found. Two years later
mutations in the gene encoding activin like kinase type 1 (ALK1), another member of the
receptor complex were found among another subgroup of HHT patients (HHT2, Johnson
et al 1996). In most cases the mutations are unique in different families, although in some
studies evidence of a founder effect has been found (Brusgaard et al 2004, Gallione et al
2000). Later on two additional gene loci, one on chromosome 5 (HHT3) and another on
chromosome 7 (HHT4) were linked to HHT (Cole et al 2005, Bayrak-Toydemir et al
2006). Both of these two genes still remain unknown. In addition, some families show no
linkage to these loci.

A genotype-phenotype correlation has been found in HHT. Endoglin and ALK1 mutations
lead to HHT1 and HHT2, respectively. Patients with a mutation in Endoglin gene (HHT1)
are more likely to have pulmonary (PAVM) and cerebral (CAVM) arteriovenous
malformations while arteriovenous malformations of liver (HAVM) are more common in patients with ALK1 mutations (HHT2, Kjeldsen et al 2005, Lesca et al 2007, Sabbà et al 2007, Sabbà et al 2007). The data on gastrointestinal bleeding is controversial (Kjeldsen et al 2005, Lesca et al 2007). There is also variation in symptoms between men and women (Letteboer et al 2006). PAVMs are more common in women than men with HHT1. Both in HHT1 and HHT2 the HAVMs are more common in women. The penetrance of HHT2 may also be lower and the phenotype milder compared to HHT1 which can make diagnosis difficult (Johnson et al 1995).

Homozygosity of the ALK1 disease allele is also thought to be lethal in a large Arab family where both of the parents were affected with HHT and carried ALK1 mutation (El-Harit, E-HA et al 2005). Three of the pregnancies ended in spontaneous abortion, four in early neonatal death and only in 5 out of 12 pregnancies the offspring survived. In animal studies both ALK1 and endoglin have been found to be essential for vascular development. In a mouse model homozygous endoglin knockout animals died during gestation due to defect in heart and vascular development (Bordeau et al 1999). In a mouse model the mouse embryos heterozygous for the endoglin mutation sometimes had abnormal and dilated blood vessels, and smooth muscle cells surrounding the vessels were disorganized, while mice with homozygous endoglin mutation knock out embryos showed severe defect in angiogenesis of the yolk sac, in cardiogenesis and in hematopoiesis and failed to progress beyond 10.5 days postcoitum (Arthur et al 2000). In a mouse model mice with homozygous inactivated ALK1 embryos died at midgestation due to severe vascular abnormalities (Oh et al 2000).

In functional studies some ALK1 mutations caused a loss of protein expression or function while the others had a dominant negative effect and they suppressed the activity of the wild-type allele (Gu et al 2006). When endoglin mutations were studied both truncating and missense mutations retained intracellularly and were not significantly expressed on the cell surface. The normal endoglin was found at reduced levels on the cell surface. This data suggests that haploinsufficiency rather than dominant negative to be a molecular mechanism in HHT1 with endoglin mutations (Pece et al 1997, Pece-Barbara et al 1999). However, another study showed that whether the mutation acts through haploinsufficiency or dominant negative effect depends on the type of the mutation (Lux et al 2000). In addition, mutations of ALK1 and endoglin are found to downregulate and upregulate a number of genes involved in angiogenesis, cytoskeleton, cell migration, proliferation and NO synthesis (Fernandez-Lopez et al 2007).

PPH and HHT together create an interesting spectrum of pleiotropic diseases of the TGF-β-signaling pathway (Figure 3). Although at present BMPR2 remains the main gene for classical PPH, there is a subgroup of patients with ALK1 mutation who have pulmonary hypertension clinically indistinguishable from PPH and in addition, clinical symptoms of HHT. Family members of these patients may have classical HHT without pulmonary hypertension (Trembath et al 2001, Abdalla et al 2004). A child with PPH with ALK1 mutation who had no signs of HHT by the age of 5, has also been reported (Harrison et al
So far no *Endoglin* mutations have been found among PPH patients, but HHT and PAVM-related pulmonary hypertension patients with *Endoglin* mutation have been reported (Harrison 2005). *SMAD4* (MIM 600993) and germline mutations of bone morphogenetic receptor type 1A (*BMPR1A* or *ALK3*, MIM 601299), another members of TGF-β-signaling pathway are found in patients with juvenile polyposis syndrome (JPS, MIM 174900, Zhou *et al* 2001, Sweet *et al* 2005). *SMAD4* mutations have also been found in some young patients with JPS who have no signs of HHT (Sweet *et al* 2005) and in some patients with HHT without previous history of JPS. In some cases asymptomatic colonic polyps may be found in endoscopy in these patients suggesting that screening of *SMAD4* is important in patients who tested negative for *ALK1* and *Endoglin* mutations because of the risk of colon cancer related to JPS (Gallione *et al* 2006). *BMPR2* mutations have also been found in some patients with PAH and congenital heart disease and also in pulmonary veno-occlusive disease (Roberts *et al* 2004, Runo *et al* 2003).

Figure 3  Diseases related to TGF-β signaling pathway
3. **AIMS OF THE STUDY**

Only a few studies on the Finnish PPH and HHT patients have been published. The main goal of this study was to provide epidemiological and genetic data about Finnish PPH and HHT patients and families.

The specific aims were:

1. to identify and collect DNA samples and clinical data of all the Finnish PPH patients and/or their first-degree family members diagnosed in Finland between 1986-1999 (I)

2. to study the clinical picture and geneology of familial PPH of all cases nationwide (I)

3. to characterize the mutation spectrum of $BMPR2$ in Finnish PPH patients (I)

4. to study possible novel candidate genes and biological mechanisms of the identified mutations behind PPH (II,IV)

5. to identify HHT patients seen at the Department of Otorhinolaryngology at Helsinki University Hospital between the years 1990-2005 and collect DNA samples for $Endoglin$ and $ALK1$ mutation screening (III)
4. MATERIALS AND METHODS

4.1 Patient identification

Subjects with a diagnosis of PPH were retrospectively identified nationwide from the hospital discharge registries of all five Finnish university hospitals, which are the major centers for the diagnosis and treatment of these patients. Searches were performed under the ICD-9 disease category 4160A (Hypertonia pulmonalis idiopathica) for the years 1987-95 and the ICD-10 disease category I27.0 (Hypertonia pulmonalis primaria) for the years 1996-99. Medical records of all identified patients were reviewed by the author to assure the diagnosis using the diagnostic criteria presented below. All the patients with defined diagnosis were contacted to participate the study along with their first-degree family members. In the case that the patient was deceased the spouse/children or parents were contacted when possible. The names and birthplaces of the parents and grandparents/great-grandparents were also queried and when not known, the data was collected from church records. Studies I, II and IV are based on this study population.

To identify potential HHT patients in the Helsinki and Uusimaa Hospital District all the patients who had visited the Department of Otorhinolaryngology in the Helsinki University Central Hospital during the years 1990-2005 were screened retrospectively. The electronic hospital discharge records were screened for the diagnoses for hemangioma (ICD9 code 2280A or ICD10 code D18), Morbus Osler (ICD9 code 4480A or ICD10 code I78.0), and capillary hemangioma (ICD10 code Q82.58). The complete medical records of the identified patients were further reviewed by docent Petri Mattila to confirm the clinical suspicion of HHT. The patients who fulfilled the clinical criteria of HHT were interviewed by the author by phone to obtain a detailed family history and information about the patient’s symptoms. The index cases and his/her first-degree family members or family members with HHT were then invited to participate in the study and they donated a blood sample for DNA extraction (Study III).

The research protocol was approved by the ethics committees of all five Finnish university hospitals, the ethics committees of Seinäjoki Central Hospital and University of Helsinki. Permission to study the tissue samples of the deceased patients was given by the National Authority of Medicolegal Affairs. All the patients and family members were informed about the aims of the study and their rights to withdraw from the study. All participants donating a blood sample gave written consent.
4.2  Diagnostic criteria

The following criteria were used for PPH diagnosis (modified from Rich et al 1987): (1) the mPAP $\geq 25$ mmHg measured by right heart catheterization or systolic right ventricle-right atrium pressure gradient $>50$ mmHg measured by Doppler echocardiography, and (2) exclusion of other known causes of pulmonary hypertension (Table 1). Heart diseases predisposing to pulmonary hypertension were excluded by clinical and echocardiographic studies; respiratory diseases by lung function testing, chest X-rays and high resolution computed tomography when available; and pulmonary thromboembolic disease by ventilation-perfusion scanning, computed tomography or pulmonary angiography. Laboratory data including results of the autoantibody testing and liver enzymes were evaluated to exclude the patients with connective tissue and liver diseases. When available, autopsy data were used to confirm the diagnosis of PPH.

For HHT diagnosis the Curaçao clinical criteria (Shovlin et al 2000) were used: 1) recurrent nosebleeds, 2) a first-degree relative diagnosed with HHT, 3) diagnosed visceral arteriovenous lesions (gastrointestinal telangiectasia, pulmonary AV malformations, hepatic AV malformations, cerebral AV malformations, spinal AV malformations), 4) telangiectasia of lips, oral cavity, fingers or nose. At least 3 out of four criteria had to be fulfilled for the clinical diagnosis of HHT.

4.3  Genes studied

For all the PPH patients and parents of the familial patients available the coding regions of the $BMPR2$ gene were studied by direct sequencing. Genomic DNA from EDTA blood was used as a template. For the deceased, the tissue samples obtained during autopsy were used to get the genomic DNA (Study I). $ALK1$, $Endoglin$, $BMPR1A$, $SMAD4$ and $SLC6A4$ were also studied in the same study population using single-strand conformational (polymorphism) SSCP method. When the fragments showed altered motility, the samples were sequenced. Except for $SLC6A4$, all the candidate genes encode members of the TGF-$\beta$ signaling pathway. $SLC6A4$ was included because a polymorpfia of the promotor region of the gene has been found to predispose to PPH (Eddahibi et al 2001)(Study IV).

All the index cases of the HHT families were studied using direct sequencing of $ALK1$ and $Endoglin$ genes. If the mutation was found the exon was also sequenced among the other affected family members. The aim was to identify the possible mutations of these genes known to predispose to HHT in previous studies and find the potential founder effect among Finnish patients (Study III).
Mutation analysis of BMPR2, Endoglin and ALK1 by direct sequencing

DNA was extracted from whole blood using non-enzymatic methods (Lahiri and Nurnberg 1991). DNA was extracted from paraffin blocks after deparaffination by xylene. The entire protein-coding region and intron/exon boundaries of the genes (BMPR2 has 13 coding exons, ALK1 9 and Endoglin 14) studied were amplified by PCR. The PCR assays were carried out in a reaction volume of 20-50 μl and 40-100 ng of genomic DNA was used as a template. PCR products for sequencing were purified using Qiaquick (Qiagen, Germany) GenElute PCR purification Kit (Sigma-Aldrich Chemie, Germany) or Exo-SAP-IT (USB, USA). ABI Prism3100 (Applied Biosystems, CA) was used for sequencing (Studies I, II, III).

Genotyping

Microsatellite markers D2S2309, D2S2214, D2S2217, and D2S2289 were selected from the physical map on 2q33 (Machado et al 2000). Primer sequences were obtained from the Genome Database (www.gdb.org/). An additional microsatellite repeat was genotyped from intron 1 of BMPR2 (Microsat1)(Machado et al 2001). PCR products were electrophoresed on denaturing 7 M urea/6% polyacrylamide gel with alleles visualized by silver staining. Within the BMPR2 locus, three additional single nucleotide polymorphisms (SNP) were genotyped: in AC009960 in positions 36854T>G (upstream, SNP1), and 5406A>C (intron 4, SNP2), and in NM_001204 in the position 2811G>A (exon 12, SNP3) using appropriate restriction enzyme digestions (Study I).

Haplotype analysis

For the association analysis of microsatellite markers and SNPs, the haplotypes were constructed manually in each family. Haplotyping was performed within each nuclear family and in cases where the phase of the chromosome could not be determined unambiguously, the responsible alleles were discarded. If the child was affected, the transmitted chromosomes were considered disease-associated and the non-transmitted chromosomes as controls. If one of the parents was affected, his/her chromosomes were considered disease-associated and the spouse’s chromosomes as controls. Additional 11 population-based control families (father, mother, child) were genotyped for the same markers to increase the pool of control chromosomes. These haplotypes were used as input for Haplotype Pattern Mining (Toivonen et al 2000)(Study I).
4.7 Restriction fragment length polymorphism (RFLP) and Southern blot hybridization

Genomic DNA was digested using three restriction enzymes: EcoRI, HindIII, and PstI (New England Biolabs, MA) according to the recommendations by the manufacturer. The digested DNA fragments were separated by agarose gel electrophoresis and transferred to nylon membrane (Hybond N+, Amersham Pharmacia Biotech, Ireland). The probes for hybridization were produced from full length BMPR2 cDNA cloned in a pcDNA3 vector. The probe was labeled with $^{32}$P using Rediprime labeling kit (Amersham Pharmacia Biotech, UK). The labeled probe was then purified using Nick Spin Column (Amersham Pharmacia Biotech, Sweden). The filters were prehybridized for 2-3 h at 65 °C in a solution consisting of 1% BSA, 7% SDS, 0.5M Na$_2$HPO$_4$, and 1 mM EDTA with 100 μg of herring sperm DNA. After prehybridization, the $^{32}$P labeled probe was added and the filters were hybridized overnight at 65 °C. After hybridization, the filters were washed with 2-0.1X SSC + 0.1% SDS at room temperature and exposed to X-ray films (Study I).

4.8 Functional analysis of cytoplasmic tail missense mutation of BMPR2 c.2696G>C encoding R899P

Using both untagged and 3’ green fluorescent protein (GFP)-tagged BMPR2 as templates the R899P mutants were prepared using Quikchange Site-directed mutagenesis kit (Strangene, UK). For Luciferase reporter gene assays normal mouse mammary gland epithelial cells (NMuMG) were co-transfected with the mutant pcDNA and a luciferase reporter plasmid, 3GC2wt-Lux, which contains a BMP-responsive element derived from the mouse Smad6 promoter. The cell supernatant was assayed for luciferase activity.

For p38MAPK immunoblotting, the NmuMG cells were transfected with the mutant construct. BMP-4 was added and the protein was harvested by washing cells in cold PBS and freezing in an ethanol/dry ice bath. For western blotting the samples were electrophoresed by SDS-PAGE and transferred to microcellulose membranes. Bands were visualized by chemiluminescence. Blots were then stripped and reprobed using an antibody to p38MAPK (Study I).

4.9 Single-strand Conformational Polymorphism (SSCP) analysis of ALK1, Endoglin, BMPR1A, SMAD4 and SLC6A4

All the five genes were studied using the same protocol. DNA was extracted from whole blood using non-enzymatic methods. The PCR fragments were designed to cover all the coding exons and corresponding exon-intron boundaries of the genes. ALK1 has 9 coding exons, Endoglin 14, BMPR1A 11, SMAD4 11, and SLC6A4 13. The lengths of the PCR fragments in SSCP analysis varied between 154 bp and 310 bp and the long exons were
studied in two or more pieces. PCR reactions for SSCP analysis were carried out in a reaction volume of 10μl and 20 ng of DNA was used as a template.

7.5 μl of bromophenolblue and formamide-containing stop solution was added to 10 μl of PCR product. After denaturation the samples were loaded on a non-denaturing 0.5X MDE polyacrylamide gel. The samples were electrophoresed at 3-4W constant power for 16-19 h at room temperature and visualized by silver staining. 10 healthy blood donors were used as controls in every SSCP gel. If a new unknown mutation or polymorphism was found at least 59 additional control samples were screened (118 control chromosomes) in addition to the positive control. The samples showing altered motility in SSCP were further studied by direct sequencing as described in section 5.4. (Study IV)
5. RESULTS AND DISCUSSION

5.1 PPH epidemiology in Finland

During the years 1987-1999 59 patients altogether in Finland were treated with the confirmed diagnosis of PPH. The male:female ratio of 1:4. In addition we found 18 patients whose PPH diagnosis was considered possible, because inadequate diagnostic testing or documentation prevented the verification of the PPH diagnosis.

During the follow-up period the annual incidence of verified PPH varied between 0.2 to 1.3 cases per million (mean 0.8 cases per million). This means that approximately 4 new PPH cases were diagnosed per year in Finland. In 1999, 30 patients with confirmed PPH were alive giving a nationwide prevalence of 5.8 per million.

Figure 4 Birthplaces of parents of PPH patients
circle=sporadic PPH, square=familial PPH, triangle= PPH and HHT in the same family
Based on interviews of the index cases or their family members, of the 59 confirmed PPH cases, four patients (7%) had familial background and one patient (2%) hereditary hemorrhagic telangiectasia in the family. All multiplex families with PPH originated from different regions of Finland. When geographic distribution of the sporadic families was studied, their origin showed no evidence of distortion from what was expected when compared to the Finnish population density (Figure 4).

The present work is the first study on the epidemiology of PPH in Finland and also very few studies have been published elsewhere. In addition, the numbers usually presented in the literature have only been estimates. We found a total of 59 patients with confirmed diagnosis of PPH making the mean annual incidence 0.8 million cases /million people/ year (0.2-1.3/million/year during the study period) or approximately 4 new cases per year in Finland and the prevalence in this study was 5.8/million people. These figures are lower than reported in Israel (Appelbaum et al 2001). However, strict diagnostic criteria were used and if the 19 patients considered as possible PPH cases were included, the mean annual incidence would have reached 1.1 cases/million people/year.

The problem as to all the prevalence and incidence and especially genetic studies of PPH is the difficulty of making the diagnosis. Although the diagnostic methods have improved over the years PPH is still a diagnosis of exclusion. Other diseases resembling PPH have to be excluded step-by-step which is time-consuming and requires physicians that are familiar with the disease. The difficulty of making the diagnosis was also faced in this study. The diagnostic tests of the patients with the diagnosis codes of PPH were not profound enough to exclude all the other possible diagnoses with the same clinical picture. This means that some patients that in fact have the right diagnosis might have been left outside the study and more PPH cases may have been found with less strict criteria. In addition, although the patients were collected from the whole country, only the university hospitals were studied. The severity of the disease varies and the diagnosis may be difficult. Some patients may not have been referred to bigger units for diagnosis and treatment and thus may have been left outside the study.

The study of HHT patients was not designed to collect nationwide epidemiological data. However, based on the figures obtained here the Helsinki University Hospital are serving approximately 1.5 million people, one new HHT family was diagnosed every year during 1990-2005. The present clinical data suggests that HHT patients with epistaxis should also be screened for other complications of the disease and that family members may also benefit from the diagnostics.

5.2  **BMPR2 mutation, haplotype, and functional analysis in PPH**

The study population for mutation and haplotype analysis was based on the same cohort of patients and family members who were identified in the epidemiological study and whose
DNA samples were available. The study population for BMPR2 sequencing consisted of the patients, whose whole blood (N=21) or paraffin block (n=7) extracted DNA was available, and two sets of parents of familial patients. The RFLP analysis included only the patients whose whole blood extracted DNA was available (n=21) and the parents of the familial cases. Patients whose family members were not available were not included in the haplotype analysis. The number of PPH patients studied using different methods is presented in Table 5.

By direct sequencing of the exons and exon-intron boundaries of BMPR2, we identified a total of 4 mutations in the Finnish cohort consisting of 26 sporadic and two familial patients and parents of two diseased, affected sibpairs (Table 4). All observed genetic variations in BMPR2 were screened in the study cohort and among 96 Finnish healthy blood donors using altered restriction sites. Each family or patient had a different mutation. In the kinase domain of the BMPR2 receptor three mutations occured, two missense (994C>T, 1472G>A) and one frameshift (1376–1377delGA) mutation. The fourth mutation was located in the C-terminal cytoplasmic domain (2696G>C). Two of the mutations (994C>T, 1376–1377delGA) led to premature termination of the transcript. The mutations were not found among the control group of Finnish blood donors (n=96) when tested using restriction enzymes to identify altered restriction sites generated by the mutation. In addition to the mutations we identified two synonymous polymorphisms. 600A>C allele frequency in exon 5 was 5% among the patients and 11% among the controls while the frequency of 2811G>A in exon 12 was 29% and 25%, respectively.

<table>
<thead>
<tr>
<th>Family history</th>
<th>Exon</th>
<th>Nucleotide change</th>
<th>Amino acid Change</th>
<th>Restriction enzyme used in mutation screening</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporadic</td>
<td>8</td>
<td>c.994C&gt;T</td>
<td>R332X</td>
<td>TaqI</td>
</tr>
<tr>
<td>Sporadic</td>
<td>10</td>
<td>c.1376-1377delGA</td>
<td>R459fs+10aa</td>
<td>BsaI</td>
</tr>
<tr>
<td>Familial</td>
<td>11</td>
<td>c.1472G&gt;A</td>
<td>R491Q</td>
<td>AluI</td>
</tr>
<tr>
<td>Sporadic</td>
<td>12</td>
<td>c.2696G&gt;C</td>
<td>R899P</td>
<td>Sau96I</td>
</tr>
</tbody>
</table>

Table 4 Mutations of BMPR2 among the Finnish PPH patients

To exclude potential large deletions that could have been missed by our sequencing approach, we performed Southern blotting using three different restriction enzymes among the patients. Restriction fragment length polymorphisms for EcoRI and HindIII, were identical among all the study individuals. For PstI, we identified an additional restriction site both among some patients and controls suggesting that the site is polymorphic among the Finns and not disease-associated (Figure 5).
We studied the BMPR2 haplotypes to identify the potential founder mutation risen in the non-coding regions of the gene. The haplotype analysis was performed in 18 families. Additional 11 population-based control families (father, mother, child) were genotyped for the same markers to increase the pool of control chromosomes. The two haplotypes were shared by PPH patients: D2S2309*2-D2S2214*2-D2S2217*2-SNP1*1-Mircosat1*8-SNP2*1-SNP3*1-D2S2289*6 (n=3) and Microsat1*8-SNP2*1-SNP3*1-D2S2289*3 (n=3). Neither of the haplotypes was associated with the disease as they are also the most frequent haplotypes observed among controls.

In functional analysis surprisingly the cytoplasmic tail of the mutant (2696G>C) was transported normally to cell membrane in HeLa cells. In Luciferase assays with Smad responsive elements, the mutant receptor had no inhibitory effect on basal or BMP4-stimulated activity when compared to the wild-type receptor. In the mutant receptor, the p38MAPK pathway was activated also in the absence of the ligand and induced smooth muscle proliferation, which was inhibited by selective p38MAPK inhibitor, which may later on be a potential focus of the development of new medicines for the disease.

More than 150 BMPR2 mutations have been found in PPH patients worldwide so far (The Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff, http://www.hgmd.cf.ac.uk/ac/index.php). The mutations found were in all functional domains of BMPRII including missense/nonsense, splicing, small insertions and deletions, small indels and gross insertions and deletions. In this study we found a mutation in six of the 28 index cases for which we were able to get a DNA sample. Four of the mutations
were found in BMPR2. Three of the mutations were in the kinase domain of the gene. The fourth mutation was interestingly found in exon 12, previously known to be skipped in a common isoform of the gene and encoding the cytoplasmic tail of BMPRII. This mutation, R899P led to constitutive activation of the Smad independent P38MAPK pathway. Three of the BMPR2 mutations were found in sporadic PPH patients explaining 12% of the cases (altogether 26 sporadic patients studied). One BMPR2 mutation was found in a familial case (33% of all the familial cases; 4 patients or sets of parents from 3 different families studied). In addition, in one sporadic case and in one patient with a familial background mutations in ALK1 were detected as described in section 5.3. Because the diagnosis of PPH is based on the exclusion of other causes of pulmonary hypertension, it is possible that some diagnoses were incorrect. On the other hand, it is possible that some diagnoses were missed because of the strict criteria.

The amount of BMPR mutations found among this group of Finnish PPH patients was somewhat smaller than in other studies. In foreign studies mutation of BMPR2 has been found in about 55% of familial cases and 26% of sporadic PPH cases (Trembath et al 2001, Thomson et al 2000). This difference may be explained simply by the fact that although the sampling was representative, the number of PPH patients in Finland and thus the number of patients studied was small and the proportion can be different by chance only. It is also possible that in addition to coding mutations there may be other regulatory elements for the gene predisposing to the disease. Researchers have already found rearrangements and exonic deletions/duplications of BMPR2 in cases previously tested negative for mutations of this gene (Cogan et al 2005, Cogan et al 2006). Our haplotype and RFLP analysis however, did not support large deletions in Finnish patients. Some additional mutations might have been found by other methods.

5.3 Mutational analysis of ALK1, Endoglin, BMPR1A, SMAD4 and SLC6A4 among the PPH patients

The study population was the same described in section 6.2. Because of the difficulty of sequencing paraffin block extracted DNA, the genes were studied only in patients whose blood samples were available for DNA extraction (n=21) and two sets of the parents of familial patients. In addition, seven sets of parents and one single parent of sporadic patients, whose DNA samples were available, were included. Genetic variations found were screened among 59 healthy blood donors. Altogether 210 samples showed altered motility in the SSCP analysis of ALK1, Endoglin, BMPR1A, SMAD4 and SLC6A4 and were further studied by direct sequencing.

Two mutations were found in ALK1. One of the mutations was found in exon 5 (D179A, 536A>C) which encodes the GS domain of the protein. The patient was middle-aged with sporadic PPH whose family history is largely unknown. No clinical signs of HHT were found in the patient’s medical files or autopsy report. The other ALK1 mutation was found
in exon 8 (R374Q, 1121G>A) encoding the kinase domain of the protein in a parent with HHT whose child was diagnosed with PPH in adolescence and who was at the time of the study already deceased. There were no signs of HHT in the child’s hospital records or autopsy report. The same mutation however was found when paraffine block extracted DNA of the child was studied.

In Endoglin a SNP 14C>T was found also causing an amino acid change (T5M) in exon 1 in two unrelated PPH patients (allele frequency 4.7%). This nucleotide change was not found among the Finnish 136 control chromosomes. However, the nucleotide change was found in one control we used (Centre d’Etude de polymorphisme Humaine, CEPH). It has been reported also as a polymorphism in international databases. We found three other polymorphisms in the coding region of the Endoglin. In exon 8 we found two different polymorphisms (1029c>t; 1060c>t) which did not cause amino acid changes (T343T; L354L). One or the other of these polymorphisms was found among 4/21 patients and 3/59 controls. In exon 11 we found a polymorphism (1374a>g, P458P, allele frequency among patients 7% and 2.5 % among controls). Although we did not systematically study the non-coding regions of the endoglin gene, we found a nucleotide change in intron 2 (rs7847860 g>t). All of these nucleotide changes (1029c>t; 1060c>t, 1374a>g, rs7847860 g>t) have been previously reported as polymorphic sites.

In BMPR1A we found nucleotide change 4 c>a causing an aminoacid change P2T. This nucleotide change was found in 16 patients or parents and in five of them as homozygous. In exon one of SLC6A4 a nucleotide change 167g>a was found. This causes an amino acid change G56A and was found in 1 patient and 1 control (allele frequencies 4.7 vs 0.8%). Both changes, P2T in BMPR1A and G56A in SLC6A4, have been reported as polymorphisms in international databases and were also found among our control chromosomes. No mutations or polymorphisms in SMAD4 were found in this study among PPH patients or parents.

<table>
<thead>
<tr>
<th>Genotyping (PPH)</th>
<th>14 patients</th>
<th>19 family members</th>
</tr>
</thead>
<tbody>
<tr>
<td>Restriction fragment length polymorphism (RFLP, PPH)</td>
<td>21 patients</td>
<td>1 set of parents of familial PPH</td>
</tr>
<tr>
<td>Direct sequencing (PPH)</td>
<td>28 patients (whole blood or paraffine block extracted DNA)</td>
<td>2 sets of parents of familial PPH patients</td>
</tr>
<tr>
<td>Single-strand Conformational Polymorphism (SSCP, PPH)</td>
<td>21 patients</td>
<td>2 sets of parents of familial and 7 sets of parents of sporadic PPH patients, one single parent</td>
</tr>
<tr>
<td>Direct sequencing (HHT)</td>
<td>8 patients</td>
<td>10 affected family members studied</td>
</tr>
</tbody>
</table>

Table 5  Number of PPH and HHT patients and family members studied with different methods
Clearly other genes and other signaling pathways may also be involved in the pathogenesis of PPH. So far overexpression of a certain variant of the serotonin transporter gene \textit{SLC6A4} has been associated with PPH supporting the theory of the role of serotonin in the pathogenesis of PPH (Eddahibi \textit{et al} 2001). In addition, the disease locus has been linked to a more proximal location on chromosome 2q31 in certain PPH families (Rindermann \textit{et al} 2003).

In this study mutations in \textit{ALK1} were found in two PPH patients. The other in a middle aged patient who did not have symptoms or signs of HHT and with unknown family history. The other mutation was found in a young adult patient with a positive family history of HHT. It has been reported that 62 \% of the HHT patients become symptomatic by the age of 16 and all the patients by the age of fourty (Portenous \textit{et al} 1992). One may speculate, that in our cohort the younger patient may have had symptoms of HHT during the following years and should be considered as HHT-related pulmonary hypertension. The older patient, however, should have developed symptoms already, and potentially had the \textit{ALK1} mutation-related PPH.

5.4 \textbf{HHT and Endoglin and ALK1 mutations}

Altogether 14 patients fulfilling the Curaçao diagnostic criteria were identified and a total of 8 patients were screened for \textit{ALK1} and \textit{Endoglin} mutations using direct sequencing. We found 7 mutations altogether, 3 in \textit{ALK1} gene and 4 in endoglin gene (Table 6). All the mutations in endoglin and one in \textit{ALK1} gene were truncating. One of the \textit{ALK1} mutations caused a deletion of one amino acid and one was a missense mutation. All the mutations were exclusive to each family and the mutation status correlated with the symptoms of family members studied.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exon</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoglin</td>
<td>1</td>
<td>65-66delCA</td>
<td>22fs+24aa</td>
</tr>
<tr>
<td>Endoglin</td>
<td>3</td>
<td>259 C&gt;T</td>
<td>Q87X</td>
</tr>
<tr>
<td>Endoglin</td>
<td>5</td>
<td>553 G&gt;T</td>
<td>E185X</td>
</tr>
<tr>
<td>Endoglin</td>
<td>11</td>
<td>1434-1435delAG</td>
<td>478fs+61aa</td>
</tr>
<tr>
<td>ALK-1</td>
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<td>W50X</td>
</tr>
<tr>
<td>ALK-1</td>
<td>7</td>
<td>1040-1042delCCG</td>
<td>347delA</td>
</tr>
<tr>
<td>ALK-1</td>
<td>8</td>
<td>1231 C&gt;T</td>
<td>R411W</td>
</tr>
</tbody>
</table>

Table 6 \textit{ALK-1 and Endoglin mutations among a group of Finnish HHT patients}

A mutation was found in as many as seven (87\%) of the eight index patients with HHT and all of their affected family members. As in PPH all the mutations were different.
Although in international studies of both PPH and HHT most of the mutations have been different and family-specific, there has been some evidence of a founder effect in HHT in some geographic isolates (Brusgaard et al 2004, Gallione et al 2000). Based on the knowledge we have about some monogenic diseases in Finland the lack of a founder mutation in both PPH and HHT was a bit surprising. On the other hand recurrent mutation in PPH and HHT seem to be rare after all. In most cases HHT does not affect fertility and by the time a diagnosis is made, many PPH patients may already have children of their own. However, BMPR2 mutations may act on fetal viability and the mutations are under negative selective pressure (Loyd et al 1995). This suggests, that \textit{BMPR2}, \textit{Endoglin} and \textit{ALK1} become mutated easily. In conclusion, among Finns the use of genetic diagnostics of PPH and HHT is as difficult as among other populations. A mutation can be found in many cases, more commonly among the HHT than PPH cases. However, there is not a simple genetic test to diagnose the diseases but rather more time-consuming sequencing and even other methods are needed.
6. CONCLUDING REMARKS

Based on the results of the present study in Finland primary pulmonary hypertension (PPH) seems to be as common as in other countries. No large pedigrees were found and the families were relatively small. BMPR2 mutation was found in 33 % of the familial and 12 % of the sporadic patients and each patient was found to harbor a private mutation. Based on haplotyping findings there is no evidence of a founder mutation in BMPR2 for PPH in Finland. Although large deletions of BMPR2 were excluded in this study, it is possible that small duplications or deletions were missed. In addition to BMPR2, four important genes (ALK1, BMPR1A, Endoglin, SMAD4) of the TGF-β-signaling pathway were studied, but only two mutations in ALK1 were found. It is possible, that other genes and signaling pathways are involved in the pathogenesis of PPH.

In HHT patients studied the ALK1 and Endoglin mutations were found in equal numbers and all the patients had different mutations. Based on these findings sequencing whole genes remains the method of choice for both of these disorders. For PPH patients the analysis of BMPR2 gene is warranted if clinical criteria presented above are fulfilled. If no BMPR2 mutation is found, screening of ALK1 and Endoglin should be considered. For HHT patients in addition to ALK1 and Endoglin genes, the screening of SMAD4 should be considered.
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