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**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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CONTENTS

List of original publications	5
Abbreviations	6
Abstract	8
1. Introduction	10
2. Review of the literature	12
2.1 Finns as a study population in genetic studies	12
2.2 Primary pulmonary hypertension (PPH) and hereditary hemorrhagic telangiectasia (HHT) as clinical entities	13
2.2.1 Diagnosis and clinical classification of the disease	13
2.2.2 Epidemiology of PPH and HHT	16
2.2.3 Etiology of PPH and HHT	17
2.2.4 Pathological findings in arterial walls and pathophysiology of PPH and HHT	18
2.2.5 Treatment of PPH and HHT	20
2.2.6 Structure of <i>BMPR2</i> , <i>ALK1</i> and <i>Endoglin</i> genes	24
2.2.7 TGF- β -signaling pathway	25
2.2.8 Molecular genetics of PPH and HHT	27
3. Aims of the study	31
4. Materials and methods	32
4.1 Patient identification	32
4.2 Diagnostic criteria	33
4.3 Genes studied	33

4.4 Mutation analysis of <i>BMPR2</i> , <i>Endoglin</i> , and <i>ALK1</i> by direct sequencing	34
4.5 Genotyping	34
4.6 Haplotype analysis	34
4.7 Restriction fragment length polymorphism (RFLP) and Southern blot hybridization	35
4.8 Functional analysis of cytoplasmic tail missense mutation of <i>BMPR2</i> c.2696G>C encoding R899P	35
4.9 Single-strand Conformational Polymorphism (SSCP) analysis of <i>ALK1</i> , <i>Endoglin</i> , <i>BMPRIA</i> , <i>SMAD4</i> , and <i>SLC6A4</i>	35
5. Results and discussion	37
5.1 PPH epidemiology in Finland	37
5.2 <i>BMPR2</i> mutation, haplotype, and functional analysis	38
5.3 Mutation analysis of <i>ALK1</i> , <i>Endoglin</i> , <i>BMPRIA</i> , <i>SMAD4</i> and <i>SLC6A4</i> among PPH patients	41
5.4 HHT and <i>Endoglin</i> and <i>ALK1</i> mutations	43
6. Concluding remarks	45
7. Acknowledgements	46
8. References	49
Original publications	

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

- I Sankelo M, Flanagan JA, Machado R, Harrison R, Rudarakanchana N, Morrell N, Dixon M, Halme M, Puolijoki H, Kere J, Elomaa O, Kupari M, Räisänen-Sokolowski A, Trembath RC, Laitinen T: BMPR2 mutations have short lifetime expectancy in primary pulmonary hypertension. *Hum Mutat* 2005;26:119-24.
- II Harrison RE, Flanagan J, Sankelo M, Abdalla SA, Rowell J, Machado RD, Elliot CG, Robbins IM, Olschewski H, McLaughlin V, Gruenig E, Kermeen F, Halme M, Räisänen-Sokolowski A, Laitinen T, Morrell NW, Trembath RC: Molecular and functional analysis identifies ALK-1 as the predominant cause of pulmonary hypertension related to hereditary haemorrhagic telangiectasia. *J Med Genet* 2003; 40:865-71. Erratum in: *J Med Genet* 2004;41:576.
- III Sankelo M, Halme M, Laitinen T, Mattila PS: Hereditary hemorrhagic telangiectasia type 1 and 2 mutations in Finland. *Acta Otolaryngol.* 2008;128:1238-41.
- IV Sankelo M, Halme M, Laitinen T: Mutational analysis of ALK1, Endoglin, BMPR1A, SMAD4, and Serotonine 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
BMPRI1A	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
IPAH	idiopathic pulmonary arterial hypertension
JPS	juvenile polyposis syndrome
K ⁺	potassium
1.5K _v	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
PDGF α	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
<i>Smad4</i>	<i>Sma</i> gene and <i>mothers against decapentaplegic homolog 4</i>
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 (BMPRIA or ALK3)*, *Mothers Against Decapentaplegic Homolog 4 (SMAD4)* and *Serotonine 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 *BMP2*, *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 *BMP2* 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, Dolará *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, eg. 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 its 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)

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 (Table1). 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 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 hemoptysis, 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.

Curaçao criteria	
1. Epistaxis	Spontaneous, recurrent nosebleeds
2. Teleangiectasies	Multiple, at characteristic sites: lips, oral cavity, fingers, nose
3. Visceral lesions	Such as: Gastrointestinal teleangiectasia (with or without bleeding), pulmonary AVM, hepatic AVM, cerebral AVMs, spinal AVM
4. Positive family history	A first-degree relative with HHT according to these criteria

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

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 the 1990s was related to long term use of fenfluramine derivatives (Simonneau *et al* 1998, Rich *et al* 2000, Abenheim *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 (PDGF α), 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-HTT* 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 trombin, 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 (McGoan 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 (Hervè *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 telangiectatic lesions which are histologically locally dilated 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 telangiectatic 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 responded 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 aaPPH 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 (McLaughlin *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 Kraus 1986, Hoeper *et al* 2000, Olschewski *et al* 2000). However, iloprost has relatively short duration of action and analogues with longer half-lives 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 \pm 80 m to 392 \pm 61m (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	Mechanism of action
CCB	Vasodilator (patients with positive vasoreactivity test)
Prostacyclin and prostacyclin analogues: Epoprostenol (continuous i.v) Trepstinil (continuous s.c.) Sodium beraprost (p.o) Iloprost (inhaled)	Multifactorial: Vasodilation, Smooth muscle relaxation, Inhibition of platelet aggregation, Clearance of ET1 Inotropic effect Cytoprotective and antiproliferative properties
ET-antagonist: Bosentan (oral) Sitaxentan (oral) Ambrisentan (oral)	Dual ETA/ETB antagonist Selective ETA antagonist Selective ETA antagonist
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 telangiectasias 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).

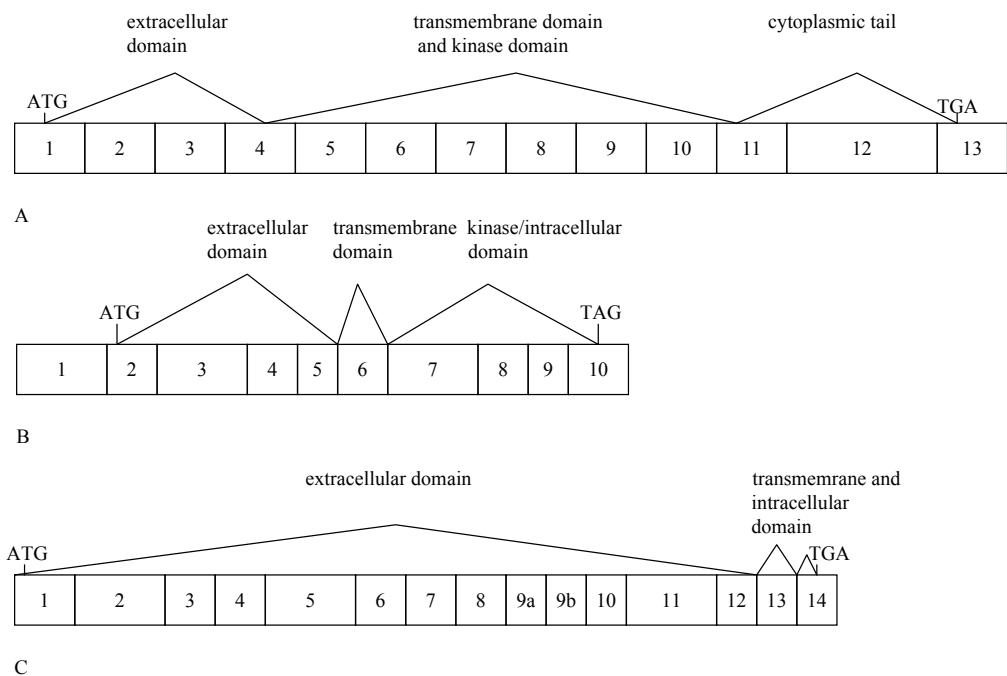


Figure 1 Structure of *BMPR2* (A), *ALK1* (B) and *Endoglin* (C) genes

2.2.7 TGF- β -signaling pathway

TGF β -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- β s, 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- β s 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, Massague 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 p38^{MAPK} kinases (Massague 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.

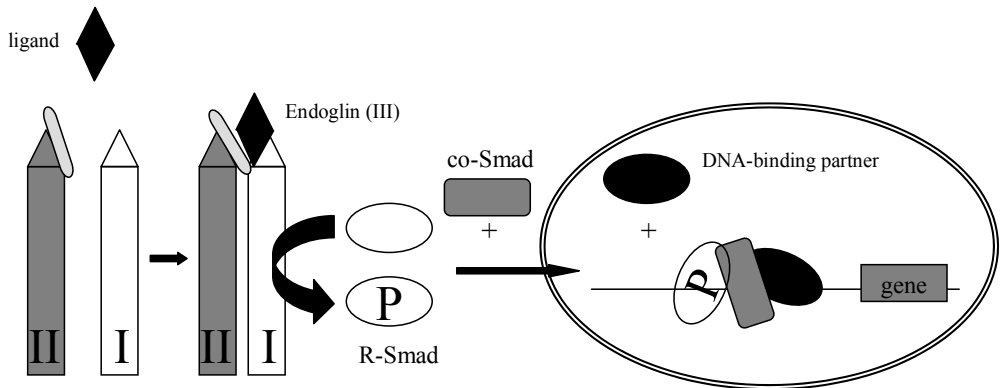


Figure 2 *TGF- β signaling pathway. Endoglin(III) presents ligand to type II receptor, which recruits and phosphorylates type I receptor. Type I receptor in turn phosphorylates Smads. Smad-complex moves to nucleus to regulate gene transcription.*

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 (Trebath 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, p38^{MAPK} -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 (K_v) 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^{2+} channels caused by reduced $K_v1.5$ 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 *BMP2* 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*

2005). 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).

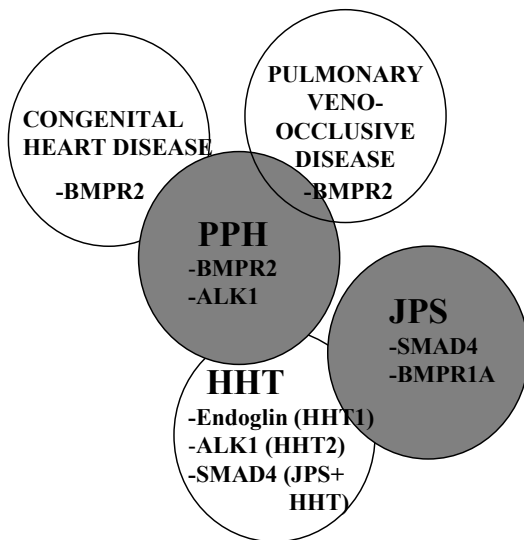


Figure 3 Diseases related to TGF- β signaling pathway

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 genealogy of familial PPH of all cases nationwide (I)
3. to characterize the mutation spectrum of *BMP2* 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 ≥ 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- β signaling pathway. *SLC6A4* was included because a polymorphism of the promoter 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).

4.4 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).

4.5 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).

4.6 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 ³²P 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 Na₂HPO₄, and 1 mM EDTA with 100 µg of herring sperm DNA. After prehybridization, the ³²P 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 p38^{MAPK} 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 p38^{MAPK} (Study I).

4.9 Single-strand Conformational Polymorphism (SSCP) analysis of *ALK1*, *Endoglin*, *BMPRIA*, *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, *BMPRIA* 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 10 μ l and 20 ng of DNA was used as a template.

7.5 μ l of bromophenolblue and formamide-containing stop solution was added to 10 μ l 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.

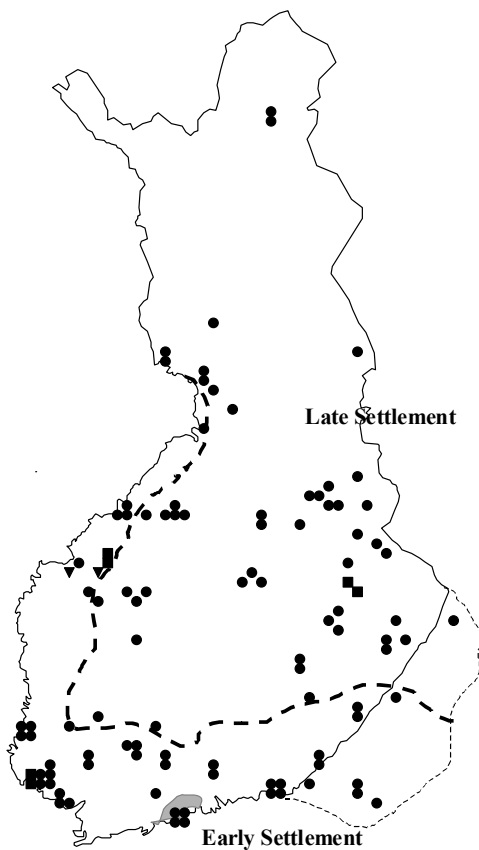


Figure 4 Birthplaces of parents of PPH patients
circle=sporadic PPH, square=familial
PPH, triangle= PPH and HHT in the
same family

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 *BMP2* 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 occurred, 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.

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

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).

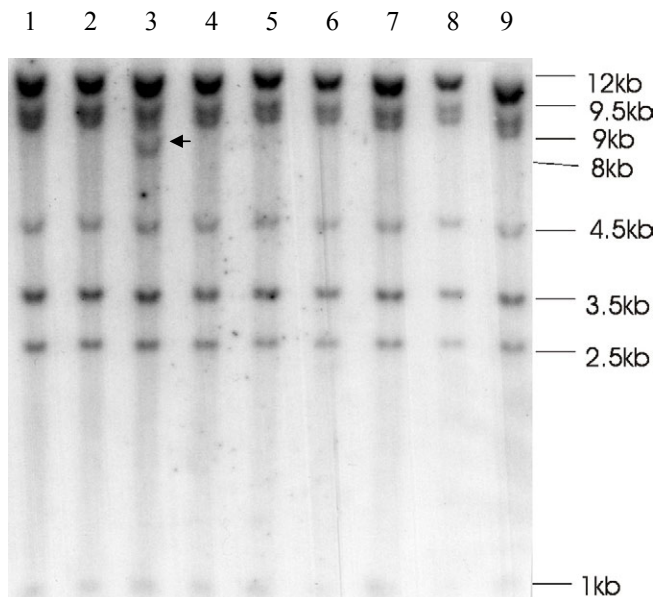


Figure 5 Southern blot analysis of *BMPR2* using *PstI*. The additional restriction site is indicated by arrow

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 p38^{MAPK} pathway was activated also in the absence of the ligand and induced smooth muscle proliferation, which was inhibited by selective p38^{MAPK} 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 *BMPR2* 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 change 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*, *BMPRIA*, *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*, *BMPRIA*, *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 *BMPRIA* we found nucleotide change 4 c>a causing an amino acid 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 *BMPRIA* 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.

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

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 *SLC6A4* has been associated with PPH supporting the theory of the role of serotonin in the pathogenesis of PPH (Eddahibi *et al* 2001). In addition, the disease locus has been linked to a more proximal location on chromosome 2q31 in certain PPH families (Rindermann *et al* 2003).

In this study mutations in *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 forty (Portenous *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 *ALK1* mutation-related PPH.

5.4 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 *ALK1* and *Endoglin* mutations using direct sequencing. We found 7 mutations altogether, 3 in *ALK1* gene and 4 in *endoglin* gene (Table 6). All the mutations in *endoglin* and one in *ALK1* gene were truncating. One of the *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.

Gene	Exon	Nucleotide change	Amino acid change
Endoglin	1	65-66delCA	22fs+24aa
Endoglin	3	259 C>T	Q87X
Endoglin	5	553 G>T	E185X
Endoglin	11	1434-1435delAG	478fs+61aa
ALK-1	3	150 G>A	W50X
ALK-1	7	1040-1042delCCG	347delA
ALK-1	8	1231 C>T	R411W

Table 6 *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, *BMP2* mutations may act on fetal viability and the mutations are under negative selective pressure (Loyd et al 1995). This suggests, that *BMP2*, *Endoglin* and *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 a 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*, *BMPRIA*, *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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8. REFERENCES

- AAssar OS, Friedman CM, White RI Jr: The natural history of epistaxis in hereditary hemorrhagic telangiectasia. *Laryngoscope*. 1991;101:977-80.
- Abdalla SA, Cymerman U, Johnson RM, Deber CM, Letarte M: Disease-associated mutations in conserved residues of ALK-1 kinase domain. *Eur J Hum Genet*. 2003;1:279-87.
- Abdalla Sa, Gallione CJ, Barst RJ, Horn EM, Knowles JA, Marchuk DA, Letarte M, Morse JH: Primary pulmonary hypertension in families with hereditary haemorrhagic telangiectasia. *Eur Respir J*. 2004;23:373-7.
- Abdelkader M, Leong SC, White PS: Endoscopic control of the sphenopalatine artery for epistaxis: long-term results. *J Laryngol Otol*. 2007;121:759-62.
- Abenhaim L, Moride Y, Brenot F, Rich S, Benichou J, Kurz X, Higenbottam T, Oakley C, Wouters E, Aubier M, Simonneau G, Begaud B: Appetite-suppressant drugs and the risk of primary pulmonary hypertension. *N Engl J Med* 1996;335:609-16.
- Aizawa K, Hanaoka T, Kasai H, Kogashi K, Kumazaki S, Koyama J, Tsutsui H, Yazaki Y, Watabane N, Kinoshita O, Ikeda U: Long-term vardenafil therapy improves hemodynamics in patients with pulmonary hypertension. *Hypertens Res* 2006;29:123-128.
- Aldred MA, Vijayakrishnan J, James V, Soubrier F, Gomez-Sanchez MA, Martensson G, Galie N, Manes A, Corris P, Simonneau G, Humbert M, Morrell NW, Trembath R: BMPR2 gene rearrangements account for a significant proportion of mutations in familial and idiopathic pulmonary arterial hypertension. *Hum Mut* 2006;
- Appelbaum L, Yigla M, Bendayan D, Reichart N, Fink G, Priel I, Schwartz Y, Richman P, Picard E, Goldman S, Kramer MR: Primary pulmonary hypertension in Israel: a national survey. *Chest*. 2001;119:1801-6.
- Arthur HM, Ure J, Smith AJ, Renforth G, Wilson DI, Torsney E, Charlton R, Parums DV, Jowett T, Marchuk DA, Burn J, Diamond AG: Endoglin, an ancillary TGFbeta receptor, is required for extraembryonic angiogenesis and plays a key role in heart development. *Dev Biol*. 2000;217:42-53.
- Barst RJ, Rubin LJ, Long WA, McGoon MD, Rich S, Badesch DB, Groves BM, Tapson VF, Bourge RC, Brundage BH, Koerner SK, Langleben D, Keller CA, Murali S., Uretsky BF, Clayton LM., Jöbsis MM, Blackburn SD, Shortino D, Crow JW, for the primary pulmonary hypertension study group. A comparison of continuous intravenous epoprostenol (prostacyclin) with conventional therapy for primary pulmonary hypertension. *N Engl J Med* 1996;334:296-301.
- Barst RJ, McGoon M, McLaughlin V, Tapson V, Rich S, Rubin L, Wasserman K, Oudiz R, Sharipo S, Robbins IM, Channick R, Badesch D, Rayburn BK, Flinchbaugh R, Sigman J, Arneson C, Jeffs R, Beraprost Study Group: Beraprost therapy for pulmonary arterial hypertension. *J Am Coll Cardiol* 2003;41:2119-25.

- Barst RJ, Galie N, Naeije R, Simonneau G, Jeffs R, Arneson C, Rubin LJ: Long-term outcome in pulmonary arterial hypertension patients treated with subcutaneous treprostinil. *Eur Respir J* 2006;28:1073-5.
- Bayrak-Toydemir P, McDonald J, Akarsu N, Toydemir RM, Calderon F, Tuncali T, Tang W, Miller F, Mao R: A fourth locus for hereditary hemorrhagic telangiectasia maps to chromosome 7. *Am J Med Genet A*. 2006;140:2155-62.
- Bellón T, Corbí A, Lastres P, Calés C, Cebrián M, Vera S, Cheifetz S, Massague J, Letarte M, Bernabéu C: Identification and expression of two forms of the human transforming growth factor-beta-binding protein endoglin with distinct cytoplasmic regions. *Eur J Immunol*. 1993;23:2340-5.
- Beppu H, Kawabata M, Hamamoto T, Chytil A, Minowa O, Noda T, Miyazono K: BMP type II receptor is required for gastrulation and early development of mouse embryos. *Dev Biol*. 2000;221:249-58.
- Beppu H, Minowa O, Miyazono K, Kawabata M: cDNA cloning and genomic organization of the mouse BMP type II receptor. *Biochem Biophys Res Commun*. 1997;235:499-504.
- Berg JN, Gallione CJ, Stenzel TT, Johnson DW, Allen WP, Schwartz CE, Jackson CE, Porteous MEM, Marchuk DA: The activin receptor-like kinase 1 gene: genomic structure and mutations in hereditary hemorrhagic telangiectasia type 2. *Am J Hum Genet*. 1997;61:60-7.
- Blobe GC, Schieman WP, Lodish HF.: Role of transforming growth factor beta in human disease. *N Engl J Med*. 2000;342:1350-8. Review.
- Bonderman D, Nowotny R, Skoro-Sajer N, Adlbrecht C, Lang IM: Bosentan therapy for pulmonary arterial hypertension associated with hereditary haemorrhagic telangiectasia. *Eur J Clin Invest*. 2006;36 Suppl 3:71-2.
- Bourdeau A, Dumont DJ, Letarte M: A murine model of hereditary hemorrhagic telangiectasia. *J Clin Invest*. 1999;104:1343-51.
- Bourdillon PDV, Oakley CM: Regression of pulmonary hypertension. *British Heart Journal* 1976;38:264-270.
- Braman SS, Eby E, Kuhn C, Rounds S: Primary pulmonary hypertension in the elderly. *Arch Intern Med* 1991;151:2433-2438.
- Braverman IM, Keh A, Jacobson BS: Ultrastructure and three-dimensional organization of the telangiectases of hereditary hemorrhagic telangiectasia. *J Invest Dermatol*. 1990;95:422-7.
- Brusgaard K, Kjeldsen AD, Poulsen L, Moss H, Vase P, Rasmussen K, Kruse TA, Hørdér M: Mutations in endoglin and in activin receptor-like kinase 1 among Danish patients with hereditary haemorrhagic telangiectasia. *Clin Genet*. 2004;66:556-61.

- Buonamico P, Suppressa P, Lenato GM, Pasculli G, D'Ovidio F, Memeo M, Scardapane A, Sabbà C: Liver involvement in a large cohort of patients with hereditary hemorrhagic telangiectasia: echo-color-Doppler vs multislice computed tomography study. *J Hepatol.* 2008;48:811-20.
- Chaouat A, Coulet F, Favre C, Simonneau G, Weitzenblum E, Soubrier F, Humbert M.: Endoglin germline mutation in a patient with hereditary haemorrhagic telangiectasia and dexfenfluramine associated pulmonary arterial hypertension. *Thorax.* 2004;59:446-8.
- Christman BW, McPherson CD, Newman JH, King GA, Bernard GR, Groves BM, Loyd JE: An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. *N Engl J Med* 1992; 327:70-5.
- Cogan JD, Vnencak-Jones CL, Phillips JA 3rd, Lane KB, Wheeler LA, Robbins IM, Garrison G, Hedges LK, Loyd JE: Gross BMPR2 gene rearrangements constitute a new cause for primary pulmonary hypertension. *Genet Med.* 2005;7:169-74.
- Cogan JD, Pauciulo MW, Batchman AP, Prince MA, Robbins IM, Hedges LK, Stanton KC, Wheeler LA, Phillips JA 3rd, Loyd JE, Nichols WC: High frequency of BMPR2 exonic deletions/duplications in familial pulmonary arterial hypertension. *Am J Respir Crit Care Med.* 2006;174:590-8.
- Cole SG, Begbie ME, Wallace GM, Shovlin CL: A new locus for hereditary haemorrhagic telangiectasia (HHT3) maps to chromosome 5. *J Med Genet.* 2005;42:577-82.
- Conte JV, Borja MJ, Patel CB, Yang SC, Jhaveri RM, Orens JB: Lung transplantation for primary and secondary pulmonary hypertension. *Ann Thorac Surg.* 2001;72:1673-9.
- Cottin V, Dupuis-Girod S, Lesca G, Cordier JF: Pulmonary vascular manifestations of hereditary hemorrhagic telangiectasia (rendu-osler disease). *Respiration.* 2007;74:361-78. Review.
- Cottin V, Chinet T, Lavolé A, Corre R, Marchand E, Reynaud-Gaubert M, Plauchu H, Cordier JF; Groupe d'Etudes et de Recherche sur les Maladies "Orphelines" Pulmonaires (GERM"O"P): Pulmonary arteriovenous malformations in hereditary hemorrhagic telangiectasia: a series of 126 patients. *Medicine (Baltimore).* 2007;86:1-17.
- D'Alonzo GE, Barst RJ, Ayres SM, Bergofsky EH, Brundage BH, Detre KM., Fishman AP, Goldring RM, Groves BM, Kernis JT, Levy PS, Pietra GG, Reid LM, Reeves JT, Rich S, Vreim CE, Williams GW, Wu M: Survival in patients with primary pulmonary hypertension. Results from a national prospective registry. *Ann Intern Med.* 1991;115:343-9.
- de la Chapelle A.: Disease gene mapping in isolated human populations: the example of Finland. *J Med Genet.* 1993;30:857-65. Review.
- de la Chapelle A, Wright FA.: Linkage disequilibrium mapping in isolated populations: the example of Finland revisited. *Proc Natl Acad Sci U S A.* 1998;95:12416-23.

- Deng Z, Morse JH, Slager SL, Cuervo N, Moore KJ, Venetos G, Kalachikov S, Cayanis E, Fischer SG, Barst RJ, Hodge SE, Knowles JA: Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. *Am J Hum Genet.* 2000;67:737-44.
- de Nucci G, Thomas R, D'Orleans-Juste P, Antunes E, Walder C, Warner TD, Vane JR: Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc Natl Acad Sci U S A.* 1988;85:9797-800.
- Derynck R, Gelbart WM, Harland RM, Heldin CH, Kern SE, Massagué J, Melton DA, Mlodzik M, Padgett RW, Roberts AB, Smith J, Thomsen GH, Vogelstein B, Wang XF. Nomenclature: vertebrate mediators of TGFbeta family signals. *Cell.* 1996 Oct 18;87(2):173.
- Dolaro A, Camerini F, Menotti A, Thiene G: Primary pulmonary hypertension: an Italian multicenter study. A retrospective epidemiological survey in period 1975-1985. *G Ital Cardiol.* 1988;18:115-120.
- Dupuis J, Goresky CA, Fournier A: Pulmonary clearance of circulating endothelin-1 in dogs in vivo: exclusive role of ETB receptors. *J Appl Physiol.* 1996;81:1510-5.
- Eddahibi S, Humbert M, Fadel E, Raffestin B, Darmon M, Capron F, Simonneau G, Dartevielle P, Hamon M, Adnot S: Serotonin transporter overexpression is responsible for pulmonary artery smooth muscle hyperplasia in primary pulmonary hypertension. *J Clin Invest.* 2001;108:1141-50
- Ehrenreich H, Rieckmann P, Sinowatz F, Weih KA, Arthur LO, Goebel F-D, Burd PR, Coligan JE, Cluse KA: Potent stimulation of monocytic endothelin production by HIV-1 glycoprotein 120. *J Immunol* 1993; 150:4601-4609.
- Eisenberg PR, Lucore C, Kaufman L, Sobel BE, Jaffe AS, Rich S: Fibrinopeptide A levels indicative of pulmonary vascular thrombosis in patients with primary pulmonary hypertension. *Circulation.* 1990;82:841-7.
- El-Harith el-HA, Kühnau W, Schmidtke J, Gadzicki D, Ahmed M, Krawczak M, Stuhmann M: Hereditary hemorrhagic telangiectasia is caused by the Q490X mutation of the ACVRL1 gene in a large Arab family: support of homozygous lethality. *Eur J Med Genet.* 2006 ;49:323-30.
- Elliott G, Alexander G, Leppert M, Yeates S, Kerber R.: Coancestry in apparently sporadic primary pulmonary hypertension. *Chest.* 1995;108:973-7.
- Fernández-Jorge B, Del Pozo Losada J, Paradela S, Martínez-González C: Treatment of cutaneous and mucosal telangiectases in hereditary hemorrhagic telangiectasia: Report of three cases. *J Cosmet Laser Ther.* 2007;9:29-33.
- Fernandez-L A, Garrido-Martin EM, Sanz-Rodriguez F, Pericacho M, Rodriguez-Barbero A, Eleno N, Lopez-Novoa JM, Düwell A, Vega MA, Bernabeu C, Botella LM: Gene

expression fingerprinting for human hereditary hemorrhagic telangiectasia. *Hum Mol Genet.* 2007;16:1515-33.

- Fernandez-L A, Garrido-Martin EM, Sanz-Rodriguez F, Ramirez JR, Morales-Angulo C, Zarrabeitia R, Perez-Molino A, Bernabéu C, Botella LM: Therapeutic action of tranexamic acid in hereditary haemorrhagic telangiectasia (HHT): regulation of ALK-1/endothelin pathway in endothelial cells. *Thromb Haemost.* 2007;97:254-62.
- Fernández-Ruiz E, St-Jacques S, Bellón T, Letarte M, Bernabéu C: Assignment of the human endoglin gene (END) to 9q34-->qter. *Cytogenet Cell Genet.* 1993;64:204-7.
- Follath F, Burkart F, Schweizer W.: Drug-induced pulmonary hypertension? *Br Med J.* 1971;1:265-6.
- Franke U, Wiebe K, Harringer W, Franke T, Wittwer T, Wahlers T, Haverich A: Ten years experience with lung and heart-lung transplantation in primary and secondary pulmonary hypertension. *Eur J Cardiothorac Surg.* 2000;18:447-52.
- Gaine SP, Rubin LJ: Primary pulmonary hypertension. *Lancet* 1998; 352: 719-25.
- Galiè N, Humbert M, Vachiéry JL, Vizza CD, Kneussl M, Manes A, Sitbon O, Torbicki A, Delcroix M, Naeije R, Hoeper M, Chaouat A, Morand S, Besse B, Simonneau G; Arterial Pulmonary Hypertension and Beraprost European (ALPHABET) Study Group: Effects of beraprost sodium, an oral prostacyclin analogue, in patients with pulmonary arterial hypertension: a randomized, double-blind, placebo-controlled trial. *J Am Coll Cardiol.* 2002 ;39:1496-502.
- Galiè N, Seeger W, Naeije R, Simonneau G, Rubin LJ: Comparative analysis of clinical trials and evidence-based treatment algorithm in pulmonary arterial hypertension. *J Am Coll Cardiol.* 2004;43(12 Suppl S):81S-88S. Review.
- Galiè N, Badesch D, Oudiz R, Simonneau G, McGoon MD, Keogh AM, Frost AE, Zwicke D, Naeije R, Shapiro S, Olschewski H, Rubin LJ: Ambrisentan therapy for pulmonary arterial hypertension. *J Am Coll Cardiol* 2005;46:529-35.
- Galiè N, Ghofrani HA, Torbicki A, Barst RJ, Rubin LJ, Badesch D, Fleming T, Parpia T, Burgess G, Branzi A, Grimminger F, Kurzydina M, Simonneau G for the Sildenafil Use in Pulmonary Arterial Hypertension(SUPER) Study Group: Sildenafil citrate therapy for pulmonary arterial hypertension. *N Engl J Med* 2005; 353:2148-57.
- Gallione CJ, Scheessele EA, Reinhardt D, Duits AJ, Berg JN, Westermann CJ, Marchuk DA: Two common endoglin mutations in families with hereditary hemorrhagic telangiectasia in the Netherlands Antilles: evidence for a founder effect. *Hum Genet.* 2000;107:40-44.
- Gallione CJ, Repetto GM, Legius E, Rustgi AK, Schelley SL, Tejpar S, Mitchell G, Drouin E, Westermann CJ, Marchuk DA: A combined syndrome of juvenile polyposis and hereditary haemorrhagic telangiectasia associated with mutations in MADH4 (SMAD4). *Lancet.* 2004;363:852-9.

- Gallione CJ, Richards JA, Letteboer TG, Rushlow D, Prigoda NL, Leedom TP, Ganguly A, Castells A, Ploos van Amstel JK, Westermann CJ, Pyeritz RE, Marchuk DA: SMAD4 mutations found in unselected HHT patients. *J Med Genet.* 2006;43:793-7.
- Garcia-Tsao G.: Liver involvement in hereditary hemorrhagic telangiectasia (HHT). *J Hepatol.* 2007;46:499-507. Review.
- Geisthoff UW, Fiorella ML, Fiorella R: Treatment of recurrent epistaxis in HHT. *Curr Pharm Des.* 2006;12:1237-42. Review.
- Geraci MW, Moore M, Gesell T, Yeager ME, Alger L, Golpon H, Gao B, Loyd JE, Tuder RM, Voelkel NF: Gene expression patterns in the lungs of patients with primary pulmonary hypertension: a gene microarray analysis. *Circ Res.* 2001;88:555-62.
- Giaid A, Yanagisawa M, Langleben D, Michel RP, Levy R, Shennib H, Kimura S, Masaki T, Duguid WP, Stewart DJ: Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. *N Engl J Med.* 1993;328:1732-9.
- Giaid A, Saleh D: Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N Engl J Med.* 1995;333:214-21.
- Gougos A, Letarte M: Identification of a human endothelial cell antigen with monoclonal antibody 44G4 produced against a pre-B leukemic cell line. *J Immunol.* 1988;141:1925-33.
- Gougos A, Letarte M: Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells. *J Biol Chem.* 1990;265:8361-4.
- Gu Y, Jin P, Zhang L, Zhao X, Gao X, Ning Y, Meng A, Chen YG: Functional analysis of mutations in the kinase domain of the TGF-beta receptor ALK1 reveals different mechanisms for induction of hereditary hemorrhagic telangiectasia. *Blood.* 2006;107:1951-4.
- Gurtner HP.: Aminorex and pulmonary hypertension. *Cor Vasa* 1985;27:160-71.
- Guttmacher AE, McKinnon WC, Upton MD. : Hereditary hemorrhagic telangiectasia: a disorder in search of the genetics community. *Am J Med Genet.* 1994 Aug 15;52(2):252-3.
- Halank M, Marx C, Hoeffken G: Long term survival in primary pulmonary hypertension. *Heart* 2004; 90:e40.
- Harrison RE, Berger R, Haworth SG, Tulloh R, Mache CJ, Morrell NW, Aldred MA, Trembath RC. Transforming growth factor- β receptor mutations and pulmonary arterial hypertension in childhood. *Circulation.* 2005;111:435-41.
- Herbert F, Mlczoch J, Huber K, Schuster E, Gurtner HP, Kneussl M: The effect of anticoagulant therapy in primary and anorectic drug-induced pulmonary hypertension. *Chest* 1997;112:714-721.

- Herve P, Drouet L, Dosquet C, Launay JM, Rain B, Simonneau G, Caen J, Duroux P: Primary pulmonary hypertension in a patient with a familial platelet storage pool disease: role of serotonin. *Am J Med.* 1990;89:117-20.
- Hoepfer MM, Sosada M, Fabel H: Plasma coagulation profiles in patients with severe primary pulmonary hypertension. *Eur Respir J.* 1998;12:1446-9.
- Hoepfer MM, Schwarze M, Ehlerding S, Adler-Schuermeyer A, Spiekerkoetter E, Niedermeyer J, Hamm M, Fabel H: Long-term treatment of primary pulmonary hypertension with aerosolized iloprost, a prostacyclin analogue. *N Engl J Med* 2000; 342:1866-70.
- Hoepfer MM, Faulenbach C, Golpon H, Winkler J, Welte T, Niedermeyer J: Combination therapy with bosentan and sildenafil in idiopathic pulmonary arterial hypertension. *Eur Respir J.* 2004 ;24:1007-10.
- Humbert M, Monti G, Fartoukh M, Magnan A, Brenot F, Rain B, Capron F, Galanaud P, Duroux P, Simonneau G, Emilie D: Platelet-derived growth factor expression in primary pulmonary hypertension: comparison of HIV seropositive and HIV seronegative patients. *Eur Respir J* 1998; 11:554-559.
- Humbert M, Labrune P, Sitbon O, Le Gall C, Callebert J, Hervé P, Samuel D, Machado R, Trembath R, Drouet L, Launay JM, Simonneau G: Pulmonary arterial hypertension and type-I glycogen-storage disease: the serotonin hypothesis. *Eur Respir J.* 2002;20:59-65.
- Humbert M, Barst RJ, Robbins IM, Channick RN, Galiè N, Boonstra A, Rubin LJ, Horn EM, Manes A, Simonneau G: Combination of bosentan with epoprostenol in pulmonary arterial hypertension: BREATHE-2. *Eur Respir J.* 2004;24:353-9.
- Huusko P, Pääkkönen K, Launonen V, Pöyhönen M, Blanco G, Kauppila A, Puistola U, Kiviniemi H, Kujala M, Leisti J, Winqvist R: Evidence of founder mutations in Finnish BRCA1 and BRCA2 families. *Am J Hum Genet.* 1998;62:1544-8.
- Ianora AA, Memeo M, Sabba C, Cirulli A, Rotondo A, Angelelli G: Hereditary hemorrhagic telangiectasia: multi-detector row helical CT assessment of hepatic involvement. *Radiology.* 2004;230(2):250-9.
- Itoh S, Itoh F, Goumans MJ, Ten Dijke P: Signaling of transforming growth factor-beta family members through Smad proteins. *Eur J Biochem.* 2000;267:6954-67. Review.
- Jameson JJ, Cave DR: Hormonal and antihormonal therapy for epistaxis in hereditary hemorrhagic telangiectasia. *Laryngoscope.* 2004;114:705-9. Review.
- Johnson DW, Berg JN, Gallione CJ, McAllister KA, Warner JP, Helmbold EA, Markel DS, Jackson CE, Porteous ME, Marchuk DA: A second locus for hereditary hemorrhagic telangiectasia maps to chromosome 12. *Genome Res.* 1995;5:21-8.
- Johnson DW, Berg JN, Baldwin MA, Gallione CJ, Marondel I, Yoon SJ, Stenzel TT, Speer M, Pericak-Vance MA, Diamond A, Gutmacher AE, Jackson CE, Attisano L, Kucherlapati

- R, Porteous ME, Marchuk DA: Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. *Nat Genet.* 1996;13:189-95.
- Karapantzos I, Tsimpiris N, Goulis DG, Van Hoecke H, Van Cauwenberge P, Danielides V: Management of epistaxis in hereditary hemorrhagic telangiectasia by Nd:YAG laser and quality of life assessment using the HR-QoL questionnaire. *Eur Arch Otorhinolaryngol.* 2005;262:830-3.
- Kataoka M, Satoh T, Manabe T, Anzai T, Yoshikawa T, Mitamura H, Ogawa S: Oral sildenafil improves primary pulmonary hypertension refractory to epoprostenol. *Circ J.* 2005;69:461-5.
- Kawut SM, Horn EM, Berekashvili KK, Widlitz AC, Rosenzweig EB, Barst RJ: von Willebrand factor independently predicts long-term survival in patients with pulmonary arterial hypertension. *Chest.* 2005;128:2355-62.
- Kere J: Human population genetics: lessons from Finland. *Annu Rev Genomics Hum Genet.* 2001;2:103-28. Review.
- Kim H, Yung GL, Marsh JJ, Konopka RE, Pedersen CA, Chiles PG, Morris TA, Channik RN: Endothelin mediates pulmonary vascular remodelling in a canine model of chronic embolic pulmonary hypertension. *Eur Respir J.* 2000;15:640-8.
- Kjeldsen AD, Vase P, Green A: Hereditary haemorrhagic telangiectasia: a population-based study of prevalence and mortality in Danish patients. *J Intern Med.* 1999;245:31-9.
- Kjeldsen AD, Oxhøj H, Andersen PE, Green A, Vase P: Prevalence of pulmonary arteriovenous malformations (PAVMs) and occurrence of neurological symptoms in patients with hereditary haemorrhagic telangiectasia (HHT). *J Intern Med.* 2000;248:255-62.
- Kjeldsen AD, Kjeldsen J: Gastrointestinal bleeding in patients with hereditary hemorrhagic telangiectasia. *Am J Gastroenterol.* 2000;95:415-8.
- Kjeldsen AD, Møller TR, Brusgaard K, Vase P, Andersen PE: Clinical symptoms according to genotype amongst patients with hereditary haemorrhagic telangiectasia. *J Intern Med.* 2005;258:349-55.
- Kleiger RE, Boxer M, Ingham RE, Harrison DC. : Pulmonary hypertension in patients using oral contraceptives. A report of six cases. *Chest.* 1976;69:143-7.
- Koulumies R and Mustakallio M: Ayerza's Syndrome. *Annales Medicinae Internae Fenniae* 1952;41:70-78.
- Krause W, Kraus T: Pharmacokinetics and pharmacodynamics of the prostacyclin analogue iloprost in man. *Eur J Clin Pharmacol.* 1986;30:61-8.
- .Lahiri DK, Nurnberger JI Jr: A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. *Nucleic Acids Res.* 1991;19:5444.

- Lane KB, Machado RD, Pauciulo MW, Thomson JR, Phillips JA 3rd, Loyd JE, Nichols WC, Trembath RC: Heterozygous germline mutations in *BMPR2*, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. The International PPH Consortium. *Nat Genet.* 2000;26:81-4.
- Langleben D, Brock T, Dixon R, Barst R; STRIDE-1 study group: STRIDE 1: effects of the selective ET(A) receptor antagonist, sitaxsentan sodium, in a patient population with pulmonary arterial hypertension that meets traditional inclusion criteria of previous pulmonary arterial hypertension trials. *J Cardiovasc Pharmacol.* 2004;44 Suppl 1:S80-4.
- Layton KF, Kallmes DF, Gray LA, Cloft HJ: Endovascular treatment of epistaxis in patients with hereditary hemorrhagic telangiectasia. *AJNR Am J Neuroradiol.* 2007;28:885-8.
- Lerut J, Orlando G, Adam R, Sabbà C, Pfitzmann R, Klempnauer J, Belghiti J, Pirenne J, Thevenot T, Hillert C, Brown CM, Gonze D, Karam V, Boillot O; European Liver Transplant Association: Liver transplantation for hereditary hemorrhagic telangiectasia: Report of the European liver transplant registry. *Ann Surg.* 2006;244:854-62.
- Lesca G, Olivieri C, Burnichon N, Pagella F, Carette MF, Gilbert-Dussardier B, Goizet C, Roume J, Rabilloud M, Saurin JC, Cottin V, Honnorat J, Coulet F, Giraud S, Calender A, Danesino C, Buscarini E, Plauchu H; French-Italian-Rendu-Osler Network: Genotype-phenotype correlations in hereditary hemorrhagic telangiectasia: data from the French-Italian HHT network. *Genet Med.* 2007;9:14-22.
- Lesnik GT, Ross DA, Henderson KJ, Joe JK, Leder SB, White RI Jr: Septectomy and septal dermoplasty for the treatment of severe transfusion-dependent epistaxis in patients with hereditary hemorrhagic telangiectasia and septal perforation. *Am J Rhinol.* 2007;21:312-5.
- Letteboer TG, Mager JJ, Snijder RJ, Koeleman BP, Lindhout D, Ploos van Amstel JK, Westermann CJ: Genotype-phenotype relationship in hereditary haemorrhagic telangiectasia. *J Med Genet.* 2006;43:371-7.
- Lilienfeld DE, Rubin LJ.: Mortality from primary pulmonary hypertension in the United States, 1979-1996. *Chest.* 2000;117:796-800.
- Liu F, Ventura F, Doody J, Massagué J: Human type II receptor for bone morphogenic proteins (BMPs): extension of the two-kinase receptor model to the BMPs. *Mol Cell Biol.* 1995;15:3479-86.
- Loyd JE, Butler MG, Foroud TM, Conneally PM, Phillips JA 3rd, Newman JH: Genetic anticipation and abnormal gender ratio at birth in familial primary pulmonary hypertension. *Am J Respir Crit Care Med.* 1995;152:93-7.
- Lunze K, Gilbert N, Mebus S, Miera O, Fehske W, Uhlemann F, Mühler EG, Ewert P, Lange PE, Berger F, Schulze-Neick I: First experience with an oral combination therapy using bosentan and sildenafil for pulmonary arterial hypertension. *Eur J Clin Invest.* 2006;36 Suppl 3:32-8.

- Lux A, Gallione CJ, Marchuk DA: Expression analysis of endoglin missense and truncation mutations: insights into protein structure and disease mechanisms. *Hum Mol Genet.* 2000;9:745-55.
- Machado RD, Pauciulo MW, Fretwell N, Veal C, Thomson JR, Vilariño Güell C, Aldred M, Brannon CA, Trembath RC, Nichols WC: A physical and transcript map based upon refinement of the critical interval for PPH1, a gene for familial primary pulmonary hypertension. *The International PPH Consortium. Genomics.* 2000;68:220-8.
- Machado RD, Pauciulo MW, Thomson JR, Lane KB, Morgan NV, Wheeler L, Phillips JA 3rd, Newman J, Williams D, Galiè N, Manes A, McNeil K, Yacoub M, Mikhail G, Rogers P, Corris P, Humbert M, Donnai D, Martensson G, Tranebjaerg L, Loyd JE, Trembath RC, Nichols WC: BMPR2 haploinsufficiency as the inherited molecular mechanism for primary pulmonary hypertension. *Am J Hum Genet.* 2001;68:92-102.
- Machado RD, James V, Southwood M, Harrison RE, Atkinson C, Stewart S, Morrell NW, Trembath RC, Aldred MA: Investigation of second genetic hits at the BMPR2 locus as a modulator of disease progression in familial pulmonary arterial hypertension. *Circulation.* 2005;111:607-13.
- Maher CO, Piegras DG, Brown RD Jr, Friedman JA, Pollock BE: Cerebrovascular manifestations in 321 cases of hereditary hemorrhagic telangiectasia. *Stroke.* 2001;32:877-82.
- Mason NA, Springall DR, Burke M, Pollock J, Mikhail G, Yacoub MH, Polak JM: High expression of endothelial nitric oxide synthase in plexiform lesions of pulmonary hypertension. *J Pathol.* 1998;185:313-8.
- Massagué J, Chen YG: Controlling TGF-beta signaling. *Genes Dev.* 2000;14:627-44. Review.
- McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrell J, et al. Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. *Nat Genet.* 1994;8:345-51.
- McGoon MD, Vanhoutte PM: Aggregating platelets contract isolated canine pulmonary arteries by releasing 5-hydroxytryptamine. *J Clin Invest.* 1984 ;74:828-33.
- McLaughlin VV, Shillington A, Rich S.: Survival in pulmonary hypertension:the impact of epoprostenol therapy. *Circulation* 2002;106:1477-82.
- McLaughlin VV, Sitbon O, Badesch DB, Barst RJ, Black C, Galiè N, Rainiso M, Simonneau G, Rubin LJ: Survival with first-line bosentan in patients with primary pulmonary hypertension. *Eur Respir J* 2005;25:244-49.
- McLaughlin VV, Oudiz RJ, Frost A, Tapson VF, Murali S, Channick RN, Badesch DB, Barst RJ, Hsu HH, Rubin LJ: Randomized study of adding inhaled iloprost to existing bosentan in pulmonary arterial hypertension. *Am J Respir Crit Care Med.* 2006;174:1257-63.

- Minai OA, Rigelsky C, Eng C, Arroliga AC, Stoller JK: Long-term outcome in a patient with pulmonary hypertension and hereditary hemorrhagic telangiectasia. *Chest*. 2007;131:984-7.
- Mitchell A, Adams LA, MacQuillan G, Tibballs J, vanden Driesen R, Delriviere L: Bevacizumab reverses need for liver transplantation in hereditary hemorrhagic telangiectasia. *Liver Transpl*. 2008;14:210-3.
- Morse JH, Jones AC, Barst RJ, Hodge SE, Wilhelmsen KC, Nygaard TG: Mapping of familial primary pulmonary hypertension locus (PPH1) to chromosome 2q31-q32. *Circulation*. 1997;95:2603-6.
- Morse JH, Jones AC, Barst RJ, Hodge SE, Wilhelmsen KC, Nygaard TG: Familial primary pulmonary hypertension locus mapped to chromosome 2q31-q32. *Chest*. 1998;114(1 Suppl):57S-58S.
- Morse JH, Horn EM, Barst RJ: Hormone replacement therapy: a possible risk factor in carriers of familial primary pulmonary hypertension. *Chest*. 1999;116:847.
- Nakonechnicov S, Gabbasov Z, Chazova I, Popov E, Belenkov Y: Platelet aggregation in patients with primary pulmonary hypertension. *Blood Coagul Fibrinolysis*. 1996;7:225-7.
- Nall KC., Rubin LJ., Lipskind S., Sennesh JD: Reversible pulmonary hypertension associated with anorexigen use. *Am J Med*. 1991; 91:97-99.
- Newman JH, Wheeler L, Lane KB, Loyd E, Gaddipati R, Phillips JA 3rd, Loyd JE: Mutation in the gene for bone morphogenetic protein receptor II as a cause of primary pulmonary hypertension in a large kindred. *N Engl J Med*. 2001;345:319-24.
- Nichols WC, Koller DL, Slovis B, Foroud T, Terry VH, Arnold ND, Siemieniak DR, Wheeler L, Phillips JA 3rd, Newman JH, Conneally PM, Ginsburg D, Loyd JE: Localization of the gene for familial primary pulmonary hypertension to chromosome 2q31-32. *Nat Genet*. 1997;15:277-80.
- Nohe A, Hassel S, Ehrlich M, Neubauer F, Sebald W, Henis YI, Knaus P: The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. *J Biol Chem*. 2002;277:5330-8.
- Nootens M, Kaufmann E, Rector T, Toher C, Judd D, Francis GS, Rich S: Neurohormonal activation in patients with right ventricular failure from pulmonary hypertension: relation to hemodynamic variables and endothelin levels. *J Am Coll Cardiol*. 1995;26:1581-5.
- Norio R, Nevanlinna HR, Perheentupa J: Hereditary diseases in Finland; rare flora in rare soul. *Ann Clin Res*. 1973 Jun;5(3):109-41. Review.
- Oh SP, Seki T, Goss KA, Imamura T, Yi Y, Donahoe PK, Li L, Miyazono K, ten Dijke P, Kim S, Li E: Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proc Natl Acad Sci U S A*. 2000;97:2626-31.

- Okano Y, Yoshioka T, Shimouchi A, Satoh T, Kunieda T: Orally active prostacyclin analogue in primary pulmonary hypertension. *Lancet* 1997; 349:1365.
- Olschewski H, Walmrath D, Schermuly R, Ghofrani A, Grimminger F, Seeger W: Aerosolized prostacyclin and iloprost in severe pulmonary hypertension. *Ann Intern Med.* 1996;124:820-4.
- Olschewski H, Ghofrani A, Schmehl T, Winkler J, Wilkens H, Höper MM, Behr J, Kleber F-X., Seeger W., for the German PPH Study Group: Inhaled iloprost to treat severe pulmonary hypertension. An uncontrolled trial. *Ann Intern Med* 2000; 132:435-443.
- Orlando G, Sabbà C, De Liguori Carino N, Scelzo C, Tariciotti L, Bonanni L, D'Angelo M, Baiocchi L, Lerut J, Tisone G: Non-transplant surgical approach to liver-based hereditary haemorrhagic telangiectasia: a first report. *Liver Int.* 2008;28:574-7.
- Pagella F, Semino L, Olivieri C, Corno S, Dore R, Draghi F, Lanzarini L, Vespro V, Buscarini E, Danesino C: Treatment of epistaxis in hereditary hemorrhagic telangiectasia patients by argon plasma coagulation with local anesthesia. *Am J Rhinol.* 2006;20:421-5.
- Paul GA, Gibbs JS, Boobis AR, Abbas A, Wilkins MR: Bosentan decreases the plasma concentration of sildenafil when coprescribed in pulmonary hypertension. *Br J Clin Pharmacol.* 2005;60:107-12.
- Pece N, Vera S, Cymerman U, White RI Jr, Wrana JL, Letarte M: Mutant endoglin in hereditary hemorrhagic telangiectasia type 1 is transiently expressed intracellularly and is not a dominant negative. *J Clin Invest.* 1997;100:2568-79.
- Pece-Barbara N, Cymerman U, Vera S, Marchuk DA, Letarte M: Expression analysis of four endoglin missense mutations suggests that haploinsufficiency is the predominant mechanism for hereditary hemorrhagic telangiectasia type 1. *Hum Mol Genet.* 1999;8:2171-81.
- Peltonen L, Jalanko A, Varilo T: Molecular genetics of the Finnish disease heritage. *Hum Mol Genet.* 1999;8:1913-23. Review.
- Peltonen L, Palotie A, Lange K: Use of population isolates for mapping complex traits. *Nat Rev Genet.* 2000;1:182-90.Review.
- Petiprez P, Brenot F, Azarian R, Parent F, Rain B, Herve P, Simonneau G: Pulmonary hypertension in patients with human immunodeficiency virus infection. Comparison with primary pulmonary hypertension. *Circulation* 1994; 89:2722-2727.
- Pietra GG, Edwards WD, Kay JM, Rich S, Kernis J, Schloo B, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, et al: Histopathology of primary pulmonary hypertension. A qualitative and quantitative study of pulmonary blood vessels from 58 patients in the National Heart, Lung, and Blood Institute, Primary Pulmonary Hypertension Registry. *Circulation.* 1989;80:1198-206. (tarkista juttu ja kaikki kirjoittajat)

- Piippo K, Swan H, Pasternack M, Chapman H, Paavonen K, Viitasalo M, Toivonen L, Kontula K: A founder mutation of the potassium channel KCNQ1 in long QT syndrome: implications for estimation of disease prevalence and molecular diagnostics. *J Am Coll Cardiol.* 2001;37:562-8.
- Pizzo CJ: Type I glycogen storage disease with focal nodular hyperplasia of the liver and vasoconstrictive pulmonary hypertension. *Pediatrics.* 1980;65:341-3.
- Plauchu H, de Chadarevian JP, Bideau A, Robert JM: Age-related clinical profile of hereditary hemorrhagic telangiectasia in an epidemiologically recruited population. *Am J Med Genet.* 1989;32:291-7.
- Porteous ME, Burn J, Proctor SJ: Hereditary haemorrhagic telangiectasia: a clinical analysis. *J Med Genet.* 1992;29:527-30.
- Puolikoki HJ, Niemelä KO, Siitonen LO: Unexplained severe pulmonary hypertension in two brothers. *Eur respir J.* 1990;3:349-353.
- Rich S, Danzker DR, Ayres SM, Bergofsky EH., Brundage BH., Detre KM., Fishman AP., Goldring RM., Groves BM., Koerner SK et al.: Primary pulmonary hypertension. A national prospective study. *Ann Intern Med* 1987;107:216-23
- Rich S., Hart K: Familial Pulmonary hypertension in association with an abnormal hemoglobin. Insights into the pathogenesis of primary pulmonary hypertension. *Chest* 1991;99:1208-10.
- Rich S, Kaufmann E, Levy PS: The effect of high doses of calcium-channel blockers on survival in primary pulmonary hypertension. *N Engl J Med* 1992; 327:76-81.
- Rich S, Rubin L, Walker AM, Schneeweiss S, Abenhaim L: Anorexigens and pulmonary hypertension in the United States. Results from the surveillance of the North American pulmonary hypertension. *Chest.* 2000; 117:870-874.
- Rindermann M, Grünig E, von Hippel A, Koehler R, Miltenberger-Miltenyi G, Mereles D, Arnold K, Pauciulo M, Nichols W, Olschewski H, Hoepfer MM, Winkler J, Katus HA, Kübler W, Bartram CR, Janssen B: Primary pulmonary hypertension may be a heterogeneous disease with a second locus on chromosome 2q31. *J Am Coll Cardiol.* 2003;41:2237-44.
- Roberts KE, McElroy JJ, Wong WP, Yen E, Widlitz A, Barst RJ, Knowles JA, Morse JH: BMPR2 mutations in pulmonary arterial hypertension with congenital heart disease. *Eur Respir J.* 2004;24:371-4.
- Robledo MM, Hidalgo A, Lastres P, Arroyo AG, Bernabeu C, Sánchez-Madrid F, Teixidó J: Characterization of TGF-beta 1-binding proteins in human bone marrow stromal cells. *Br J Haematol.* 1996;93:507-14.

- Rokhlin OW, Cohen MB, Kubagawa H, Letarte M, Cooper MD: Differential expression of endoglin on fetal and adult hematopoietic cells in human bone marrow. *J Immunol.* 1995;154:4456-65.
- Rosenzweig BL, Imamura T, Okadome T, Cox GN, Yamashita H, ten Dijke P, Heldin CH, Miyazono K: Cloning and characterization of a human type II receptor for bone morphogenetic proteins. *Proc Natl Acad Sci U S A.* 1995;92:7632-6.
- Rubens C, Ewert R, Halank M, Wensel R, Orzechowski HD, Schultheiss HP, Hoeffken G: Big endothelin-1 and endothelin-1 plasma levels are correlated with the severity of primary pulmonary hypertension. *Chest.* 2001;120:1562-9.
- Rubin LJ, Badesch DB, Barst RJ, Galie N, Black CM, Keogh A, Pulido T, Frost A, Roux S, Leconte I, Landzberg M, Simonneau G: Bosentan therapy for pulmonary arterial hypertension. *N Engl J Med.* 2002;346:896-903. Erratum in: *N Engl J Med* 2002;346:1258.
- Rudarakanchana N, Flanagan JA, Chen H, Upton PD, Machado R, Patel D, Trembath RC, Morrell NW: Functional analysis of bone morphogenetic protein type II receptor mutations underlying primary pulmonary hypertension. *Hum Mol Genet.* 2002 Jun 15;11(13):1517-25.
- Runo JR, Vnencak-Jones CL, Prince M, Loyd JE, Wheeler L, Robbins IM, Lane KB, Newman JH, Johnson J, Nichols WC, Phillips JA 3rd: Pulmonary veno-occlusive disease caused by an inherited mutation in bone morphogenetic protein receptor II. *Am J Respir Crit Care Med.* 2003;167:889-94.
- Röijer E, Miyazono K, Aström AK, Geurts van Kessel A, ten Dijke P, Stenman G: Chromosomal localization of three human genes encoding members of the TGF-beta superfamily of type I serine/threonine kinase receptors. *Mamm Genome.* 1998;9:266-8.
- Sabbà C, Pasculli G, Suppressa P, D'Ovidio F, Lenato GM, Resta F, Assennato G, Guanti G: Life expectancy in patients with hereditary haemorrhagic telangiectasia. *QJM.* 2006;99:327-34.
- Sabbà C, Pasculli G, Lenato GM, Suppressa P, Lastella P, Memeo M, Dicuonzo F, Guant G: Hereditary hemorrhagic telangiectasia: clinical features in ENG and ALK1 mutation carriers. *J Thromb Haemost.* 2007;5:1149-57.
- Sadick H, Bergler WF, Oulmi-Kagermann J, Naim R, Sadick M, Hörmann K, Riedel F: Estriol induced squamous metaplasia on the nasal mucosa in patients with hereditary hemorrhagic telangiectasia. *Arch Med Res.* 2005;36:468-73.
- Sadick H, Sadick M, Götte K, Naim R, Riedel F, Bran G, Hörmann K: Hereditary hemorrhagic telangiectasia: an update on clinical manifestations and diagnostic measures. *Wien Klin Wochenschr.* 2006;118:72-80. Review.
- Saksela N, Helske E, Saxén E: Hereditary hemorrhagic telangiectasia and its pathologic anatomy. *Annales Medicinae Internae Fenniae.* 1959;38:132-146.

- Saksela N, Helske E: Oslerin taudista. *Duodecim*. 1949; 69:363-375.
- Sandoval J, Bauerle O, Gomez A, Palomar A, Martinez Guerra ML, Furuya ME: Primary pulmonary hypertension in Children: Clinical characterization and survival. *J Am Coll Cardiol* 1995; 25:466-74.
- Sandoval J, Gaspar J, Pulido T, Bautista E, Martínez-Guerra ML, Zeballos M, Palomar A, Gómez A: Graded balloon dilation atrial septostomy in severe primary pulmonary hypertension. A therapeutic alternative for patients nonresponsive to vasodilator treatment. *J Am Coll Cardiol*. 1998;32:297-304.
- Sauvageau S, Thorin E, Caron A, Dupuis J: Endothelin-1-induced pulmonary vasoreactivity is regulated by ET(A) and ET(B) receptor interactions. *J Vasc Res*. 2007;44:375-81.
- Sawabe M, Arai T, Esaki Y, Tsuru M, Fukazawa T, Takubo K: Three-dimensional organization of the hepatic microvasculature in hereditary hemorrhagic telangiectasia. *Arch Pathol Lab Med*. 2001;125:1219-23.
- Seki T, Yun J, Oh SP: Arterial endothelium-specific activin receptor-like kinase 1 expression suggests its role in arterialization and vascular remodeling. *Circ Res*. 2003;93:682-9.
- Shovlin CL, Guttmacher AE, Buscarini E, Faughnan ME, Hyland RH, Westermann CJ, Kjeldsen AD, Plauchu H: Diagnostic criteria for hereditary hemorrhagic telangiectasia (Rendu-Osler-Weber syndrome). *Am J Med Genet* 2000;91:66-7.
- Simonneau G, Fartoukh M, Sitbon O, Humbert M, Jagot J-L, Herve P: Primary pulmonary hypertension associated with the use of fenfluramine derivatives. *Chest* 1998; 114:195S-199S.
- Simonneau G, Galiè N, Rubin LJ, Langleben D, Seeger W, Domenighetti G, Gibbs S, Lebrec D, Speich R, Beghetti M, Rich S, Fishman A: Clinical classification of pulmonary hypertension. *J Am Coll Cardiol*. 2004;43(12 Suppl S):5S-12S. Review.
- Sipilä W, Taipale E, Heikel PE, Kyllönen KEJ, Larmi TKI: Itsenäisestä paineen kohoamisesta pienessä verenkielrossa (essentiellistä pulmonaalihypertensiosta). *Duodecim* 1953;69:631-643.
- Sitbon O, Humbert M, Simonneau G: Primary pulmonary hypertension: Current therapy. *Prog Cardiovasc Dis*. 2002;45:115-28. Review.
- Sitbon O, Lascoux-Combe C, Delfraissy J-F, Yeni PG, Raffi F, De Zuttere D, Gressin V, Clerson P, Sereni D, Simonneau G: Prevalence of HIV-related pulmonary arterial hypertension in the current antiretroviral therapy era. *Am J Respir Crit Care Med* 2008; 177:108-113.
- Souza R, Humbert M, Sztrymf B, Jaïs X, Yaïci A, Le Pavec J, Parent F, Hervé P, Soubrier F, Sitbon O, Simonneau G: Pulmonary arterial hypertension associated with fenfluramine exposure: report of 109 cases. *Eur Respir J*. 2008;31:343-8. Erratum in: *Eur Respir J*. 2008;31:912.

- Speich R, Jenni R, Opravil M, Pfab M, Russi EW: Primary pulmonary hypertension in HIV infection. *Chest*. 1991;100:1268-71.
- Stewart DJ, Levy RD, Cernacek P, Langleben D: Increased plasma endothelin-1 in pulmonary hypertension: marker or mediator of disease? *Ann Intern Med*. 1991;114:464-9.
- Sweet K, Willis J, Zhou XP, Gallione C, Sawada T, Alhopuro P, Khoo SK, Patocs A, Martin C, Bridgeman S, Heinz J, Pilarski R, Lehtonen R, Prior TW, Frebourg T, Teh BT, Marchuk DA, Aaltonen LA, Eng C: Molecular classification of patients with unexplained hamartomatous and hyperplastic polyposis. *JAMA*. 2005;294:2465-73.
- Tada Y, Majka S, Carr M, Harral J, Crona D, Kuriyama T, West J: Molecular effects of loss of BMPR2 signaling in smooth muscle in a transgenic mouse model of PAH. *Am J Physiol Lung Cell Mol Physiol*. 2007;292:L1556-63.
- ten Dijke P, Ichijo H, Franzén P, Schulz P, Saras J, Toyoshima H, Heldin CH, Miyazono K: Activin receptor-like kinases: a novel subclass of cell-surface receptors with predicted serine/threonine kinase activity. *Oncogene*. 1993;8:2879-87.
- Thenappan T, Shah SJ, Rich S, Gomberg-Maitland M: A USA-based registry for pulmonary hypertension: 1982-2006. *Eur Respir J* 2007;30:1103-1110.
- Thomson JR, Machado RD, Pauciulo MW, Morgan NV, Humbert M, Elliott GC, Ward K, Yacoub M, Mikhail G, Rogers P, Newman J, Wheeler L, Higenbottam T, Gibbs JS, Egan , Crozier A, Peacock A, Allcock R, Corris P, Loyd JE, Trembath RC, Nichols WC: Sporadic primary pulmonary hypertension is associated with germline mutations of the gene encoding BMPR-II, a receptor member of the TGF-beta family. *J Med Genet*. 2000;37:741-5.
- Toivonen HT, Onkamo P, Vasko K, Ollikainen V, Sevon P, Mannila H, Herr M, Kere J: Data mining applied to linkage disequilibrium mapping. *Am J Hum Genet*. 2000;67:133-45.
- Trembath RC, Thomson JR, Machado RD, Morgan NV, Atkinson C, Winship I, Simonneau G, Galie N, Loyd JE, Humbert M, Nichols WC, Morrell NW, Berg J, Manes A, McGaughran J, Pauciulo M, Wheeler L: Clinical and molecular genetic features of pulmonary hypertension in patients with hereditary hemorrhagic telangiectasia. *N Engl J Med*. 2001;345:325-34.
- Trembath RC, Harrison R: Insights into the genetic and molecular basis of primary pulmonary hypertension. *Pediatr Res*. 2003;53:883-8.
- Tuder RM, Cool CD, Geraci MW, Wang J, Abman SH, Wright L, Badesch D, Voelkel NF: Prostacyclin synthase expression is decreased in lungs from patients with severe pulmonary hypertension. *Am J Respir Crit Care Med*. 1999;159:1925-32.
- Vachharajani A, Saunders S: Allelic variation in the serotonin transporter (5HTT) gene contributes to idiopathic pulmonary hypertension in children. *Biochem Biophys Res Commun*. 2005;334:376-9.

- Wang C, Wang J, Zhao L, Wang Y, Liu J, Shi L, Xu M, Wang C: Sildenafil inhibits human pulmonary artery smooth muscle cell proliferation by decreasing capacitative Ca^{2+} entry. *J Pharmacol Sci.* 2008;108:71-78.
- Varilo T, Laan M, Hovatta I, Wiebe V, Terwilliger JD, Peltonen L.: Linkage disequilibrium in isolated populations: Finland and a young sub-population of Kuusamo. *Eur J Hum Genet.* 2000;8:604-12.
- Werner A, Bäuml W, Zietz S, Kühnel T, Hohenleutner U, Landthaler M: Hereditary haemorrhagic telangiectasia treated by pulsed neodymium:yttrium-aluminium-garnet (Nd:YAG) laser (1,064 nm). *Lasers Med Sci.* 2008;23:385-91.
- West J, Fagan K, Steudel W, Fouty B, Lane K, Harral J, Hoedt-Miller M, Tada Y, Ozimek J, Tudor R, Rodman DM: Pulmonary hypertension in transgenic mice expressing a dominant-negative BMPRII gene in smooth muscle. *Circ Res.* 2004;94:1109-14.
- Westermann CJ, Rosina AF, De Vries V, de Coteau PA: The prevalence and manifestations of hereditary hemorrhagic telangiectasia in the Afro-Caribbean population of the Netherlands Antilles: a family screening. *Am J Med Genet A.* 2003;116A:324-8.
- Wharton J, Strange JW, Møller GMO, Growcott EJ, Ren X, Franklyn AP, Phillips SC, Wilkins MR: Antiproliferative effects of phosphodiesterase type 5 inhibition in human pulmonary artery cells. *Am J Respir Crit Care Med.* 2005;172:105-113.
- Whyte RI, Robbins RC, Altinger J, Barlow CW, Doyle R, Theodore J, Reitz BA: Heart-lung transplantation for primary pulmonary hypertension. *Ann Thorac Surg.* 1999;67:937-41.
- Wilenius R: Ett fall av angiomatosis hereditaria haemorrhagica (Osler). *Finska läkaresällskapets handlingar.* 1935;77:291-296.
- Wille RT, Krishnan K, Cooney KA, Bach DS, Martinez F: Familial association of primary pulmonary hypertension and a new low-oxygen affinity β -chain hemoglobinopathy, Hb-Washtenaw. *Chest* 1996; 109:848-50.
- Willemsse RB, Mager JJ, Westermann CJ, Overtoom TT, Mauser H, Wolbers JG: Bleeding risk of cerebrovascular malformations in hereditary hemorrhagic telangiectasia. *J Neurosurg.* 2000;92:779-84.
- Voelker NF, Tudor RM: Cellular and molecular mechanisms in the pathogenesis of severe pulmonary hypertension. *Eur Respir J.* 1995;8:2129-2138.
- Wolf M, Boyer-Neumann C, Parent F, Eschwege V, Jaillet H, Meyer D, Simonneau G: Thrombotic risk factors in pulmonary hypertension. *Eur Respir J.* 2000;15:395-9.
- Woswinckel R, Enke B, Reichenberger F, Kohstall M, Kreckel A, Krick S, Gall H., Gessler T, Schmehl T, Ghofrani HA, Schermuly RT, Grimminger F, Rubin LJ, Seeger W, Olschewski H: Favorable effects of inhaled treprostinol in severe pulmonary hypertension. Results from randomized controlled pilot studies. *JACC* 2006; 48:1672-81.

- Vuorio AF, Turtola H, Piilahti KM, Repo P, Kanninen T, Kontula K.: Familial hypercholesterolemia in the Finnish north Karelia. A molecular, clinical, and genealogical study. *Arterioscler Thromb Vasc Biol.* 1997;17:3127-38.
- Yeager ME, Halley GR, Golpon HA, Voelkel NF, Tudor RM: Microsatellite instability of endothelial cell growth and apoptosis genes within plexiform lesions in primary pulmonary hypertension. *Circ Res.* 2001;88:E2-E11.
- Young KA, Ivester C, West J, Carr M, Rodman DM: BMP signaling controls PASMC KV channel expression in vitro and in vivo. *Am J Physiol Lung Cell Mol Physiol.* 2006;290:L841-8.
- Yuan JX, Aldinger AM, Juhaszova M, Wang J, Conte JV Jr, Gaine SP, Orens JB, Rubin LJ: Dysfunctional voltage-gated K⁺ channels in pulmonary artery smooth muscle cells of patients with primary pulmonary hypertension. *Circulation.* 1998;98:1400-6.
- Zhou XP, Woodford-Richens K, Lehtonen R, Kurose K, Aldred M, Hampel H, Launonen V, Virta S, Pilarski R, Salovaara R, Bodmer WF, Conrad BA, Dunlop M, Hodgson SV, Iwama T, Järvinen H, Kellokumpu I, Kim JC, Leggett B, Markie D, Mecklin JP, Neale K, Phillips R, Piris J, Rozen P, Houlston RS, Aaltonen LA, Tomlinson IP, Eng C: Germline mutations in BMPRIA/ALK3 cause a subset of cases of juvenile polyposis syndrome and of Cowden and Bannayan-Riley-Ruvalcaba syndromes. *Am J Hum Genet.* 2001;69:704-11.
- Åström AK, Jin D, Imamura T, Röijer E, Rosenzweig B, Miyazono K, ten Dijke P, Stenman G: Chromosomal localization of three human genes encoding bone morphogenetic protein receptors. *Mamm Genome.* 1999;10:299-302.