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GENETIC AND EPIDEMIOLOGICAL STUDIES OF INFANTILE HYPERTROPHIC PYLORIC STENOSIS

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To my family and friends

ABSTRACT

Infantile Hypertrophic Pyloric Stenosis (IHPS) is a condition of early infancy characterized by thickening of the pyloric muscle resulting in obstruction of gastric outflow. It affects 1-3/1000 live births and is one of the most common causes of gastrointestinal obstruction in infants. IHPS is developed under influence of both genetic and environmental factors, but the exact pathogenesis is unknown. The aim of this thesis was to elucidate genetic and environmental factors contributing to development of IHPS.

In study *I* and *II* we used a candidate gene approach to study association of two different genes with potential involvement in pathogenesis of IHPS. Since treatment with the motilin agonist erythromycin gives an increased risk for IHPS, we proposed the GI-hormon motilin as an IHPS candidate gene (study *I*). Sequencing of the *MLN* coding exons revealed three previously not reported sequence variants occurring in both cases and controls, though without obvious association to the disease. In addition, no significant association was found between the rs2281820 (p.Val15Ala) polymorphism and IHPS. Neuronal nitric oxide synthase encoded of *NOS1* is key player in the pathway mediating pyloric relaxation, and is suggested as a IHPS susceptibility gene. A functional *NOS1C* promoter polymorphism (c.-84G>A; rs41279104) regulating expression of the gene was genotyped in cases and controls (study *II*). We could not confirm a previously reported association of the A-allele with IHPS in our material.

In *study III* we used an unbiased strategy, genome wide linkage analysis, to search for chromosomal regions harboring candidate genes. 37 Swedish IHPS families were analysed in an initial genome wide scan revealing seven regions of interest for fine mapping. Fine mapping using a statistical model with equal weight to all families identified significant linkage to 2q24.3, and suggestive linkage to 7p22.2 and 12q24.23. The statistical model using a weighting scheme to compensate for different pedigree structures identified significant linkage to 2q24.3 and 7p21.3, and suggestive linkage to 6p21.31. Interestingly, these candidate regions harbor the previously suggested candidate genes *MLN* and *NOS1*. Two novel proposed candidate genes, *NPY* and *GCG*, were sequenced without obvious pathological findings. Extending the material with additional 31 British IHPS families in the fine mapped regions did not enhance the NPL score.

In *study IV* we performed a nationwide register study based on all IHPS cases in Sweden 1973-2008, yielding a study cohort of 3608 cases and 17588 matched controls. Incidence fell from 2.25 to 0.43/1000 between years 1988-1998 with a relatively constant sex ratio of 5:1 boys to girls. Study variables associated with an increased risk for IHPS in our material were caesarian delivery, prematurity, neonatal jaundice and being first born. Maternal smoking, low maternal age and Nordic ethnicity were also associated with increased IHPS risk. Maternal smoking and low birth rank were stronger risk factors during period with low incidence. Prematurity was a stronger risk factor when analysis was stratified for late onset of disease.

In summary we have identified candidate gene regions on chromosome 2, 6, 7 and 12 and identified several epidemiological risk factors for IHPS.

LIST OF PUBLICATIONS

This thesis is based on the following four publications, which will be referred to in the text by their Roman numerals (*I-IV*).

I. Svenningsson A, Lagerstedt K, Omrani MD, Nordenskjöld A.
    Absence of motilin gene mutations in infantile hypertrophic pyloric stenosis.
    J Pediatr Surg. 2008 Mar;43(3):443-6

II. Lagerstedt-Robinson K, Svenningsson A, Nordenskjöld A.
    No association between a promoter NOS1 polymorphism (rs41279104) and Infantile Hypertrophic Pyloric Stenosis.
    J Hum Genet. 2009 Dec;54(12):706-8

III. Svenningsson A, Söderhäll C, Persson S, Luthman H, Chung E, Gardiner M, Kockum I, Nordenskjöld A.
    Genome-wide linkage analysis in families with Infantile Hypertrophic Pyloric Stenosis indicates novel susceptibility loci.
    Submitted

IV. Svenningsson A, Svensson T, Akre O, Nordenskjöld A.
    Changes in risk factors after decreased incidence of IHPS.
    Submitted

CONTENTS

LIST OF ABBREVIATIONS

ARPs	Affected relative pairs
BH4	Tetrahydrobiopterin
BMI	Body mass index
CNS	Central nervous system
CI	Confidence interval
cM	centiMorgan
DNA	Deoxyribonucleic acid
DZ	Dizygotic
ENS	Enteric nervous system
GI	Gastrointestinal
IBD	Identical by descent
IBS	Identical by state
IHPS	Infantile hypertrophic pyloric stenosis
mRNA	Messenger RNA
MZ	Monozygotic
nNOS	Neuronal nitric oxide synthase
NPL	Non parametric linkage
NPR	The Swedish national patient register
NO	Nitric oxide
OR	Odds ratio
PAH	Phenylalanine hydroxylase
PCR	Polymerase chain reaction
PKU	Phenylketonuria
SIDS	Sudden infant death syndrome
SLOS	Smith-Lemli-Opitz syndrome
SNP	Single nucleotide polymorphism

1   INTRODUCTION

The pyloric muscle is a sphincter defining the transition between the stomach and duodenum. The term pylorus means "gatekeeper" formed by the greek words *pyle* (gate) and *ourus* (guard), giving a good description of the pylorus two main functions: control the outflow from the stomach preventing passage of large pieces of food to the duodenum, and to prevent backflow of intestinal content to the stomach.
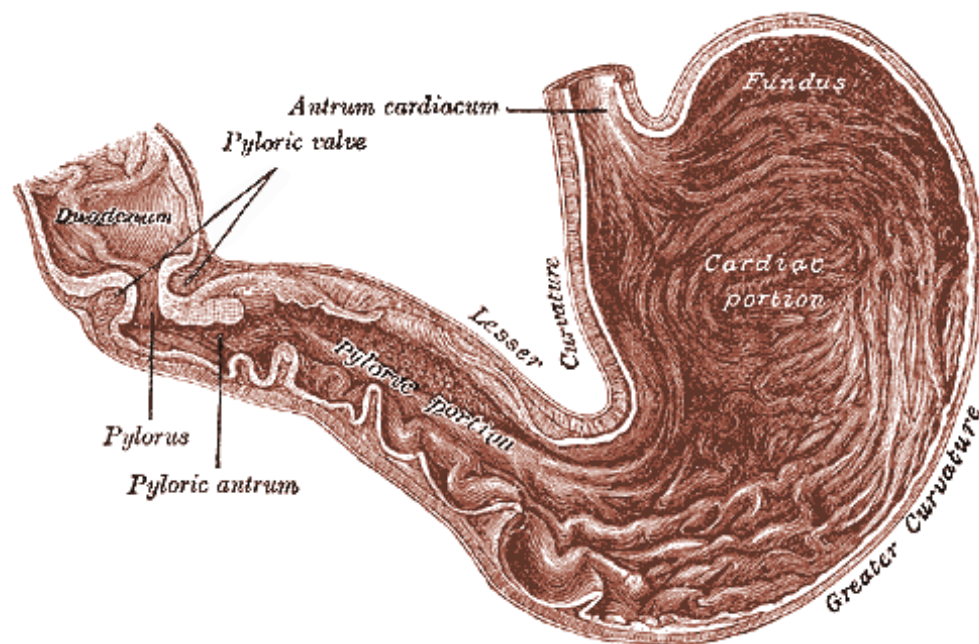
Figure 1. Sagittal section illustrating the relationship between stomach, pylorus and duodenum. *Gray's anatomy of the Human Body, 1918*

The pyloric sphincter consists of two smooth muscle layers, a thin outer longitudinal layer and a thicker inner circular layer exerting the sphincter function (Ramkumar and Schulze 2005). Its activity is controlled by a complex mechanism including both neuronal and hormonal pathways (Daniel et al 1994, Fisher et al. 1973, Shafik et al 2007).

Infantile hypertrophic pyloric stenosis (IHPS) is a paediatric condition, in which the pylorus becomes abnormally thickened caused by pyloric muscle hypertrophy as shown in Figure 2. Histological studies of IHPS samples describe true muscle hypertrophy mainly of the circular layer with little or no hyperplasia (Tam 1985, Abel et al. 1998). The disease occurs only during a limited age span, with onset ranging from

one to approximately twelve weeks of age. The thickening of the pyloric sphincter causes a nearly complete obstruction of the gastric outlet, which prevents gastric emptying. Consequently, the affected infant presents with projectile vomiting after feeding which untreated can cause severe illness within a few days. In the past IHPS often had a lethal outcome, but today mortality is negligible. The surgical procedure relieving the pyloric obstruction, Ramstedt's pyloromyotomy presented in 1912, has been described as one of the greatest advances in pediatric surgical history since it has saved so many lives.
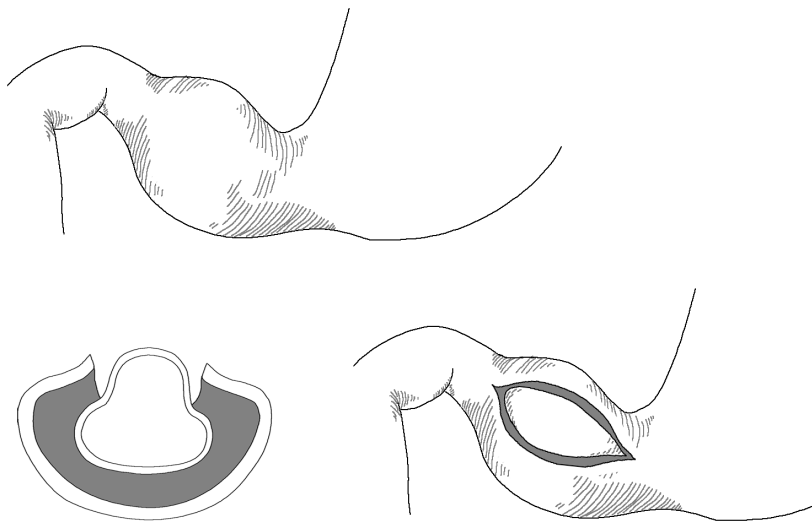
Figure 2. Schematic illustration of pyloric hypertrophy before and after Ramstedt's pyloromyotomy. The hypertrophic pyloric muscle is incised down to the mucosa, which bulges out through the muscular incision relieving the obstruction.

The incidence of IHPS varies between populations, and is most common in Caucasians with a frequency of to 1.5-3 per 1000 births (Persson et al 2001, Mitchell and Risch 1993). Interestingly, there is a striking predominance of affected boys with a sex ratio of 4-5 affected boys per one girl, giving an incidence of 1 per 2-300 male births and 1 per 1-2000 female births (Rimoin et al 2007, Krogh 2010). A trend of decreasing incidence is reported from several European countries including Sweden during the last decades (Persson et al 2001, Hedbäck et al 2001, Sommerfield 2008, Pedersen et al 2008). However, IHPS is still one of the most common causes of gastrointestinal obstruction in infants and one of the most common diagnoses requiring surgery during the first months of life.

Although IHPS is sometime referred to as a congenital disease, it has been shown that the pyloric muscle is normal at birth in infants later presenting with the disease

(Wallgren 1946, Rollins et al 1989). An interesting aspect is that the pyloric hypertrophy, and subsequently the symptoms, spontaneously resolves after a few weeks to months if the infant meanwhile can be cared for and kept alive with conservative treatment (Tallerman 1951, Swift and Prossor 1991).

The underlying mechanism causing the disease is still unknown, but a multifactorial background is well established where a combined influence of genetic and environmental factors contributes to the disease (Mitchell and Risch 1993, Chakraborty 1986). Studying both the molecular background and the environmental factors involved, can give a better understanding of the pathophysiology and possibly also lead to identification of environmental risk factors which both can be targets for preventive measures.

HISTORICAL BACKGROUND

The first clinical report of a probable case of IHPS was published in 1627 by Hildanus, who described an infant suffering from "spastic vomiting". Anatomical descriptions of pyloric hypertrophy can be found back in the early 1700s in a publication by Blair who in 1717 reported the clinical history and autopsy record of a case of pyloric stenosis (Hernanz-Schulman 2003, Kellett 1933). In 1788 Beardsley wrote the report "A case of scirrus in the pylorus of an infant", which was the first American publication describing the disease (Caulfield 1930). However, it was not until 100 years later IHPS became a clinical entity that attracted scientific attention. This after the Danish pediatrician Hirschsprung reported two patients with convincing clinical and pathological features of the disease in his seminal article in 1888 (Hirschsprung 1888, Hernanz-Schulman 2003).

In the beginning of the 1900s, IHPS was "more or less equal to a death sentence" and mortality rates exceeded 50% well into the 1920s (Carter 1961). Efforts were made to keep the infants alive by frequent small feedings until the pyloric muscle hypertrophy, and so the symptoms, spontaneously resolved after a few weeks to several months. Yet, the surviving children often suffered from consequences of the severe malnutrition associated with the disease (Klein and Forbes 1975). Oral atropine treatment with purpose to relieve the pyloric spasm was to some extent successful (Svensgaard 1935). However, it was the introduction of pyloromyotomy, together with improvements in anaesthetic technique and perioperative care, that significant improved the prognosis of survival and led to the decreased rate of mortality (Carter 1961).

The first report describing an attempt to operate IHPS dates back to 1892 when Cordua performed a jejunostomy in a patient with IHPS that unfortunately did not survive (Cleon and Nafe 1947). In 1898 Meyer successfully introduced gastro-enterostomy as an IHPS treatment (Solowiejczyk et al 1980). It became a quite successful strategy and the most frequent used procedure until the early 1900s, even though it was associated with over 50% mortality (Taylor 1959). The gastro-enterostomy era was followed by a less extensive procedure, pyloroplasty that was introduced in 1903 by Dent. The pylorus was then incised longitudinally down through the muscle and mucosa, and sutured transversally (Taylor 1959). Burghard in 1906 introduced a different technique by using Hegar's dilators introduced through an incision in the stomach to induce dilation, or rather divulsion, of the pyloric muscle from the inside (Taylor 1959). In 1907 Fredet performed the first extra-mucous pyloroplasty, but still suturing the muscle layer transversely as in the original pyloroplasty. Meanwhile and independently from Fredet, an almost identical technique was developed in 1908 by Weber. The Fredet-Weber procedure was modified by Conrad Ramstedt in 1911, partly by coincidence, when he left the pyloric muscle unclosed since the sutures cut through the muscle edges. In 1912 his method "Ramstedt pyloromyotomy" was published (Ramstedt 1912), and is still today used as the golden standard procedure and treatment of choice.

CLINICAL ASPECTS

IHPS usually presents at 2-8 weeks of age with intense, projectile nonbilious vomiting as dominating symptom. Onset beyond three months of age is rare, and the disease virtually does not exist after 1 year of age. Since the affected infant only can pass very limited amounts of fluid to the intestine for absorption, dehydration and weight loss are rapidly encountered. The combination of dehydration and loss of gastric juice promptly results in disturbed electrolyte balance and metabolic acidosis. A few percent of the patients develops jaundice due to adverse effect of starvation on hepatic glucuronyl transferase activity, which resolves after surgery (O'Neill et al 2004). If left untreated, the infant can become severe ill within only a few days.

The typical history of sudden onset of projectile, nonbilious vomiting in an infant within the age span of one to a few months, gives a strong clinical suspicion of IHPS. Occasionally, the hypertrophic pyloric muscle can be palpated as an "olive " when the infant is examined. In the past, this was a reliable diagnostic sign occurring in 70-90% of the cases (Pollock et al 1957, Scharli et al 1969, Grimes et al 1950, Hulka et al

1997). Today, only 20% of cases exhibit a palpable pyloric tumor and diagnostics rely more on radiologic investigation (Hulka 1997). It has been suggested the decreased frequency of palpable finding is due to earlier admission and thus a healthier and less emaciated infant (Hulka et al 1997). The standard diagnostic method is abdominal ultrasound, which can visualize the thick and elongated pyloric muscle. If ultrasound is inconclusive, an upper gastrointestinal study with contrast can be diagnostic.

The preoperative care consists of intravenous rehydration and correction of electrolyte disturbances to get the infant in condition for surgery. Typically the infant can be operated on within one day after admission. The standard procedure is Ramstedt's pyloromyotomy in which the pyloric muscle is incised down to the mucosa, and then left unclosed. This immediately relieves the obstructed gastric outflow and allows passage of stomach content to the duodenum. The pyloromyotomy can be performed either with laparotomy preferable through a small incision in the supraumbilical fold (Tan and Bianchi 1986), or as a laparoscopic procedure (Jia et al 2011). The postoperative course is usually uncomplicated with the infant reaching full enteral feeding within a few days. Most infants are discharged from the hospital within 2-3 days post operatively.
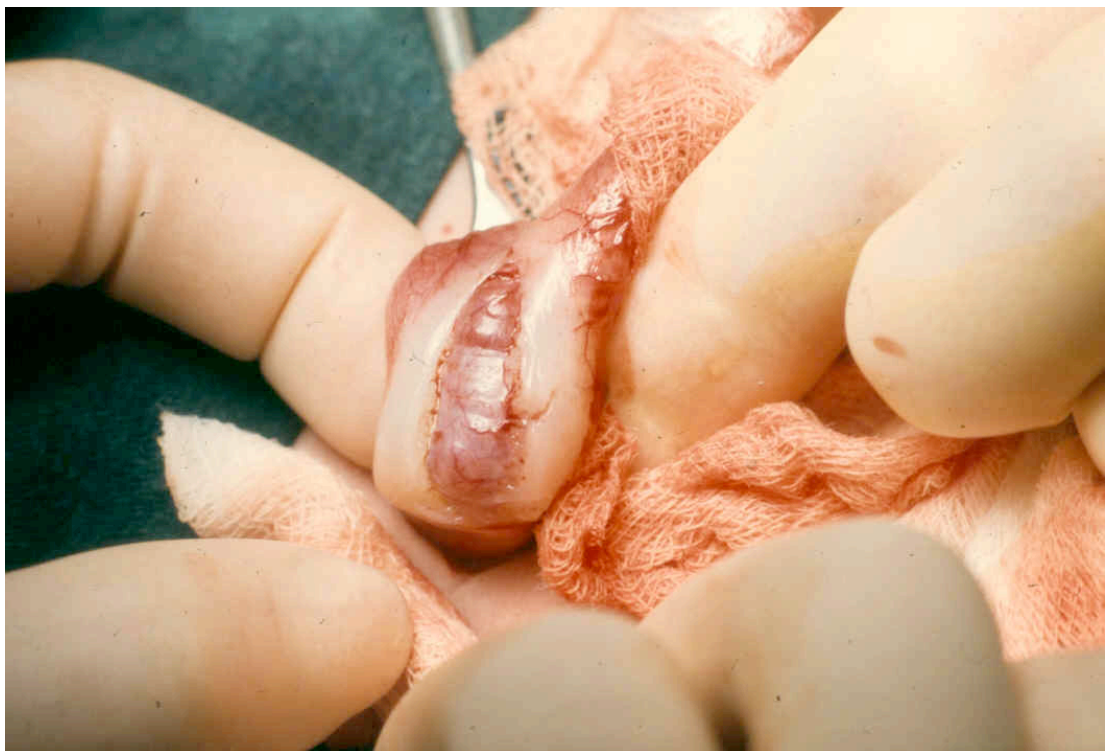

Figure 3. Hypertrophic pyloric stenosis after pyloromyotomy. Photo: Henrik Ehrén.

2 GENETICS IN IHPS

Determining the genetic background of a disease includes observations of familial aggregation, estimation of relative risk and heritability, twin studies or finding of the genetic aberration causing the disease (Haines and Pericak-Vance 1998). IHPS exhibit obvious clustering in families, even though the majority of cases are sporadic (Krogh et al 2011, Carter 1961). Moreover, the inheritance pattern in familiar cases most often does not follow the laws of Mendelian inheritance (Chakraborty 1986). Twin studies of IHPS support the genetic background of the disease, but also imply the importance of environmental factors for the disease to occur (Krogh et al 2011, MacMahon 2006). These observations are all typical features of a complex disease and IHPS was already in the 1950s postulated to belong to this group of disorders. In fact, IHPS was one of the first and most thoroughly studied complex diseases when described in the today classic publications from Carter et al (Carter and Powell 1954, Carter 1961, Carter and Evans 1969)

COMPLEX DISEASES

In contrast to monogenic diseases caused by alterations in a single gene transmitted with simple Mendelian inheritance, complex disorders depends on interaction of multiple susceptibility genes with environmental factors and are not passed with Mendelian transmission. The features of multiple susceptibility genes and interaction with non-genetic factors explain why complex diseases often are referred to as a multifactorial, or polygenic diseases.

A single susceptibility gene is neither necessary nor enough for causing a complex disease. Instead, susceptibility genes interact through an additive effect or through gene-gene interactions (epistasis) to reach the threshold for manifest disease. Susceptibility genes also interact, to a greater or smaller degree, with environmental factors. The genetic predisposition (liability) for the disease determines the response and effect of exposure to environmental factors that might trigger disease (Strachan and Read 2004).

The concept of susceptibility and threshold for complex diseases is summarized in Figure 4. All individuals in a population have a susceptibility for a certain complex condition determined by multiple genes. Susceptibility is a continuous variable ranging

from high to low, and is in a population distributed as a Gaussian curve. Individuals whose susceptibility exceeds the threshold for disease, gets affected. As in the case for IHPS where the disease is more common in boys, the threshold value for girls is higher resulting in fewer affected girls. If the population studied consists of relatives to an affected individual, the whole Gaussian curve is shifted towards the threshold value. This results in a higher number of affected individuals in the "population of relatives to an affected" compared to the general population. The Gaussian curve of a population representing relatives to an affected girl is (in the case of IHPS) shifted more toward the threshold value compared the curve representing relatives to an affected boy (Strachan and Read 2004).
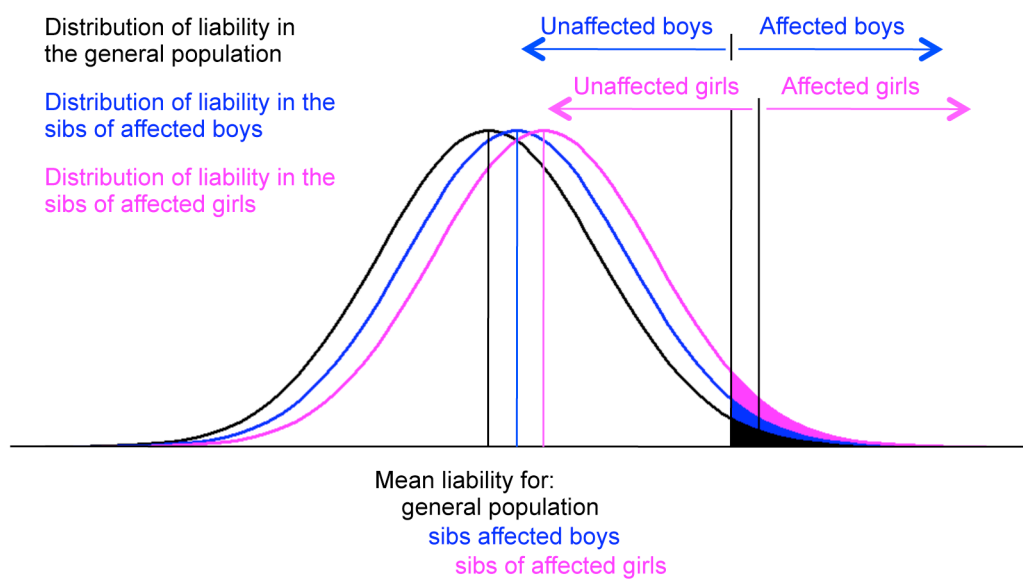
Figure 4. A polygenic threshold model for a complex disease with sex-specific thresholds. (Figure adapted from Strachan and Read 2004).

The number of susceptibility genes involved can range from just a few to a large number. The higher number of susceptibility genes involved the smaller contribution of each gene to the total risk of disease. From a gene mapping point of view, genes only contributing with a small risk are difficult to detect. Another phenomenon that complicates gene mapping is genetic heterogeneity, i.e. when two or more genes act independently in different individuals to cause an identical disease. (Ott 1990, Weeks and Lathrop 1995, Todd 2000).

The recurrence risk of a monogenic disease can be calculated from the combination of transmission mode (dominant, recessive, X-linked) and the probability of the disease gene being passed from parents to the offspring. For complex diseases, the situation is

quite different, and calculation of recurrence risk is replaced by an empiric risk based on observations in the population (as in Table 1). This of course has a very low, or negligible correct predictive value. To foresee who will get affected will in most cases therefore not be better than a qualified guess (Strachan and Read 2004).

Relative risk ($\lambda_{R)}$ is commonly used to describe to which extent a relative of an index patient has an increased risk to be affected compared to the general population. λ_R is calculated as the ratio between the incidence among relatives to an affected individual and the general population incidence. R denotes the kind of relationship to the affected (sibling, son, daughter etc) and separate values of λ_R is calculated for each such group of relatives (R).

Both genetic and environmental factors shared in a family can cause aggregation of a disease. Twin studies can be helpful to distinguish these two, and estimate to which extent the genetic and environmental factors contributes to the disease, respectively. Twins share environmental factors to a very large extent, and have either identical genetic background (monozygotic twins, MZ) or equaling a sib pair that in average share half of their genes (dizygotic twins, DZ). Higher concordance (both twins affected) in MZ twin pairs compared to DZ twins demonstrates the importance of genetic factors for development of the disease. Concordance not depending on zygosity suggests instead environmental factors of importance for development of the disease.

FAMILIAL AGGREGATION AND TRANSMISSION OF IHPS

Familial aggregation of IHPS is reported to occur in 13-14% of the IHPS cases (Persson et al 2001). As expected from a complex disease, IHPS transmission in general does not follow the Mendelian laws of inheritance. However, there are reports of families with a dominant transmission of the disease, sometimes referred to as families with monogenic form of IHPS (Fried et al 1981, Finsen 1979, Capon et al 2006, Everett et al 2008a). Linkage studies in a few of these families have identified loci with evidence of linkage, but a specific disease causing gene has not been found in these regions (Capon 2006 et al, Everett et al 2008a).

According to the polygenic threshold model summarized in Figure 4, children of IHPS affected mothers have a higher risk to be affected compared to children of affected fathers. Especially sons of affected mothers should have the highest risk, and this was also confirmed by Carter (1961) and Fuhrmann (1976) showing 21-23% of sons of

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IHPS affected mothers were affected compared to 1-3 % of daughters of affected fathers.

Table 1. Data of IHPS recurrence risks stratified for gender and relationship with the index patient (Data from Carter 1961).

Index patient	Sons	Daughters	Brothers	Sisters
Male	6.8%	1.2%	3.2%	3.0%
Female	20.5%	11.1%	13.2%	2.5%

With an incidence of 0.5% in boys and 0.1% in girls, data from Table 1 gives a relative risk $\lambda \approx 40$ for sons to an affected mother and $\lambda \approx 12$ for daughters to an affected father.

Krogh et al found a concordance rate of 46% in monozygotic twins compared to 8% in dizygotic twins, i.e. an almost 6-fold higher risk among MZ twins supporting the genetic background of the disease (Krogh et al 2011). Furthermore, IHPS showed a strong familial aggregation with heritability estimated to 87% and risk ratios of 19 for siblings, 29 for DZ twins and 182 for MZ twins, if an older sibling was affected. However, in this material no difference was found when inheritance was compared between male and female index patients (Krogh et al 2011).

IHPS SUSCEPTIBILITY LOCI AND CANDIDATE GENES
Genome wide linkage studies
A few genome wide linkage studies are reported in the search for chromosomal loci for IHPS. Linkage was found to chromosome 16p12-p13 and 16q24 in two different multiplex pedigrees with dominant transmission (Capon et al 2006, Everett et al 2008a). However, analysing these regions in other extended families did not confirm linkage, supporting IHPS being a disease with locus heterogeneity. The only genome wide linkage study of multiple families so far identified linkage to chromosome 11q14-q22 and Xq23 in a material of 81 pedigrees (Everett et al 2008b).

NOS1 gene
To date, the neuronal nitric oxide synthase (nNOS) gene (*NOS1*) is the only gene reported with evidence as an IHPS susceptibility gene (IHPS1 MIM ID*163731), both by linkage and a proposed functional promoter SNP associated to the disease. The enzyme nNOS catalyzing synthesis of NO in enteric neurons, is a key player in the pathway mediating pyloric relaxation, which is thought to be deficient in IHPS patients.

Expression studies from pyloric tissue have indicated a down regulation of *NOS1* (Kusafuka and Puri 1997). Evidence of genetic linkage to *NOS1* has been reported in a material of 27 IHPS families in a publication studying the *NOS1* locus exclusively (Chung et al 1996). However, linkage could not be confirmed in a subsequent study comprising three IHPS families from a different population (Söderhäll and Nordenskjöld 1998).

NOS1 has an extremely complex regulation of transcription with alternate promoters, alternative splicing, cassette insertions and deletions, varied sites for 3'-UTR cleavage and polyadenylation that produces different mRNA transcripts. This results in several different possible nNOS isoforms with different structural and functional properties (Wang et al 1999). A possible link between a functional *NOS1* promoter polymorphism and IHPS was suggested by Saur et al (2004). Following the observation that predominantly the exon 1c mRNA isoform was decreased in IHPS pyloric tissue, the *NOS1* exon 1c promoter region was sequenced revealing a previously uncharacterized SNP (c.-84A/G). This -84SNP A-allele had a 30% decreased promoter activity and was associated with IHPS, however only investigated in a small material consisting of 16 patients.

IHPS IN CHROMOSOMAL SYNDROMES AND GENETIC DISORDERS

When a disease is associated with a chromosomal aberration or a genetic disorder, it can indicate a chromosomal region harboring susceptibility genes or in which pathways to search for these. IHPS is associated with a few chromosomal syndromes and disorders:

Smith-Lemli-Opitz syndrome (SLOS) is an uncommon severe autosomal recessive disorder caused by mutations in the 7-dehydrocholesterol reductase gene (*DHCR7*) resulting in defective cholesterol synthesis (Kelley et al 2000). SLOS is characterized by facial dysmorphia, microcephaly, congenital heart disease, limb malformation, cholestatic liver disease, adrenal insufficiency and mental retardation (Craigie et al 2005). IHPS was included as one of the SLOS characteristics in the original description of the syndrome, and occurs among 14-30% of the SLOS patients. This equals a 157-fold increased risk compared to the population (Kelley 2000, Schechter et al 1997). Interestingly, early supplementary treatment with cholesterol seems to reduce the number of SLOS patients being affected of IHPS (Kelley 2000). If it is the defective

cholesterol synthesis itself that results in increased IHPS risk, or if it is some other kind of secondary SLOS manifestation is unclear (Schechter et al 1997).

Partial duplication of chromosome 9q. IHPS is reported to be frequent among patients with a partial duplication of proximal chromosome 9q (Yamamoto et al 1988, Heller et al 2000, Maraschio et al 1998, Chung et al 1993). In general, genes for associated diseases are likely to be found at the breakpoints, which in this case would be at 9q22 and 9q31. A linkage study was also performed focusing on the 9q region in a material of 20 IHPS families but without providing evidence for an IHPS locus (Chung et al 1993).

Trisomy 21. In trisomy 21, duodenal atresia is a frequently occurring gastrointestinal malformation (Choudry et al 2009). There are conflicting results whether infants with trisomy 21 also have an increased risk for IHPS (Knox et al 1972, Fabia and Drolette 1970, Schechter et al 1997). None one of the published genetic linkage studies so far have reported evidence of linkage to chromosome 21.

Phenylketonuria. An increased incidence of IHPS has been observed among infants with phenylketonuria (PKU) (Dodge 1975, Johnson et al 1978, Koch et al 1973). The classic form of PKU is an autosomal recessive disorder caused by mutations in the phenylalanine hydroxylase (*PAH*) gene. In presence of the co-factor terahydrobiopterin (BH4) PAH converts phenylalanine to tyrosine. Deficient PAH function results in accumulation and high levels of phenylalanine, which have a toxic effect on the brain. Consequences of untreated PKU include impaired mental development and seizures. Except from the classic form of PKU, other variants exist with mutations in genes encoding enzymes that control synthesis and recycling of BH4 (Scriver 2007). Interestingly, the BH4 deficient mouse model (hph1-mouse) exhibits a phenotype including transient pyloric hypertrophy. Supplementary BH4 has therefore been used experimentally to medically treat IHPS, however without success (Braegger et al 1997).

3 STRATEGIES FOR FINDING SUSCEPTIBILITY GENES

Different strategies can be used to search for susceptibility genes, and which one to use depends on several factors including study material available and knowledge of the pathogenesis of the disease. The *candidate gene approach* identifies a candidate gene based on a priori knowledge or hypothesis of its involvement in the pathogenesis, and explores if association to the disease can be found. *Linkage analysis* identifies unbiased and without prior knowledge of the pathogenesis, chromosomal regions linked to the disease, i.e. regions that might harbor susceptibility genes. When candidate regions are obtained from the linkage analysis, candidate genes are identified from these linked regions. Having a restricted chromosomal region to search in gives higher odds of finding the causative genes.

The study populations used for candidate gene approach and linkage analysis are different. Candidate gene studies can utilize DNA samples from unrelated cases and healthy controls, to test polymorphisms for association or to screen the gene for mutations. For linkage analysis, DNA from whole families with aggregation of the disease is used. Large multiplex families with multiple affected are preferable, but these are often rare particularly when dealing with complex diseases. However, there are also linkage methods that can utilize materials with small families including only two affected, for example sib-pair analysis. Success of finding linked regions relies then of including as many families as possible in the analysis.

LINKAGE ANALYSIS

Basic idea of linkage analysis is to identify chromosomal regions segregating together with the disease in families. These linked regions are likely to harbor susceptibility genes. A primary advantage of linkage analysis is that no prior knowledge of physiology or biology underlying the disease is needed (Kwon and Goate 2000). During meiosis, genetic material from two homolog chromosomes is exchanged in a process called recombination. The closer two loci are located on a chromosome, the less likely will they be separated by recombination and instead segregate together to the next generation. The recombination fraction theta (θ) ranging from 0 to 0.5 is a measurement of how often recombination between two loci is likely to occur. Two loci are said to be completely linked ($\theta=0$) if they always segregate together, never

separated by recombination. Two loci located far apart on a chromosome, or even at different chromosomes, are not linked with recombination fraction θ=0.5. Between this two extremes, are loci exhibiting some degree of linkage with a θ value somewhere between 0 and 0.5.

First step in a linkage study is to genotype all individuals for a number of polymorphic DNA markers with known genomic position. Polymorphic microsatellite markers or SNPs can be used for this purpose. Genotype data is then analysed using a statistical test to search for evidence of linkage between markers and a putative disease loci

Two main variants of linkage analysis exist, parametric and non-parametric linkage analysis. The classic parametric, or model-based, analysis requires specification of a number of parameters such as mode of inheritance, penetrance, allele frequency etc. The parametric method can be very powerful if these parameters can be specified correctly. However, in complex diseases a correct genetic inheritance model and reliable estimations of other parameters can seldom be specified, resulting in low power when using parametric analysis. Instead, the non-parametric "model-free" linkage analysis that makes no assumptions of inheritance model is the method of choice (Lander and Schork 1994).

Non-parametric linkage (NPL) analysis is based on observations of affected relatives pairs (ARPs) sharing identical by descent (IBD) alleles located close to a disease locus more often than expected from random allele segregation. IBD means that two relatives have inherited not only the same allele variant (identical by state, IBS), but also fulfilling the condition that this allele is inherited from the same parent (IBD). According to Mendelian laws of inheritance, the probability for a sib-pair to share 2, 1 or 0 alleles are 25%, 50% and 25% respectively (Figure 5). If the allele sharing distribution deviates from the expected, it might indicate linkage. Sib-pair analysis studies sib-pairs exclusively (Penrose 1935). The more powerful affected relative pairs method can utilize several kinds of relationship apart from sib-pairs, for example grandparent-grandchild, cousins, avuncular and half-sibs (Haines and Pericak Vance 1998).
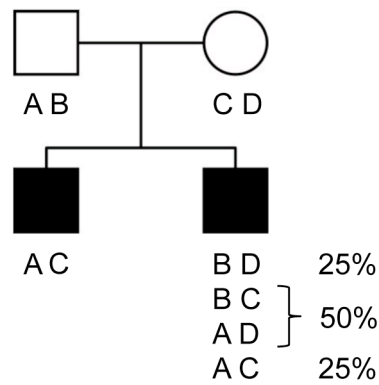
Figure 5. IBD-sharing possibilies in a sib-pair and the expected frequencies of sharing 2, 1 or 0 IBD-alleles according to Mendelian laws of inheritance.

CANDIDATE GENE APPROACH

The candidate gene approach is a straightforward method to test a gene potentially contributing to a disease, by testing for association or mutation screening. Candidate genes can be either functional or positional. Functional candidate genes are identified based on having a potential role in a disease causing mechanism. Positional candidate genes are located in a region with linkage to the disease, but usually also have a suggested functional role in the pathogenesis. Identifying one functional candidate gene that turns out to be associated with a disease, can often lead to identification of other disease associated genes in the same pathway. Compared to linkage analysis, candidate gene approach with large sample size is often a more successful strategy to detect genes contributing with only a small risk (Kwon 2000).

4 EPIDEMIOLOGY AND RISK FACTORS

CHANGING INCIDENCE

In the early 1990s the pediatric surgeons in Sweden noticed a decreased frequency of IHPS patients. This observation was confirmed in epidemiological studies describing a sharp decline of IHPS incidence to approximately one third during a ten-year period (Hedbäck et al 2001, Persson et al 2001). Previously, several reports have described temporal variations of incidence in several regions, but not as remarkable as this (Webb et al 1983, Dodge 1975, Wallgren 1960, Kerr 1980, Grant and McAleer 1984, Sule et al 2001, MacMahon 2006). The genetic background in a population can obviously not change over a few years, and cause a shift in incidence such as this. Instead, a change in the infant's perinatal environment with removal of a risk factors, or addition of a protecting factor, would explain the decline in incidence. Geographical differences with almost three times higher incidence in south of Sweden compared to the north was also reported (Hedbäck 2001). A genetic explanation for these differences could be a founder effect, but more likely differences in environment. Apart from the Swedish reports, several other European countries or regions have also reported a similar pattern of decline in incidence (Nielsen et al 2000, Sommerfield 2008, Pedersen et al 2008). However, a few studied regions with an already low IHPS incidence during the 1980s remained unchanged (Pedersen et al 2008).

RISK FACTORS

Maternal factors and birthorder

Several consistent reports have confirmed that infants to young mothers and first-borns have a higher risk to develop IHPS, also when controlling for bias between these two variables (Rasmussen et al 1989, Dodge 1975, Schechter et al 1997). Another SIDS risk factor also studied for IHPS, is maternal smoking. A Danish population based study showed a 2-fold increased IHPS risk for infants to smoking mothers compared to non-smokers (Sörensen 2002).

Feeding practices

A higher incidence of IHPS among bottle fed infants compared to breast fed has been reported in several studies (Habbick et al 1989, Webb et al 1983). However, occasional reports have also claimed the opposite (Dodge 1975). Curds formed from artificial milk formula interfering with or obstructing the pylorus is proposed as a possible mechanism

resulting in pyloric hypertrophy (Osifo and Evbuomwan 2009). A correlation between the frequency of feeding (3 or 4-hourly) and age at onset of symptoms is reported (Gerrard et al 1955). One can speculate if feeding practices, in terms of frequency and volume of each feeding, is of importance also for development of the disease and might differ between breast and bottle fed infants.

Prone position

A parallel incidence rate between SIDS and IHPS during the 1970s-1990s was previously described, suggesting a common risk factor for these disorders (Persson 2001). The "back to sleep" campaign during the 1990s encouraging parents to let the infants sleep on their back, was attributed as the most important measure for decreasing the SIDS incidence. Therefore the prone position was also suggested as an IHPS risk factor. Feeding often precedes putting the infant to sleep, and therefore can different localization of stomach content in prone or back position might be of importance as a predisposing IHPS factor. In the back position, the given contrast was located in the fundus region, not interfering with the pyloric region. In the prone position, the contrast was instead accumulated in the pyloric antrum region, interfering with the pylorus and possibly exerting mechanical pressure to this region (Persson et al 2001).
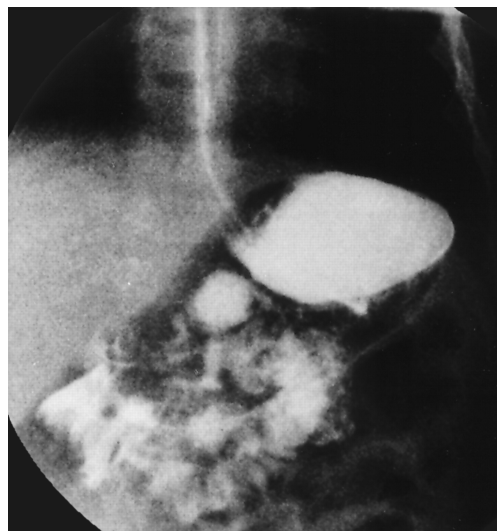
Prone position   **Back position**

Figure 6. Upper GI-study of infant lying in prone and back position. In the prone position the contrast is accumulated in the pyloric antrum. In the back position, the contrast is instead accumulated in the fundus region separated from the pyloric region. Pictures from Persson et al 2001. Reproduced with permission from Pediatrics, Vol 108, Page E70, Copyright 2001 by the AAP.

The IHPS-SIDS study was later replicated in Scotland. This showed a decreased incidence for both SIDS and IHPS during the 1990s with linear correlation. However, the fall of IHPS incidence preceded the fall of SIDS incidence with 2 years. The authors therefore doubted the hypothesis of shared risk factors of IHPS and SIDS (Sommerfield 2008). However, the time lag between decreasing IHPS and SIDS incidence does not automatically exclude that prone position is a risk factor for IHPS, since prone position could be a stronger risk factor for IHPS than SIDS. The effect in terms of decreasing incidence would then be seen earlier for IHPS compared to SIDS.

Erythromycin treatment

A sudden increased number of IHPS cases were noticed in a hospital a few weeks after the newborns had received erythromycin prophylaxis due to a pertussis outbreak (Honein et al 1999). Further studies showed an up to 10-fold increased risk of IHPS among infants treated with Erythromycin within the first 2 weeks of life (Cooper et al 2002, Mahon et al 2001).

5 PATHOGENESIS

It is well established that both genetic and environmental factors influence development of IHPS, but the underlying pathogenesis resulting in pyloric hypertrophy is still not revealed. Several observations suggest that pyloric spasm is part of the mechanism resulting in pyloric hypertrophy. Molecular studies in this field have mainly focused on abnormal innervation of the pyloric muscle and altered expression of neurotransmitters mediating pyloric relaxation.

PYLORIC SPASM

Although uncommon, there are a few reports of infants with onset of IHPS symptoms virtually from the first feeding (Andrassy et al 1977, Geer et al 1985, Hatiboglu 1996). The initial radiological investigation has in such cases shown signs of pyloric spasm, but not hypertrophy of the pyloric muscle. With a repeated radiologic study a few days after, also pyloric hypertrophy has been detected and the infant has been diagnosed and cured with pyloromyotomy (Ng and Lee 2002). It is proposed that the process with pyloric spasm can start early, even prenatally, suggested by the finding of polyhydramnios and in-utero detected gastric dilation in a few fetuses presenting with IHPS shortly after birth (Houben and Kely 1997, Powell 1962, Zenn and Redo 1993, Katz et al 1988). However, the scientific evidence of these few cases should not be overestimated. In addition, early onset cases are uncommon and for the vast majority of patients the start of enteral feeding seems obligate for the development of the disease (Chung 2008). Less is known whether pyloric spasm precedes the hypertrophy also in these cases. Beneficial effect of medical treatment of IHPS with anticholinergic substances however indicates that pyloric spasm is involved in the pathogenesis.

PYLORIC INNERVATION

The pylorus is innervated by the enteric nervous system (ENS), also called "the second brain" indicating its complexity (Gershon 1999). The ENS is competent to operate autonomously through reflex circuits with sensory neurons detecting physiological conditions and motor neurons regulating peristalsis, absorption, blood flow and release of enzymes and hormones (Gershon 2005). However, under normal circumstances the ENS operates under influence of the central nervous system (CNS).

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In functional terms, two different kinds of motor neurons mediates contraction (excitatory motor neuron) and relaxation (inhibitory motor neuron) of the gut. A large number of neurotransmitters and neuropeptides mediating these effects have been identified. Nitric oxide (NO) is the major neurotransmitter mediating relaxation (Bult et al 1990).

Nitric oxide

The neurotransmitter that has gained most attention in IHPS research is nitric oxide (NO), which is an important mediator of enteric smooth muscle relaxation (Desai et al 1991, Bult et al 1990). NO is synthesized from L-arginine by nitric oxide synthase (NOS), an enzyme family including three different isoforms. Synthesis of NO in intestinal neurons is catalyzed by the neuronal nitric oxide synthase (nNOS) requiring a few co-factors including tetrahydrobiopterin (BH4). This is an interesting connection to the hph1-mouse model for pyloric stenosis (Abel et al 2004).

Immunohistochemistry studies have shown absence or decreased density of nNOS containing nerve fibers in hypertrophic pyloric muscle, suggesting that the pyloric muscle hypertrophy is a result of abnormal innervation (reviewed by Hernanz-Schulman 2003).

ANIMAL MODELS
Genetic modified animal models

Two genetic modified animal models for IHPS are described, the *Nos1*-mouse and the *hph-1* mouse. They exhibit two different genetic alterations both resulting in a final common pathway with deficient or absent NO synthesis.

The *Nos1* knock out mouse exhibits a phenotype with enlarged distended stomach and thickened pylorus regardless of fasting or being fed. Histologic examination shows hypertrophy of the circular muscle layer of the stomach and pylorus. As in IHPS, the pyloric hypertrophy causes gastric outlet obstruction. The remainder of the intestine and peripheral NOS-expressing tissue appeared normal (Huang et al 1993).

The *hph-1* mouse was originally created as a model of phenylketonuria caused of an induced error of BH4 metabolism. BH4 is a co-factor for both phenylalanine hydroxylase and NOS enzymes why BH4 deficiency in addition to hyperphenylalaninaemia results in decreased NOS activity and production of NO (Abel

et al 2004, Brand et al 1995, Giovanelli et al 1991). The *hph1*-mouse exhibits a phenotype resembling the IHPS features in human with transient pyloric muscle hypertrophy, distended stomach and failure to thrive which resolves as the pyloric muscle normalize (Abel et al 2004). Innervation and immunoreactivity for neurotransmitters has been reported normal (Abel et al 2004).

Pentagastrin induced pyloric stenosis

Administration of pentagstrin to canine fetuses or pups can produce pyloric stenosis and to a lesser extent also pyloroduodenal ulcers. The histological feature of the pyloric hypertrophy resembles those found in human form of IHPS (Dodge and Karim 1976) The mechanism by which pentagastrin induce pyloric hypertrophy is uncertain but several mechanisms are possible. Pentagastrin is known to stimulate antral motility (Misiewicz et al 1969) which could contribute to pyloric spasm. Pentagastrin also stimulates acid secretion, which stimulates a release of secretin and cholecystokinin that stimulate gastric contractions, possibly ending up with pyloric spasm (Rogers et al 1975).

6 AIMS

The general aim of this thesis was to investigate the genetic and environmental risk factors predisposing for IHPS.

The specific aims were:

- To study two candidate genes for IHPS (MLN in study *I* and NOS1 in study *II*)

- To unbiased localize predisposing loci for IHPS by genome-wide linkage analysis (Study *III*)

- To study IHPS incidence in Sweden and analyse maternal and perinatal characteristics as risk factors for IHPS (Study *IV*)

7 MATERIALS

GENETIC STUDIES

Patients recruited to the genetic studies *I-III* were identified from the pediatric surgery clinics in Stockholm, Uppsala, Lund and Göteborg where the patients were treated for IHPS from 1971 and onward. In a questionnaire we asked for occurrence of more known IHPS affected individuals in the family. If no heredity was revealed, the patient was classified as a sporadic case. The families with positive heredity were interviewed for pedigree mapping, and all family members were offered to participate in the study. Genomic DNA was isolated from venous blood samples or from skin fibroblast cells from skin biopsies collected at surgery with pyloromyotomy.

In collaboration with University College London Institute of Child Health, the Swedish material of families in study *III* was extended with DNA samples from British IHPS families. A population based control group consisting of randomly selected healthy and anonymous blood-donors from the Stockholm region was used as controls in study *I* and *II*.

Study populations

In study *I*, 57 IHPS cases (22 familial and 35 sporadic) were used for mutation screening and genotyping of *MLN*. Also, the family of one familial case including 4 additional individuals (1 affected, 3 not affected) was analysed due to sequence variant in the proband. The control group consisted of 184 randomly selected controls.

In study *II*, 82 IHPS cases (54 familial and 28 sporadic) were genotyped for the *NOS1* exon 1c promoter polymorphism. The control group consisted of 80 randomly selected controls.

In study *III*, 37 Swedish families with at least two IHPS affected members (184 individuals, 92 affected, 58 affected relative pairs) were included for genome-wide scan. Ratio of affected boys to girls was 1.7:1. Figure 7 shows the pedigrees of the Swedish families.

To extend the Swedish material, 31 additional British families (256 individuals, 101 affected, 126 affected relative pairs) were included for specific analysis of the fine mapping regions. Ratio of affected boys to girls was 3:1.
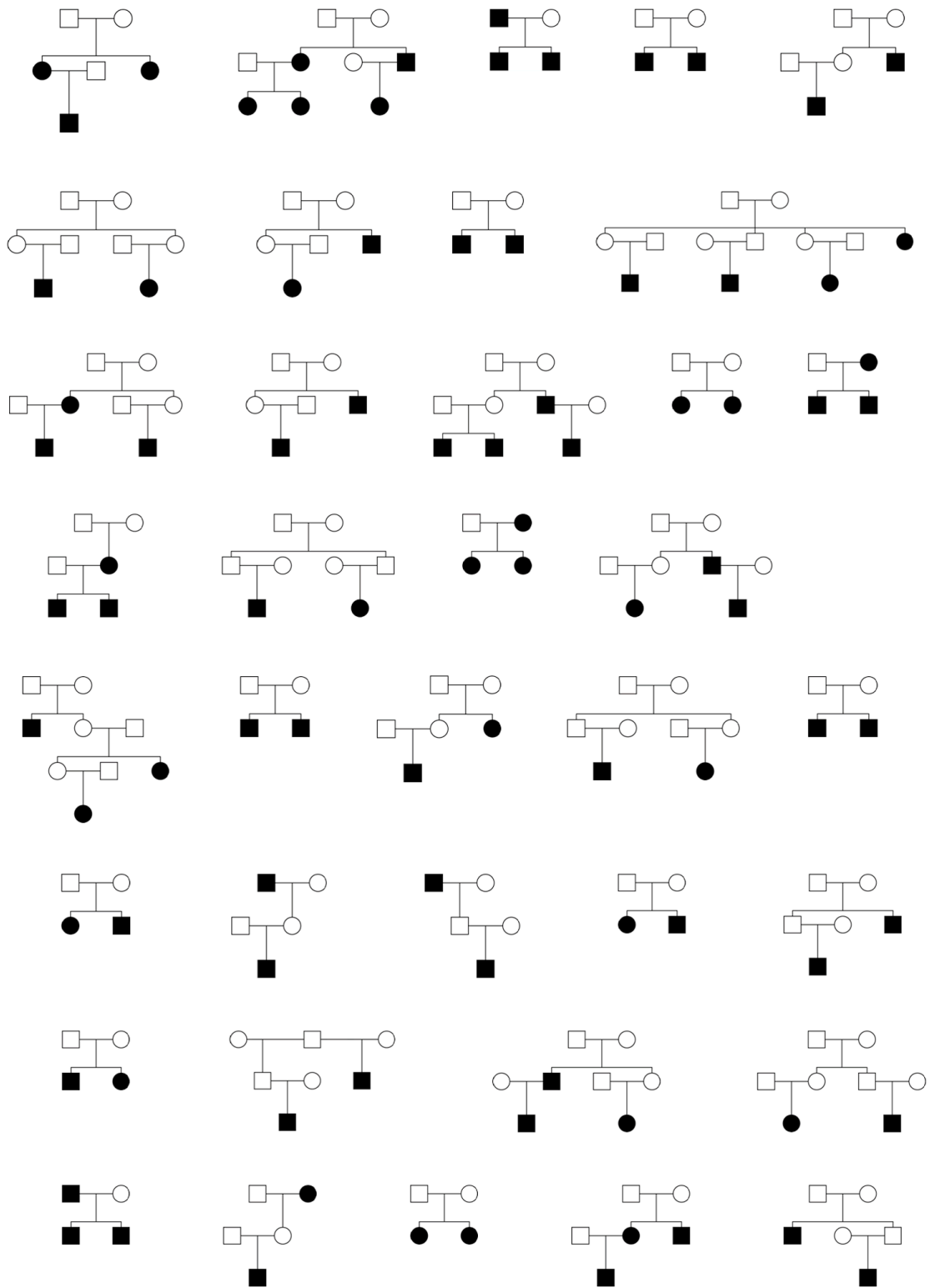

Figure 7. Pedigrees of the 37 Swedish families included in the genome-wide linkage study. DNA was available from all affected and total 184 individuals.

EPIDEMIOLOGICAL STUDY

Registers

The Swedish National Patient Register (NPR) held by the Swedish National Board of Health and Welfare, contains prospectively collected data of all hospitalizations in Sweden. NPR was founded in 1964 and included at that time only 6 of the 26 Swedish counties. Since then has the number of included counties gradually increased, and the register reached full coverage in 1987 when all 26 counties were included. The main purposes of the register are to provide data for health care evaluation, statistics and research. NPR data includes patient's personal registration number, geographical data, date of admission and discharge, procedures, and main and secondary diagnosis coded according to the ICD-classifications. The quality of data is extremely good with a drop out rate of less than 2% of all hospitalizations (Kvalitet och innehåll i patientregistret 2009).

The Swedish Medical Birth Register held by the Swedish National Board of Health and Welfare, is a nationwide register founded in 1973 containing data of all pregnancies in Sweden leading to deliveries. Register data includes prospectively collected information of both the pregnant woman and the newborn infant, registered at the antenatal clinic, delivery and neonatal units up to one month after birth. Register variables includes personal registration numbers, maternal age and parity, maternal weight, length and ethnicity, exposures during pregnancy such as smoking, chronic or relevant diseases, single or multiple birth, duration of pregnancy, delivery record, birth weight and possible diagnosis of the infant. Reporting is also good with only 1-3 % of infants missing (Medicinska Födelseregistret).

Study population

In study *IV*, from the Swedish Patient register we identified all individuals treated as in-patients with IHPS as main or secondary discharge diagnosis during the period 1973-2008 (ICD-8 code 750.10, ICD-9 code 750F, ICD-10 code Q40.0). Further inclusion criteria were diagnosis before 1 year of age, since cases beyond this age most probably have as a misclassified diagnosis, and being born in Sweden to assure data from the Medical birth register. A total of 4797 cases were identified with these criteria during the study period. Flowchart of the study participants is shown in Figure 8.

To further reduce risk of including patients with misclassified diagnosis, patients not surgically treated with pyloromyotomy or a similar procedure (surgical procedure code

4410, 4464, 4499, JDH60, JDH61, JDH63, JDH00) were excluded from the study. After exclusion of 1001 conservative treated patients, 3796 cases remained.

For multiple births, there is a known risk that a twin's medical birth register form instead contains information of its twin sibling. This motivated exclusion of twins for the case control part of the study. After exclusion of 188 twin cases and 1397 controls (twin control or control corresponding to excluded twin), 3608 cases and 17588 controls remained as the final cohort for case control study.

ETHICAL PERMISSIONS

The genetic studies were approved by the Ethics Committee of Karolinska Institutet, and the Ethics Committee of University College London Hospital. Informed consent was obtained from all Swedish and British participating individuals.

The epidemiological study was approved by the Ethics Committee of Karolinska Institutet.
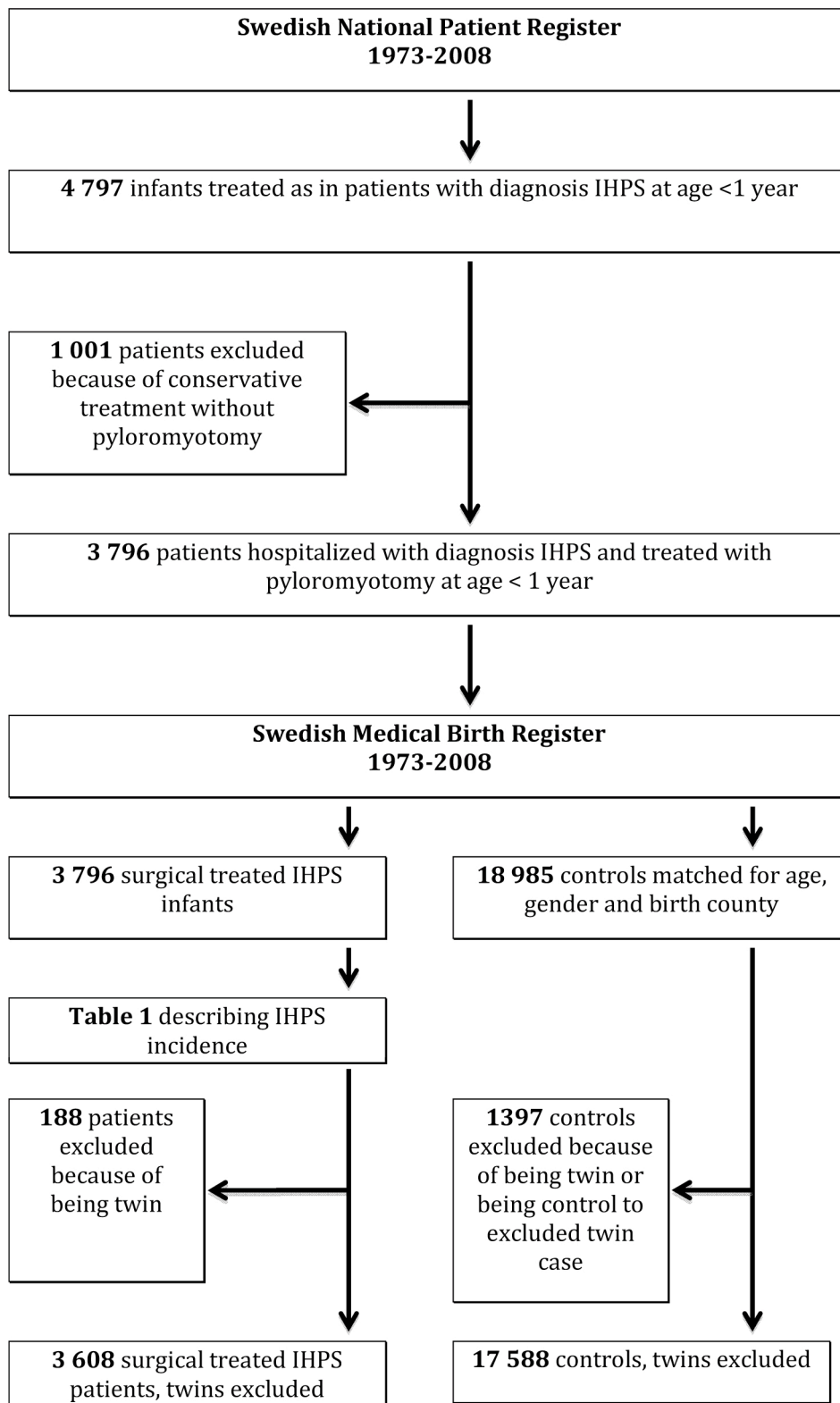
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│           Swedish National Patient Register              │
│                     1973-2008                            │
└─────────────────────────────────────────────────────────┘
                          │
                          ▼
┌─────────────────────────────────────────────────────────┐
│ 4 797 infants treated as in patients with diagnosis IHPS │
│                   at age <1 year                         │
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┌──────────────────────┐
│ 1 001 patients       │ ◄──────┐
│ excluded             │        │
│ because of           │        │
│ conservative         │        │
│ treatment without    │        │
│ pyloromyotomy        │        │
└──────────────────────┘        │
                                ▼
┌─────────────────────────────────────────────────────────┐
│ 3 796 patients hospitalized with diagnosis IHPS and      │
│           treated with pyloromyotomy at age < 1 year     │
└─────────────────────────────────────────────────────────┘
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┌─────────────────────────────────────────────────────────┐
│           Swedish Medical Birth Register                 │
│                     1973-2008                            │
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              │                           │
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┌──────────────────────┐    ┌──────────────────────────────┐
│ 3 796 surgical       │    │ 18 985 controls matched for   │
│ treated IHPS infants │    │ age, gender and birth county  │
└──────────────────────┘    └──────────────────────────────┘
              │
              ▼
┌──────────────────────┐
│ Table 1 describing   │
│ IHPS incidence       │
└──────────────────────┘

┌──────────────┐              ┌──────────────────┐
│ 188 patients │ ◄──┐         │ 1397 controls    │ ◄──┐
│ excluded     │    │         │ excluded because │    │
│ because of   │    │         │ of being twin or │    │
│ being twin   │    │         │ being control to │    │
│              │    │         │ excluded twin    │    │
│              │    │         │ case             │    │
└──────────────┘    │         └──────────────────┘    │
                    ▼                                  ▼
┌──────────────────────┐    ┌──────────────────────────────┐
│ 3 608 surgical       │    │ 17 588 controls, twins        │
│ treated IHPS         │    │ excluded                      │
│ patients, twins      │    │                               │
│ excluded             │    │                               │
└──────────────────────┘    └──────────────────────────────┘
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Figure 8. Flowchart of cases and controls included in study *IV*.

8 METHODS

GENETIC STUDIES

DNA isolation

Genomic DNA for study *I-III* was isolated from peripheral blood samples or skin fibroblast cells using a standard protocol.

DNA sequencing

Sequencing was used for genotyping and mutation screening of candidate genes in study *I, II* and *III*. Fragments to be sequenced were amplified with polymerase chain reactions (PCR) using primers flanking the fragments. Primer sequences and PCR conditions are available on request. PCR products were purified and then used for sequencing reaction using ABI PRISM BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's recommendations. Sequence data were analyzed on ABI 310, ABI 3100 ABI 3110 or ABI 3730 (Applied Biosystems). Chromatograms were generated using Sequencing analysis (Applied Biosystems).

In study *I*, all five *MLN* exons were sequenced for mutation screening and genotyping. In study *II*, the *NOS1* exon 1c promoter region was sequenced for genotyping. Mutation screening of the candidate genes *NPY* and *GCN* in study *III* was performed by sequencing all *NPY* exons and only exon 5 in *GCN* gene. This exon codes for the part of the proglucagone protein corresponding to GLP-2 after cleavage of the prohormone.

Statistical test for association

In study *I*, test for statistical significant association between *MLN* p.Val15Ala polymorphism and IHPS was performed using chi-square (χ^2) test. P-value <0.05 was considered to be statistically significant.

In study *II*, test for statistical significant association between *NOS1* 1c promoter polymorphism and IHPS was assessed using logistic regression analysis. Risks associated with variant genotypes were calculated and expressed as odds ratios (ORs) with 95% confidence intervals (CIs).

Linkage analysis

In study *III* microsatellite based genome-wide linkage analysis and fine mapping was performed. The principles and theoretical background of linkage analysis is discussed in chapter 3.

Genotyping genome-wide scan

All individuals in the Swedish families were genotyped with 353 fluorescent-labeled microsatellite markers evenly distributed over the 22 autosomes and the X chromosome. The marker set was based on the Weber 6 screening set (Sheffield et al 1995). In sparsely covered regions additional markers were added from the Genome Database and Marshfield Medical Research foundation. Mean inter marker distance for the initial genome wide scan was 10.24 cM and the mean heterozygosity rate was 0.76. Each marker was amplified in separate PCR reactions. PCR conditions and marker list are available on request. Up to five markers from each and same individual were pooled and then size fractioned on an ABI 377 DNA Sequencer or ABI 3730 DNA analyzer (Applied Biosystems). The resulting electrophoretic data was analysed with Genescan 3.1.2 / Genotyper2.0 software (Applied Biosystems). Success rate for genotyped markers were 80%.

Genotyping fine-mapping

Fine-mapping markers were selected from the Genome Database and Marshfield Medical Research foundation. For fine mapping of the loci on chromosome 2p, 2q, 6p, 7p, 8q, 12q and 13q a set of 6, 4, 11, 10, 2, 2 and 6 markers were added, respectively. Also, two markers on chromosome 16p were added due to a recently published report of linkage in this region (Capon 2006). Average marker density of the fine mapped regions was 2.9 cM. PCR, size fractioning and analysis of electrophoretic data were performed as for the initial genome wide scan. Obtained fine-mapping results were added to the genome-wide scan data set, and NPL analysis was reanalysed for the chromosomes including fine mapping regions.

Statistical analysis

Non parameteric linkage (NPL) analysis with affected relative pairs method was used since mode of inheritance is unknown. Analysis was performed in Allegro software version 1.2 (Gudbjartsson et al 2000) with option arguments for linear statistics and S_{all} scoring function. The majority of the pedigrees in the Swedish material are relatively

small and homogene in structure with 2-3 affected, which motivates the use of a statistical model with equal weight assigned to all pedigrees. However, the material also includes a few extended pedigrees with up to 5 affected individuals. If an equal weight model is used, there is a risk that these families exert excessive influence on the total result because of their higher information content. An extended pedigree alone could then give a NPL peak that falsely seems to be valid for the whole material. This motivates instead the use of a statistical model where a weighting scheme is applied to all pedigrees to compensate for different pedigree structures. We choosed to analyse data with both an equal weighted model and a model with weighting factor power 0.5, and evaluate possible differences between these two analyses.

Simulations

The threshold value for significant linkage using NPL score is dependent on the pedigree structures included in the study material. Therefore, for each study performed the specific threshold value has to be determined by either simulations or theoretical approximations. We performed simulations and defined cut off values for suggestive and significant genome-wide linkage using Lander Kruglyak's criteria (Lander and Kruglyak 1995). Significant linkage equals the NPL score occurring by chance at any chromosomal position in average 0.05 times per genome-wide scan. Threshold value for suggestive linkage equals the score randomly occurring once per genome scan.

Genotype data for 10 000 genome-wide scans were simulated using Allegro software (Gudbjartsson et al 2000). The simulation marker set included both the genome-wide scan and fine mapping markers. Pedigrees had identical structure and individuals the same affection status as in the original material. The simulated genotypes had identical, success rate and allele frequencies as in the original analysis. Only individuals having genotype data in the original analysis were assigned genotype data in the simulations.

The 10 000 simulated sets of genome-wide genotype data were analysed in Allegro software (Gudbjartsson et al 2000) using the same settings as in the original analysis. Analysis was performed with both equal weight and power0.5 statistics. The NPL-score that occurred in average 0.05 times per simulated genome-wide scan for each statistical model was defined as the threshold NPL-score for significant linkage. Threshold value for suggestive linkage was defined as the NPL-score occurring in

average once per simulated genome-wide scan. Double peaks of a NPL score were not considered independent if they occurred within 20 cM of each other.

Candidate regions and identification of candidate genes

Candidate regions for loci showing evidence in favour of linkage were pragmatically defined as the region exhibiting NPL-score exceeding the regions maximum NPL-score minus 1 unit (NPLmax-1).

The function and expression pattern of genes located in the candidate regions were investigated by mining the Ensembl and NCBI databases. In addition, the gene prioritizing software Endeavour (Aerts 2006, Tranchevent et al 2008) was used as a tool in the search of candidate genes located in the candidate regions. Candidate genes were screened for mutations as previously described.

STATISTICAL ANALYSES IN EPIDEMIOLOGY STUDY

Study design was a nationwide population based register study using data from the period 1973-2008. Analysis included both descriptive analysis and a case control study. All analyses were performed using Stata 11.0 for Mac OS software (StataCorp, College station, TX, USA).

Descriptive analysis

Annual incidence of IHPS was calculated as rate per 1000 live births. Data of annual birth numbers were obtained from Statistics Sweden. Incidence was calculated for the total cohort and stratified by gender. Nationwide data from NPR is only available from 1987 and onward. To get an indication of nationwide incidence before 1987, incidence rate in Stockholm was used as an estimate since NPR covered all Stockholm hospitals since 1973. Incidence data presented in Figure 10 was adjusted for year 2008 age distribution using Poisson regression.

Based on incidence rate, three obvious periods with high, declining and low incidence could be defined:
- 1973-1988 high incidence
- 1989-1997 declining incidence
- 1998-2008 low incidence.

Age at diagnosis was defined as age in weeks at first admission with diagnosis IHPS and categorized into two-weeks groups.

Distribution of gender, twin births and age at diagnosis were calculated for the cumulative study period, and for each of the three periods corresponding to high (1973-1988), declining (1989-1997) and low incidence (1998-2008).

Case-control study
Study variables from Medical Birth Register were categorized for statistical analysis as shown in Table 2.

Association between each of the study variables and IHPS risk was analysed using conditional logistic regression, conditioned on birth year, sex and birth county. Relative risks were calculated and expressed as odds ratios (ORs) with 95% confidence intervals (CI). Data was analysed with both a univariate and a multivariable model to include adjustment for covariates that could be possible confounders.

Three sub analyses were performed with multivariable analysis stratified by time period, gender and age at diagnosis. The breakpoint for early/late diagnosis was defined as the median value for age at diagnosis. Trends in ORs over time was analysed using logistic regression stratified by time period (time periods corresponding to high, decreasing and low incidence).

Table 2. Categorization of study variables from Medical Birth Register

Birthorder	First, second, third or fourth and more born.
Gestational age	<35 completed weeks (Very Preterm) 35-36 weeks (Preterm) 37-41 weeks (Term) ≥42 weeks (Postterm)
Birth weight for gestational age	<5th percentile 5-9.9th percentile
	10-89.9th percentile 90-94.9th percentile ≥95th percentile
Neonatal jaundice	Occurrence of ICD classification code for neonatal jaundice (haemolytic jaundice excluded) **ICD-10:** P570, P578-585, P589-590, P592-593, P598-599, R179 **ICD-9:** 773A-C, 773E-F, 774A-D, 774E-F, 774G-H **ICD-8:** 77400, 77410, 77420, 77429, 77497, 77499, 77893, 77894, 77896)
Pre-pregnant BMI	<18.5 (Underweight) 18.5-24.9 (Normal weight) 25-29.9 (Overweight) 30- (Obese)
Maternal age	<20 years 20-24 years 25-29 years 30-34 years 35 years and older
Labour	Vaginal birth Caesarean section
Maternal smoking[*] [*] **during early pregnancy**	Non-smoker < 9 daily cigarettes ≥10 daily cigarettes
Maternal birth country	Nordic Non-Nordic
Maternal diabetes	Occurrence of ICD classification code for diabetes mellitus or gestational diabetes mellitus: **ICD-10:** E100-149, O240-244, O249 **ICD-9:** 250A-H, 648A, 648W **ICD-8:** 25000-25009

9 RESULTS AND DISCUSSION

MUTATION SCREENING OF THE *MLN* GENE

We proposed motilin (*MLN*) as an IHPS candidate gene, since treatment with the motilin agonist erythromycin increases risk for IHPS (Cooper and Griffin 2002, Mahon et al 2001, Peeters et al 1989). Motilin is a gastrointestinal hormone released from intestinal mucosa cells, causing intense gastric contractions migrating to the intestine (Jadcherla et al 1997). Our hypothesis was that motilin could induce pyloric spasm in the immature pylorus, resulting in pyloric hypertrophy. All coding exons of the *MLN* gene were screened for mutations. Using a case control design, allele frequencies of the *MLN* polymorphism rs2281820 (p.Val15Ala) was assessed for association with the disease.

Sequencing of the *MLN* gene revealed three previously not reported sequence variations, all present in both patients and controls (Table 3). One of the variants, c.74C>G was a missense variation exchanging an alanine to glycine (p.Ala25Gly) at the last position in the motilin prohormone's signal peptide. The patient exhibiting this variant was a familial case, which prompted mutation testing of the c.74C>G variant in the rest of that family including one additional affected individual and three additional not affected individuals. The result showed that c.74C>G variant did not co-segregate with the disease. Screening the whole gene in all family members detected the c.-66C>T variant, which also did not segregated with the disease. Heterozygote frequencies of the detected sequence variants in cases and controls are shown in Table 3. No significant differences of the heterozygote frequencies were detected.

Table 3. Heterozygote frequencies of detected novel sequence variants

Sequence variant	Familial[*] (n=23)	Sporadic (n=35)	Total cases[*] n=58	Controls[*] n=187	Variant type	Aminoacid change
c.-66C>T	1	0	1 (1.7%)	3 (1.6%)	5'-UTR	-
c.74C>G	1	0	1 (1.7%)	1 (0.5%)	Missense	Ala25Gly
c.489C>T	0	2	2 (3.4%)	15 (8%)	3'-UTR	-

[*] Additional family members from mutation screened family included

Genotype and allele frequencies of the p.Ala25Gly polymorphism in patients and controls are listed in Table 4. C-allele homozygotes were more frequent and C-allele frequency was higher among cases than controls, but these differences were not

significant (χ^2 test p >0.05). Also, when stratifying cases into familial and sporadic cases no significant differences were detected (χ^2 test p >0.05).

Table 4. Genotype and allele frequencies of *MLN* rs2281820 polymorphism in cases and controls.

Genotype	Familial cases (n=22)	Sporadic cases (n=35)	Cases total (n=57)	Controls (n=184)
C/C	11 (50%)	15 (43%)	26 (46%)	65 (35%)
C/T	9 (41%)	13 (37%)	22 (39%)	94 (51%)
T/T	2 (9%)	7 (20%)	9 (16%)	25 (14%)
Allele				
C	31 (70%)	43 (61%)	74 (65%)	224 (61%)
T	13 (30%)	27 (39%)	40 (35%)	144 (39%)

These results do not support *MLN* gene mutations or the p.Ala25Gly polymorphism to play a major role in the IHPS pathogenesis. However, a polymorphism contributing only with a minor risk would require a large sample size to detect significant association. According to our hypothesis that motilin induces pyloric spasm, the putative *MLN* gene variation causing this would presumptively have a gain of function effect. The most probable mechanism causing this would be an alteration in the *MLN* gene's regulatory region. Therefore, the promoter region should also be investigated in a future study before *MLN* is dismissed as a candidate gene. Another feasible mechanism for involvement of the motilin pathway in IHPS pathogenesis, is changes in the motilin receptor. However, the motilin receptor gene (*MLNR)* has been screened for mutations in a British material without any positive findings (Chung, personal communication).

ASSOCIATION STUDY OF A *NOS1* PROMOTER POLYMORPHISM

A polymorphism in the *NOS1* exon 1c promoter (c.-84G>A) was previously suggested to be associated with IHPS (Saur et al 2004). This variant is a functional SNP where the A-allele reduces the expression of the *NOS1C* isoform, and it has also been associated to IHPS. This prompted us to study the promoter c.-84 SNP for association with IHPS in our Swedish material.

Genotype and allelic association for the c.-84 SNP with IHPS was assessed for the total patient group compared to controls, for familial and sporadic cases separately compared to controls, and finally in a multivariate model with adjustment for gender and familial inheritance. Homozygote G-allele carriers were more frequent among

cases compared to controls, but no significant allelic association could be detected. Comparing homozygote G-allele carriers and G/A carriers, detected with borderline significance a lower frequency of A-allele carriers among cases than controls (OR 0.47; 95% CI 0.21-1.00). No significance was found for either genotype or allelic association when cases were stratified into familiar and sporadic cases, or in the multivariate model.

Table 5. Genotype frequencies of *NOS1* SNP rs41279104 in cases and controls

Genotype	Familial cases (n=54)	Sporadic cases (n=28)	Cases total (n=82)	Controls (n=80)
G/G	46 (85%)	23 (82%)	69 (84%)	57 (71%)
A/G	7 (13%)	5 (18%)	12 (15%)	23 (29%)
A/A	1 (2%)	0	1 (1%)	0
Allele				
G	99 (92%)	51 (91%)	150 (91%)	137 (86%)
A	9 (8%)	5 (9%)	14 (10%)	23 (14%)

Contrary to previous finding of association between c.-84A SNP and IHPS (Saur et al 2004), we found with borderline significance association to the heterozygote G/G genotype when compared to A/G carriers (OR 0.47 95% CI 0.21-1.00). There are several possible explanations for the diverging results. The sample size in the study from Sauer et al was very small and included only 16 patients, that also originated from another population compared to our study. Following our publication yet another replication study was performed in an Asian population that confirmed lack of association between the c.-84 SNP and IHPS (Miao et al 2010). The *NOS1* gene is one of the most complex human genes regarding promoter diversity, why further regulatory mechanisms except from the c.-84 SNP must be considered.

GENOME-WIDE LINKAGE ANALYSIS AND FINE-MAPPING

The genetic influence on development of IHPS is well established. In order to unconditionally identify IHPS loci we performed a genome wide linkage study in Swedish IHPS families, using non-parametric linkage (NPL) analysis with affected relative pairs method. Statistical analysis was performed using two different models assigning equal weight to all pedigrees or applying a weighting scheme power 0.5 to control for different pedigree structures. Chromosomal regions exhibiting the highest NPL scores in the genome-wide scan were further studied by adding additional fine mapping markers and extension of the material with additional British families.

The initial genome-wide scan of the Swedish material revealed seven regions of interest for further fine mapping. Regions on chromosome 2q, 7p, 8q and 13q were implicated from statistical analyses with both equal weight and weighted analysis. 12q was proposed from the equal weighted analysis, and 6p from the weighted analysis.

Table 6. Fine mapping regions, position determined by flanking fine mapping markers.

Chromosome	Cytogenetic location	Position (cM)*			Flanking markers	
2	2p23.3-2p21	49.6**	-	68.84	D2S2144	D2S2298
2	2q23.3-2q31.1	159.43**	-	177.98	D2S2299	D2S335
6	6p25.2-6p21.1	11.3	-	68.9**	D6S1617	D6S1650
7	7p22.3-7p14.3	3.5**	-	47.84	D7S1532	D7S2496
8	8q24.21	132.43**	-	135.57**	D8S1774	D8S1801
12	12q24.22-12q24.23	136.23**	-	139.04**	D12S2082	D12S385
13	13q12.13-13q14.13	17.41	-	50.11**	D13S1254	D13S1312

*Based on deCODE map **Position missing in deCODE map, position estimated based on Marshfield map

Fine mapping enhanced the NPL score in five of the seven fine mapped regions, presented in Figure 2. Threshold values for suggestive and significant linkage were defined using material specific simulations according to Lander Kruglyak's criteria. This provided evidence in favor of significant or suggestive linkage to four loci:

- 2q24.3 evidence of significant linkage in equal and weighted analysis
- 7p21.3-7p22.2 , evidence of significant linkage in the weighted analysis and suggestive linkage in the equal analysis.
- 6p21.31 evidence of suggestive linkage in weighted analysis
- 12q24.23 evidence of suggestive linkage in the equal weight analysis

Table 7. Fine mapping results, equal weight analysis. Marker and highest NPL score from each fine-mapped region reaching NPL>1.5 is listed.

Chr	Cytogenetic location	Marker	Position (cM)*	NPL-score	Significance defined by simulations
2	2q24.3	D2S111	171	3.10	Significant linkage (NPL >3.04)
7	7p22.2	D7S1532	3.5**	2.98	Suggestive linkage (NPL >2.18)
12	12q24.23	D12S366	137.36	2.63	Suggestive linkage (NPL >2.18)
13	13q12.2	D13S1244	18.72	1.75	n.s
6	6p24.3	D6S470	23.75	1.65	n.s

*Based on deCODE map; **Position missing in deCODE map, estimated position based on Marshfield map; n.s non significant

**Table 8.** Fine mapping results, weighted analysis. Marker and NPL score from each fine-mapped region reaching NPL>1.5 is listed.

Chr	Cytogenetic location	Marker	Position (cM)[*]	NPL-score	Significance defined by simulations
7	7p21.3	D7S2514	14.94	4.55	Significant linkage (NPL >3.46)
2	2q24.3	D2S111	171	3.77	Significant linkage (NPL >3.46)
6	6p21.31	D6S1568	53.85	2.97	Suggestive linkage (NPL >2.69)
13	13q12.3	D13S217	22.17	2.55	n.s
2	2p22.3	D2S1788	58.96[**]	2.16	n.s

[*]Based on deCODE map; [**]Position missing in deCODE map, estimated position based on Marshfield map; n.s non significant; n.s non significant
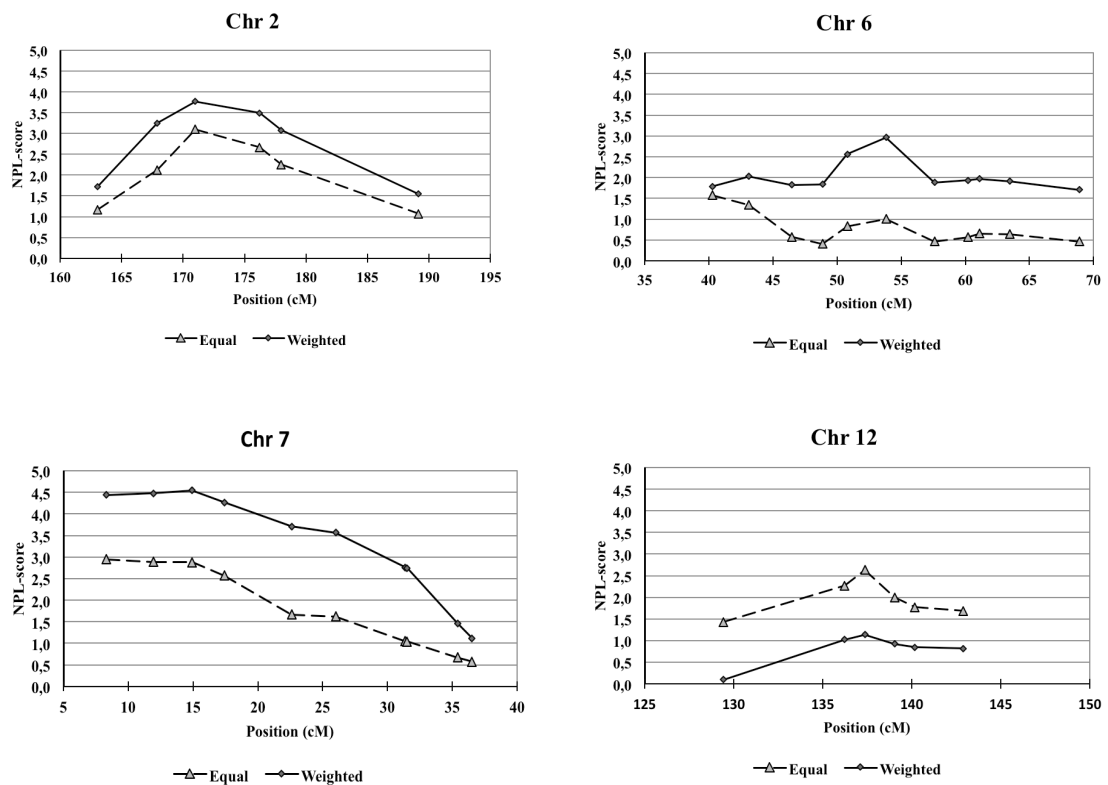
Figure 9. Chromosomal regions exhibiting significant or suggestive linkage after fine-mapping using equal weight or weighted analysis.

NPL threshold for significant linkage: 3.04 (equal analysis), 3.46 (weighted analysis)

NPL threshold for suggestive linkage: 2.18 (equal analysis), 2.60 (weighted analysis)

Interestingly, previously proposed IHPS candidate genes *NOS1* (12q24.22) and *MLN* (6p21.31) are located in two of the regions showing evidence in favour of linkage. Further mining of these loci and their most adjacent regions for novel candidate genes, identified neuropeptide Y (*NPY*) and GLP-2 (coded by the glucagon gene *GCN*) as two

interesting functional candidates. However, mutation screening of *NPY* and exon 5 *GCN* did not identify any obvious pathological mutations.

Reanalysis of the fine-mapped regions with the material extended with British families failed to reach the same level of NPL-score as in the analysis including only the Swedish material.

Two different strategies to continue mapping of potential candidate regions from a genome-wide scan are to either extend the material by adding more families, or to fine-map the regions by adding more dense markers. Additional markers gives more information that can enhance the resulting NPL-score, provided the markers from initial analysis was not fully informative and true linkage exists. It can also narrow the susceptibility region making the search for candidate genes more precise (Haines 1998, Lander Kruglyak 1995).

In our study, all available Swedish IHPS families were included already in the initial genome-wide scan. Extending the material would therefore imply inclusion of families from another population. So as a first step we performed fine mapping with more dense markers using the Swedish material. This enhanced the NPL scores in five of the seven fine-mapped regions, supporting existence of linkage in these regions. Four loci exceeded threshold value for genome-wide significant or suggestive linkage.

The definition of significant genome-wide linkage according to Lander and Kruglyak means a less than 5% risk of a NPL-score in the genome-wide scan reaching the threshold value for linkage by coincidence. The threshold for suggestive linkage is reached at one point along the chromosomes in average once per genome-wide scan (Lander and Kruglyak1995).

In our results, loci at 2q24.3 and 7p21.3-7p22.2 reached significant or suggestive linkage in both statistical models used. The locus 12q24.23 harboring the *NOS1* gene, exhibits suggestive linkage in the equal, but not weighted analysis. This indicates that possibly one or a few of the extended families alone contributes to the NPL-score peak, an effect being compensated for and therefore disappears in the weighted analysis. It is implicated to separately analyse the contribution from each family at this locus in a future study. This can possibly identify further families exhibiting linkage to the NOS1

gene as shown by Chung et al (1996). It is unclear why the locus on chromosome 6p21.31 exhibits suggestive linkage in the weighted analysis alone, and not in the equal weight analysis.

Finally, extending the material with additional British IHPS families in the regions where the Swedish material showed linkage, did not enhance the NPL-score. This could of course be due to false positive results in the Swedish study, but also due to differences between the genetic backgrounds of the two populations represented in the samples. The importance of different genes contributing to a disease can apparently differ within a population, and this is likely to be even more apparent between different populations.

In conclusion, we have identified three novel IHPS loci on chromosome 2q, 6p, and 7p. Also, a loci on chromosome 12q previously reported with evidence of linkage was confirmed with suggestive linkage in our material.

EPIDEMIOLOGICAL STUDY OF IHPS RISK FACTORS

A rapid and pronounced decline in IHPS incidence during the 1990s implies a major change of one or multiple environmental risk factors during this period (Persson 2001, Hedbäck 2001, Sommerfield 2008). We performed a nation wide population study based on register data from Swedish National Patient Register (NPR) and Swedish Medical Birth Register to study the incidence and analyse maternal and perinatal characteristics as IHPS risk factors. To evaluate any changes of risk factors possibly causing the decline in IHPS incidence, trends in ORs were analysed over time. The skew gender distribution with predominance of boys, implied analysis stratified for gender to assess if risk factors differ between boys and girls. Also, analysis was stratified for age at diagnosis to evaluate differences in characteristics for infants with early or late onset of disease.

The study cohort consisted of 3796 surgically treated IHPS cases born and diagnosed 1973-2008, and 18985 matched controls. Twins were excluded from the case-control part of the study since they do not have reliable data from Medical Birth register. After exclusion of twins, 3608 cases and 17588 controls remained for the case-control study.

Descriptive analysis

The nationwide IHPS incidence decreased to one fifth during the period 1988-1997 (2.25 to 0.43 per 1000 live births) and then remained constant at a low level around 0.6/1000. Since NPR not has full national coverage until 1987, nationwide incidence data before 1987 was omitted. Stockholm, which has complete NPR data from 1973 was instead used as a model to estimate nationwide incidence level before 1987. Incidence in Stockholm co-varied with nationwide incidence during time period 1987-2008 (Figure 10). Three periods with high, declining and low incidence respectively, was defined (high incidence 1973-1988, declining incidence 1989-1997 and low incidence 1998-2008). Incidence stratified by gender showed a relatively constant sex ratio of 5-6:1 boys to girls independent of changing incidence (Figure 11).
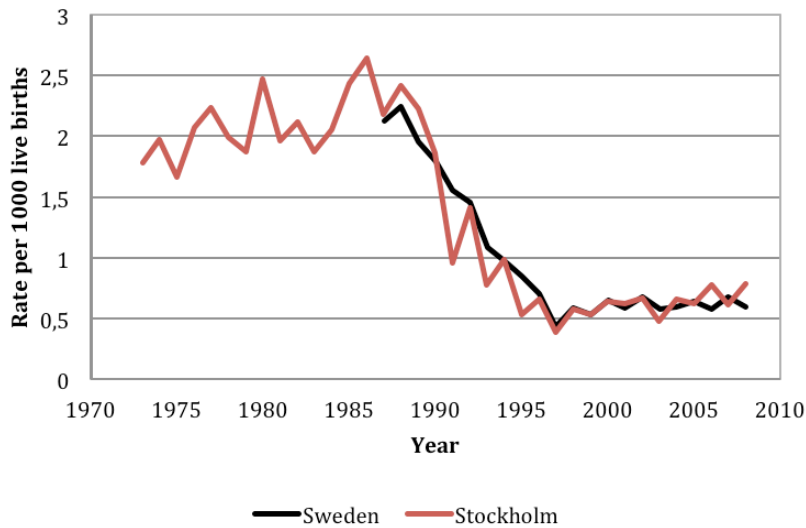
Figure 10. Incidence of IHPS in Sweden and Stockholm. Nationwide data only available from 1987 and onward.
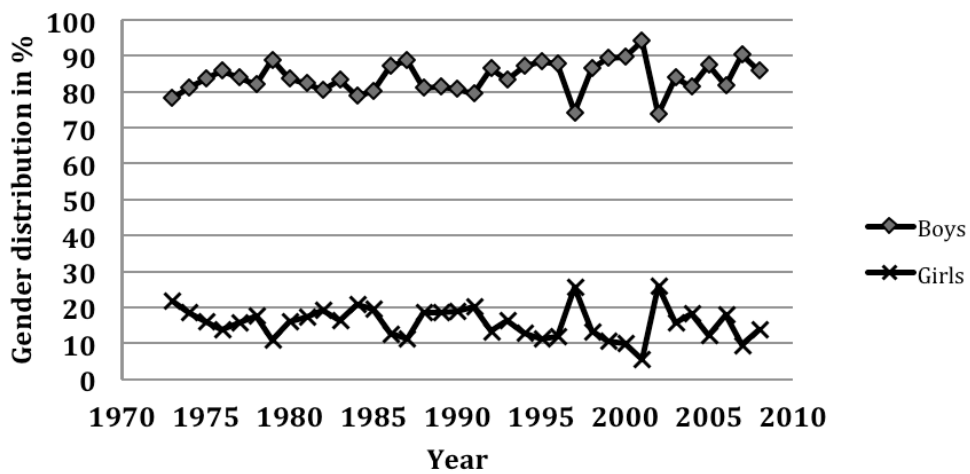

Figure 11. Proportion of boys and girls among IHPS cases in Sweden 1973-2008.

Median age at diagnosis was 5 weeks, and the majority of cases (>95%) were diagnosed before 13 weeks of age. Analysing age at diagnosis stratified for time periods, revealed a higher percentage of cases diagnosed before 5 weeks of age in recent years (Figure 12). Analysing age at diagnosis stratified for gestational age showed that very preterm infants presented later with the disease (Figure 13). There seems to be high number of very preterm infants diagnosed during first two weeks after birth. However, this is most probably due to a methodological error when registering

age at diagnosis. Very preterm infants are often admitted to the neonatal unit immediately after birth, and often stay for several weeks. If they are treated for IHPS during the hospitalization, they will falsely be registered for age at diagnosis the same day as they were admitted to the neonatal unit.
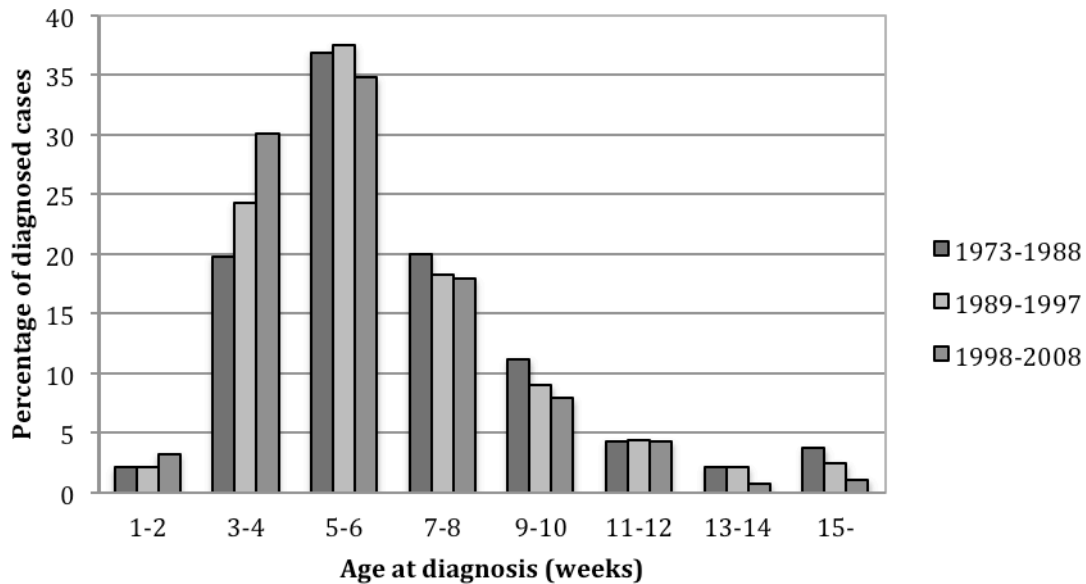
Figure 12. Age at diagnosis for different time periods.
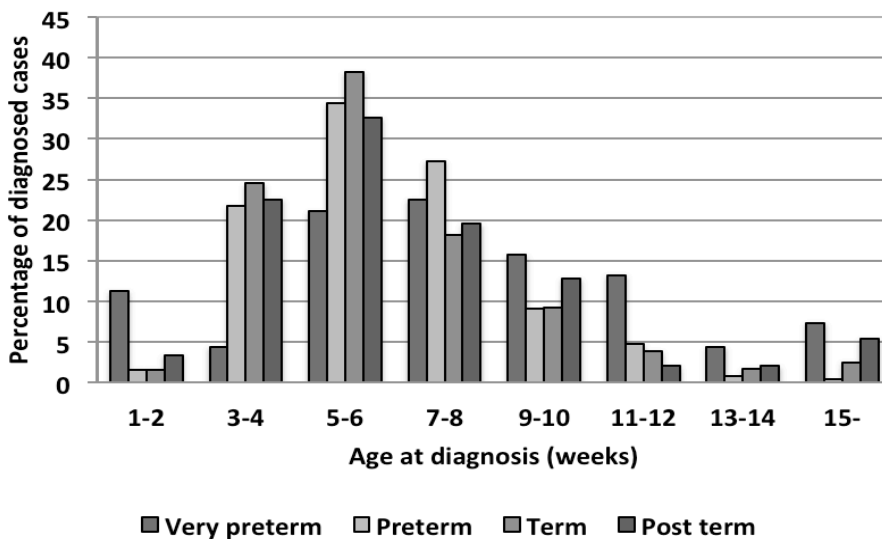
Figure 13. Age at diagnosis for infants born very preterm, preterm, term or postterm. Very preterm infants presented later with the disease than all other groups.

A higher frequency of twins was seen among cases (5%) compared to controls (2.5%). 13% of the twin cases were concordant for the disease and all of these twin pairs had

the same gender (9 male twin pairs, 3 female twin pairs). However, data regarding zygosity is not included in the register and concordance for MZ and DZ twins separately were therefore not possible to evaluate.
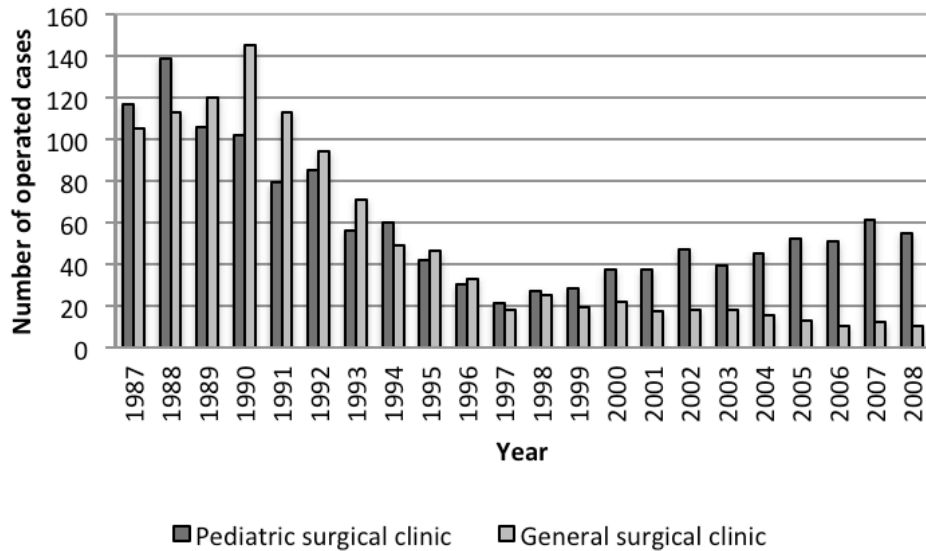
Figure 14. Annual number of IHPS cases surgically treated at a pediatric surgical clinic and general surgical clinic, respectively.

A survey of which hospitals the IHPS patients were surgically treated at since 1987 was performed. This showed a trend of increased percentage of patients treated at pediatric surgery clinics in recent years.

Case control study

To assess a number of variables from Medical Birth Register as possible IHPS risk factors, cases were compared to controls using multivariate analysis. Two different models were used because data on smoking and BMI was not available for the whole study period. Model 1 includes the whole study period 1973-2008 but not the variables smoking and BMI. Model 2 includes data from 1982-1989 plus 1992-2008, and all study variables including smoking and BMI. Results from preferably model 2 are shortly commented below:

Table 9. Selected variables from multivariate analysis with adjusted ORs

Variable	Model 1, data from 1973-2008 Cases n=3608[a] Controls n=17588[a] OR (95% CI)	Model 2, data 1982-89, 1992-2008 Cases n=2353[a] Controls n=11461[a] OR (95% CI)
Birth order		
1	1	1
2	0.78 (0.71-0.85)	0.76 (0.67-0.86)
3	0.81 (0.72-0.91)	0.77 (0.64-0.92)
≥4	0.80 (0.67-0.96)	0.80 (0.62-1.03)
Gestational age (weeks)		
Very preterm (<35)	2.09 (1.68-2.59)	1.44 (1.00-2.07)
Preterm (35-36.9)	1.53 (1.29-1.82)	1.48 (1.15-1.90)
Term (37-41.9)	1	1
Postmature (42-)	0.94 (0.83-1.08)	0.82 (0.67-1.01)
Labour		
Caesarean section	1.68 (1.52-1.86)	1.73 (1.50-2.01)
Normal/vaginal birth	1	1
Neonatal jaundice		
Yes	1.06 (0.88-1.27)	1.40 (1.07-1.82)
No	1	1
Maternal age (years)		
<20	1.42 (1.17-1.72)	1.24 (0.90-1.72)
20-24	1.34 (1.21-1.47)	1.31 (1.13-1.52)
25-29	1	1
30-34	0.94 (0.85-1.03)	0.92 (0.80-1.07)
35-	0.98 (0.86-1.12)	0.99 (0.83-1.19)
Maternal smoking[b] (daily cigarettes) **		
None	NA	1
1-9	NA	1.62 (1.40-1.88)
≥10	NA	1.66 (1.38-2.00)
Maternal birth country		
Nordic	1	1
Non-Nordic	0.50 (0.42-0.58)	0.58 (0.47-0.72)

Abbreviations:
[a] twins excluded; [b] maternal smoking during early pregnancy;
**Data of smoking available from 1982; NA, non applicable

Birth order. Being first born was associated with an increased IHPS risk. Infants born as second or third child had a 23-24% decreased IHPS risk compared to being first born.

Gestational age. Infants born before 37 weeks gestational age had an almost 50% increased IHPS risk. Categorizing this group into very preterm and preterm born infants, the very preterm group reached only borderline significance (OR 1.44; 95%; CI 1.00-2.07) but the preterm group exhibited a significantly increased IHPS risk (OR 1.48; 95% CI 1.15-1.90).

Birth weight for gestational age. No significant difference in birth weight for gestational age was observed between cases and controls.

Labour. Delivery with caesarean section was associated with 73% increased IHPS risk compared to vaginal birth. Indication (emergency or elective) for the performed caesareans was only available for a small percentage, thus further categorization and analysis of this group was not possible to perform.

Neonatal jaundice. The risk of IHPS was increased with 40% in infants diagnosed with neonatal jaundice. However, neonatal jaundice did not reach significance as a risk factor in model 1.

Maternal age. In model 1, a 34-42% increased risk for IHPS was noted for infants with mothers younger than 25 years. However, in model 2 only infants with mothers aged 20-24 had a significantly increased IHPS risk (OR 1.31; 95% CI 1.13-1.52).

Prepregnant BMI. Infants to mothers with overweight exhibited a 19% increased IHPS risk (OR 1.19; 95% CI 1.03-1.36). Infants to mothers with underweight, normal weight and obesity had no increased risk.

Maternal smoking was associated with an over 60% increased IHPS risk for the infant. A weak quantitative correlation between the number of daily cigarettes and ORs were noticed.

Ethnicity. Non-Nordic maternal birth country was associated with a 42% lower risk for IHPS.

Diabetes. No association between IHPS risk and maternal diabetes was observed.

Analysis stratified for time period, trends over time

To assess changes in risk factors related to the changed IHPS incidence during the 1990s, analysis stratified for time using the defined time periods for high, declining and low incidence, respectively, was performed. Main finding was that ORs for smoking significantly increased over time when incidence decreased. During the period with low incidence (1998-2008), infants to mothers who smoked ≥10 cigarettes daily had an over 2-fold increased risk for IHPS. Low birth rank reached not significance as a risk factor during the time period with high incidence (1982-1989), and prematurity failed to reach significance during the time period with low incidence.

Analysis stratified for gender

Analysis was stratified for gender to study if the risk factors had different influence on boys and girls. Analysis for girls rely on a very much lower number of cases compared to boys (366 girls vs. 1987 boys). Thus, results in the analysis stratified for girls are less likely to reach significance. This might explain why boys reach significance for several more study variables compared to girls.

Birth rank as a risk factor, reached only significance among boys. Very preterms did not reach significance in either group. OR for prematures was higher among girls than boys, but not significantly (Girls: OR 2.03; 95%CI 1.08-3.78, Boys: OR 1.39; 95% CI 1.05-1.83). Neonatal jaundice did not reach significance as a risk factor among girls, neither did young maternal age or maternal Nordic birth country.

Analysis stratified for age at diagnosis

Analysis stratified for age at diagnosis identified that being very preterm was a significantly higher risk factor among cases with late diagnosis (OR 2.66; 95% CI 1.55-4.56) compared to early diagnosis (OR 0.81; 95% CI 0.47-1.39).

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In summary we confirmed previous reports of a pronounced decreased incidence of IHPS in Sweden during the 1990s. The incidence has remained low and we can therefore state this not being a temporal variation. Whatever the cause for the decline in incidence, it seems to remain unchanged since end of the 1990s. The constant skew gender distribution supports a genetic background of the male predominance, even though environmental factors not can be excluded. A trend of earlier diagnosis is noted in recent years, and it is also obvious that very preterm born infants have a later onset of the disease.

We confirmed the previously suggested risk factors low birth rank, prematurity, low maternal age and maternal smoking. Also, we identified a previously not reported association to caesarean section giving an over 70% increased risk for IHPS. The mechanism can only be speculated on. However, the risk is adjusted for possible confounders and the increased risk is stable when analysed over time and not differing between groups when stratified for gender and age at diagnosis. Association of being first born and IHPS can be possibly be explained by both maternal factors and other exposures such as infections etc from siblings.

No published study so far, has analysed changing trends of risk factors over time. This could be an explanation why some studies from different time periods reach different conclusions. However, we found no major changes in environmental risk factors reflected in the study variables, that could explain the drop of incidence during the 1990s. Though, maternal smoking was identified as a relatively more important risk factor in recent years during low incidence, giving a 2-fold increased risk IHPS risk. As in previous study by Sörensen 2002, we cannot from the registered data determine if smoking registered during early pregnancy was continued during the rest of the pregnancy and after delivery. Therefore the mechanism how smoking causes an increased IHPS risk is unclear.

10 CONCLUDING REMARKS

In consistency with the multifactorial background of IHPS we have studied, and confirmed evidence of both genetic and environmental factors playing a role in the pathogenesis of IHPS.

- Three novel chromosomal loci with evidence in favour of linkage were identified on chromosome 2q24.3 (NPL=3.10), 7p21.3 (NPL=4.55) and 6p21.31 (NPL=2.95) in our Swedish material. We also confirmed a previous report of linkage to 12q24.23 (NPL=2.63) harboring the *NOS1* gene. Interestingly, the *MLN* gene previously studied in a candidate gene approach study, is located in the chromosome 6 candidate region.

- Candidate gene motilin (*MLN*) was studied without finding of mutations with obvious connection to the disease, or significant association of the p.Val15Ala polymorphism. This however, does not exclude the possibility of *MLN* playing a role in the pathogenesis of IHPS, especially since evidence of linkage was found to the chromosomal region harboring the *MLN* gene. In addition, the promoter region is not studied and also association of the p.Val15Ala polymorphism could be studied in a larger material.

- A previously described association of NOS1 exon 1c polymorphism rs41279104 could not be confirmed in our material. Due to the complexity of the *NOS1* gene, it is likely that alternative regulatory mechanisms apart from the studied polymorphism will be found that thus could explain the diverging results.

- Caesarean section was identified as a novel risk factor for IHPS associated with an almost 80% increased risk for IHPS. Previously suggested risk factors as low birth rank, prematurity, jaundice, low maternal age and maternal smoking was confirmed. Smoking and primiparity were more important risk factors in recent years when IHPS incidence is low. Maternal smoking was associated with an two-fold increased IHPS risk. Association between prematurity and late onset of disease was identified.

11 POPULÄRVETENSKAPLIG SAMMANFATTNING

Infantil hypertrofisk pylorusstenos (IHPS) är en sjukdom som drabbar spädbarn upp till några månaders ålder. Detta tillstånd innebär att den nedre magmunnen, pylorus, blir kraftigt förtjockad och därmed utgör ett näst intill totalt passagehinder mellan magsäck och tolvfingertarm. De drabbade barnen insjuknar vanligen vid 3-8 v ålder med kraftiga kräkningar efter matning till följd av den trånga passagen mellan magsäck och tarm. Relativt omgående blir de drabbade barnen svårt uttorkade med karakteristiska rubbningar i saltbalansen. Sjukdomen botas med en operation, pyloromyotomi, då pylorusmuskeln klyvs vilket omedelbart botar barnet.

IHPS drabbar omkring 1.5-3 per 1000 födda och är 5 ggr vanligare hos pojkar jämfört flickor. Förekomsten av sjukdomen minskade kraftigt under 1990-talet i Sverige och flera andra Europeiska regioner av oklar orsak. Dock är IHPS fortfarande en av de vanligaste indikationerna för kirurgi på spädbarn under första levnadsmånaderna.

Det finns väl underbyggda bevis för att IHPS är en så kallad komplex sjukdom med multifaktoriell bakgrund, där både genetiska- och omgivningsfaktorer samt ett samspel mellan dessa bidrar till att sjukdomens utvecklas. Sjukdomens uppkomstmekanism är dock ej klarlagd, men mycket tyder på att en spasm (kramp) i pylorusmuskeln kan utlösa sjukdomen. Syftet med denna avhandling var att belysa och försöka klargöra genetiska- och omgivningsfaktorer associerade till sjukdomen.

I studie *I-III* har två principiellt olika metoder använts för att leta efter sjukdomsgener, kandidatgensmetoden och kopplingsanalys. Vid kandidatgensmetoden utgår man från en tänkbar sjukdomsmekanism, och identifierar utifrån denna de sjukdomsgener som potentiellt kan vara av betydelse. Dessa så kallade kandidatgener screenas därefter för mutationer eller association mellan särskilda varianter av genen (polymorfier) och sjukdomen. Vid kopplingsanalys analyseras istället, förutsättningslöst och utan nödvändig förkunskap om sjukdomens uppkomstmekanism, hur genetiska markörer nedärvs i familjer med flera fall av sjukdomen. De kromosomregioner de sjuka ärvt gemensamt oftare än vad som kan förväntas orsakat av slumpen, kallas kandidatregioner och härbärgerar sjukdomsassocierade gener med en sannolikhet angiven som en "sannolikhets score".

Motilin är ett hormon i magtarm-kanalen som inducerar kontraktioner (muskelsammandragningar) i magsäck och tarm. I studie *I* identifierades motilin som en kandidatgen mot bakgrunden av att antibiotikabehandling med erytromycin före 2 v ålder ger en ökad risk för spädbarn att utveckla IHPS. Erytromycin har förutom dess antibakteriella effekt en sidoeffekt som motilin agonist, dvs den binder till motilinreceptorerna i tarmen och stimulerar receptorernas aktivitet. Hypotesen i studie *I* var därför att det kroppsegna motilin hormonet, liksom erytromycin, kunde vara delaktigt i uppkomsten av IHPS genom att framkalla spasm i pylorus. Mutationsscreening av motilingenen identifierade tre tidigare okända sekvensvariationer som dock ej hade någon tydlig koppling till sjukdomen. Vi kunde inte heller identifiera någon signifikant association till en tidigare känd polymorfi i motilingenen.

Kvävemonoxid (NO) är en signalsubstans som medierar relaxation (avslappning) av glatt muskulatur bland annat i mag-tarm kanalen. NO i tarmens nervsystem bildas med hjälp av enzymet neuronalt kväveoxidsyntas (nNOS) som därför spelar en nyckelroll i den signalväg där en störning kan tänkas leda till pylorusspasm. En variant i nNOS genens (*NOS1*) regulatoriska region som påverkar hur mycket genen uttrycks, har tidigare visats vara associerad till IHPS. I studie *II* analyserades denna regulatoriska *NOS1* polymorfi i vårt svenska material utan att vi kunde konfirmera tidigare påvisad association.

Kopplingsanalys i 37 svenska familjer med minst två personer som behandlats för IHPS uppvisade koppling till regioner på kromosom 2, 6, 7 och 12. I dessa regioner återfinns intressant nog både motilin genen (kromosom 6) och nNOS genen (kromosom 12). Utöver dessa identifierade vi ytterligare två kandidatgener som screenades för mutationer, *NPY* (kromosom 7) och *GCG* (kromosom 2), dock utan fynd med uppenbar koppling till sjukdomen.

Den kraftigt minskade incidensen av IHPS under 1990-talet indikerar förändrad förekomst av en eller flera riskfaktorer under denna period. I syfte att studera incidensvariation och riskfaktorer utfördes i studie *IV* en registerstudie omfattande alla opererade IHPS fall i Sverige under perioden 1973-2008. Incidensen av IHPS föll till en femtedel under perioden 1988-1998, hela tiden med en väsentligen oförändrad könskvot 5:1 mellan pojkar och flickor. Kejsarsnitt, prematuritet (för tidigt född), gulsot under nyföddhetsperioden och att vara förstfödd i en syskonskara, var associerat med en ökad risk för IHPS i vårt material. Maternell rökning, ung moder och mor med

nordisk etnicitet var också associerat med en ökad risk för IHPS. Under senare år då IHPS incidensen varit låg konstaterades en ökad betydelse av rökning och primiparitet som riskfaktorer. Prematuritet var en högre riskfaktor för patienter med sent insjuknande jämfört insjuknande vid låg ålder. Denna studie är den första som påvisat ett samband mellan kejsarsnitt och risk för IHPS.

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