Hamartomatous Polyps – A Clinical and Molecular Genetic Study

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THE SIX ORIGINAL PAPERS ARE

1. Jelsig AM, Ousager LB, Brusgaard K, Qvist N. Juvenile polyps in Denmark from 1995-2014. Accepted for publication.

2. Jelsig AM, Qvist N, Brusgaard K, Nielsen CB, Hansen TP, Ousager LB. Hamartomatous polyposis syndromes: A review. Orphanet J Rare Dis. 2014 Jul 15; 9:101.

3. Jelsig AM, Brusgaard K, Hansen TP, Qvist N, Larsen M, Bojesen A, Nielsen CB, Ousager LB. Germline variants in Hamartomatous Polyposis Syndrome-associated genes from patients with one or few hamartomatous polyps. Submitted

4. Jelsig AM, Qvist N, Brusgaard K, Ousager LB. Research participants in NGS studies want to know about incidental findings. Eur J Hum Genet. 2015 Oct; 23(10):1423-6.

5. Jelsig AM, Qvist N, Sunde L, Brusgaard K, Hansen Tvo, Wikman FP, Nielsen CB, Nielsen IK, Gerdes AM, Bojesen A, Ousager LB. Disease pattern in Danish patients with Peutz-Jeghers Syndrome Submitted.

6. Jelsig AM, Tørring PM, Kjeldsen A, Qvist N, Bojesen A, Jensen UB, Andersen MK, Gerdes AM Brusgaard K, Ousager LB. JP-HHT phenotype in Danish patients with SMAD4 mutations. Clin Genet 2015 Nov 17. doi: 10.1111/cge.12693. [Epub ahead of print]

BACKGROUND

Polyps in the gastrointestinal tract

Polyps in the gastrointestinal (GI) tract are defined as nodules or masses that project above the level of the surrounding mucosa. Polyps are most common in the colon but can be found throughout the GI tract and at extraintestinal sites. The prevalence of polyps in the general population is unknown as polyps may be asymptomatic. Autopsy studies suggest that about 30-60% of adults have colonic polyps (1-3). GI polyps vary in size from a few millimetres to several centimetres in diameter and can be described according to their gross structure: Pedunculated (with a stalk) or sessile (without a stalk). They may also be classified as non-neoplastic or neoplastic based on their histopathological appearance. The most common types of polyps in the large bowel are hyperplastic polyps and adenomas (1, 2, 4), the latter considered as having the potential to progress to cancer (neoplastic). The inflammatory polyp, which is characterized by fibromuscular hyperplasia of the lamina propria, mixed inflammatory infiltrates, erosion, and epithelial hyperplasia, is rare (5).

Hamartomatous polyps

The hamartomatous polyp (HP) is also rare. It is considered to be a non-neoplastic tumour-like growth consisting of normal tissue and normal mature cells in abnormal number or distribution. It can occur anywhere in the body, and whereas malignant tumours contain poorly differentiated cells, HPs consist of distinct cell types. HPs in the GI tract can be subdivided into different histopathological categories based on their histopathological appearance: the juvenile polyp (JP) and the Peutz-Jeghers polyp (PJP). Some also mention HPs related to ganglioneuromatosis and HPs of Cronkhite-Canada type (6). The JP was first reported by *Diamond* in 1939 (7) and *Helwig* in 1946 (8), and the histopathological distinction to adenomas was finally described by *Horrilleno et al.* in 1957 (9). Macroscopically, JPs are typically lobulated and pedunculated with surface erosion and may vary in size from a few millimetres to several centimetres (10). Histopathologically they appear cystic with dilated glands with inflammatory cells (Figure 1A and 1B). The PJPs are typically large and pedunculated with a lobulated shape with sizes that vary from few millimetres to several centimetres (Figure 3). They are histopathologically characterized by an arborizing network of smooth muscle, lamina propria, and glands lined by a normal appearing epithelium (5) (Figure 2). Although, the different subtypes of HPs and other GI polyps are well characterized, it can be difficult to distinguish them from each other at histopathological examinations. Especially inflammatory polyps and JPs can resemble each other.

Frequency and localization of hamartomatous polyps

The JP is considered rare in the general population, but the exact prevalence is difficult to determine, as some polyps may be asymptomatic throughout life. Yet, the JP is the most common type of polyp in children comprising over 90% of polyp cases (11-13). JPs are mainly found in the rectosigmoid, but are also localized in the remaining part of the colon in a significant amount of cases (14-

17). Most patients have a single JP (17, 18), but patients with multiple polyps can have Juvenile Polyposis Syndrome (JPS) as described later. The PJP is even more rare and can be found throughout the GI tract, but mainly in the small bowel as part of the Peutz-Jeghers Syndrome (PJS). Cases of solitary PJPs without additional symptoms of PJS have been described in few cases (19-21), but whether a single PJP is a clinical entity distinct from PJS is not clear at the moment (22).

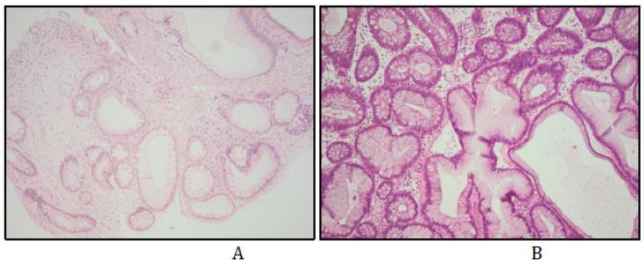

Figure 1: A: *Histopathological image of a JP from a patient with a single JP. Note the cystic appearance. Magnification x10.* ***B****: Histopathological image of a JP from a study patient (Paper VI, Family III, Patient no.1) with JPS and a germline SMAD4 mutation. There is no clear difference in appearance between sporadic JPs and those appearing in JPS, but the syndromic polyps have been described as having a non-expanded stroma and higher crypt density (23). Magnification x10.*

Symptoms

The symptoms reported in patients with JPs include rectal bleeding, abdominal pain, anal extrusion of the polyp, anaemia, diarrhoea, and/or constipation. Rectal bleeding has been reported as the most frequent symptom and is seen in over 90% of cases (13, 24). Most JPs in children are diagnosed in the first decade of life with a mean age of approximately five years, but can be diagnosed throughout childhood and adolescence (25-30). JPs in adults are less investigated: *Roth&Helwig* reported 59 adults with JPs with a mean age of 25.5 years and with rectal bleeding as the most common symptom, followed by prolapse and abdominal pain (31). *Nugent et al.* studied JPs in both adults and children and found a mean age at diagnosis of 32 years (32). For PJPs the presenting symptom can be small bowel obstruction, which is seen as the presenting symptom in 40-50% of patients with PJS (33). Other symptoms include abdominal pain and rectal bleeding. The age at first GI symptom in PJS patients varies considerably, but the median age has been reported to be in adolescence, at 12-15 years of age (34, 35). Importantly, mucocutaneous pigmentations, usually located in and around the mouth and nostrils, often precede the first GI symptoms.

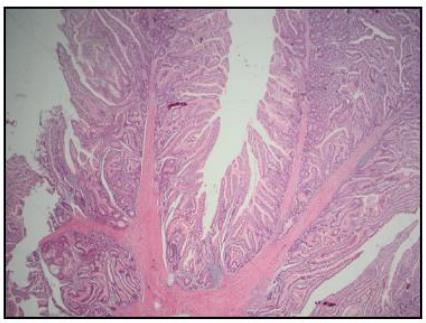

Figure 2: *Histopathological image of a PJP from a study patient with PJS. Note the chracteristic arborizing network of smooth muscle. Magnification x2*

Clinical management

The clinical management of polyps vary according to their localization and size. Polyps in the large bowel are often detected using endoscopy and removed with polypectomy concurrently. Gastric or duodenal polyps are removed concurrently with gastroscopy. For patients, who need surveillance of the stomach, duodenum, and/or the large bowel, endoscopy is the method of choice as well. Surveillance and removal of polyps in the small bowel are especially relevant in PJS patients, but is complicated. Video Capsule Endoscopy (VCE) has proven to be a good method for detection of PJPs, but the detection rate and visualization of the entire small bowel may not be complete (36-38). An alternative to VCE is MR enterography (MRE), which has been studied in PJS patients (39-41). One study showed that the accuracy of polyp localization and size was better with MRE compared to VCE, but that VCE detected smaller polyps more often (40). Yet, the detection rate for polyps >10-15 mm has been reported to be the same (39, 40), or better with VCE compared to MRE (42). Patients are also reported to prefer VCE to MRE (39, 42).

Ideally any visualized polyp should be removed to prevent complications. Push-enteroscopy has for long been the preferred method, but the depth of insertion is limited. Thus Device-assisted enteroscopy, including double-balloon enteroscopy, single-balloon enteroscopy, spiral enteroscopy, and balloon-guided endoscopy, has largely replaced push-enteroscopy, but is more labour-intensive (43). Studies have described a high diagnostic yield with successful polypectomy when using double-balloon enteroscopy in PJS patients (44). Guidelines from The European Society of Gastrointestinal Endoscopy, recommend small bowel surveillance in PJS patients with VCE and/or MRE/enteroclysis, depending on local availability, expertise, and patient preference; they also recommend Device-assisted enteroscopy with polypectomy when large polyps (>10-15 mm) are detected (43). An acute clinical presentation in the course of invagination or other complications often result in laparotomy with removal of the affected part of the bowel.

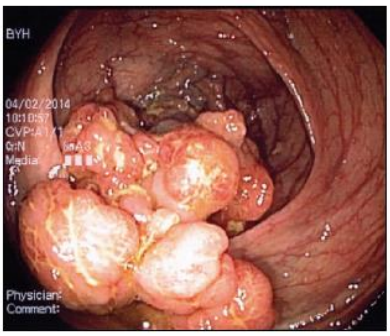

Figure 3: *Macroscopic appearance of PJPs in a study patient with PJS*

Single juvenile polyps and risk of cancer

The risk of cancer when having one or a few JP(s) is not clear. Generally, it is believed that a single or a few JP(s) do not increase the risk of cancer and do not require clinical follow-up. This assumption is based on few studies with a limited number of patients, thus *Nugent et al.* studied the survival rate and cancer occurrence in a population of 82 patients with solitary JPs and found no increased risk of cancer (32). Kapetanakis et al. investigated cancer occurrences in relatives of 24 children with a single JP and found no increased risk of more polyps or colorectal cancer (CRC) (45). Adenomatous transformations of single JPs have been reported (16, 46,

47), but no evidence-based practice guidelines exist for patients with one or a few JPs, who do not fulfil the criteria for JPS.

Hamartomatous Polyposis Syndromes

It is important to distinguish patients with one or a few HPs from patients with Hamartomatous Polyposis Syndromes (HPS). These patients typically have multiple HPs in the GI tract, a high risk of cancer from early age, and, sometimes, extraintestinal findings. The HPS account for only a small part of the inherited GI cancer syndromes and occur at approximately 1/10th of the frequency of adenomatous polyposis syndromes comprising <1% of CRC cases (48). The HPS include PJS, JPS, and the PTEN hamartoma tumour syndrome (PHTS). A high frequency of HPs has been reported in other syndromes, but these often have other features leading to the diagnosis. The syndromes include Gorlin Syndrome, Neurofibromatosis type 1, Hereditary Mixed Polyposis Syndrome, Multiple Endocrine Neoplasia type 2B, and Cronkhite-Canada Syndrome. Comprehensive reviews of the HPS have been published (48-53). In the following sections the syndromes will be briefly described.

Genetics and diagnostics of HPS

HPS are, with the exception of Cronkhite-Canada syndrome, inherited syndromes with an autosomal dominant inheritance pattern and age dependent penetrance. Diagnosis of HPS is usually based on a clinical approach, as clinical criteria for most HPS are available (52), and genetic testing is used frequently to confirm the diagnosis. When a patient has the characteristic clinical features as well as typical and numerous polyps, the diagnosis is often straightforward. Yet, the syndromes do share a phenotypic overlap and show significant inter- and intrafamiliar variation in expression, which can make diagnostics difficult. Furthermore, a significant part of HPS cases is sporadic *(de novo)* without affected family members. Candidate genes for most of the syndromes have been identified, but mutations cannot be detected in all patients with the syndromes; thus a genetic screening of HPS related genes cannot rule out the diagnosis. Still, all patients suspected of HPS should be offered genetic counselling in order to identify at-risk family members and receive information about prenatal options.

Peutz-Jeghers Syndrome

PJS (OMIM 175200) was first described by JT Connor and Hutchinson in 1885 and 1886 respectively (54, 55). The syndrome is named after Peutz, who described a family with autosomal dominant inheritance of GI polyposis and pigmented mucous membranes in 1921 (56) and Jeghers who defined the syndrome as a clinical entity (57). The syndrome is characterized by GI polyposis with PJPs (especially in the small bowel) and mucocutaneous pigmentations (see Figure 4). There is not definite international consensus about the clinical criteria, but *Beggs et al.* described a somewhat European consensus on behalf of a group of European experts (58). According to this paper a patient should fulfil one or more of following: (1) Two or more histologically confirmed PJS-type HPs, (2) any number of PJS-type polyps detected in one individual, who has a family history of PJS in a close relative(s), (3) characteristic mucocutaneous pigmentations in an individual who has a family history of PJS in a close relative(s), (4) any number of PJS-type polyps in an individual who also has characteristic mucocutaneous pigmentations (58).

The incidence of PJS is estimated to be 1:50,000 to 1:200,000 live birth (59). The first GI symptoms can present in infancy or childhood and 50-75% of patients experience GI symptoms before 20 years of age (34, 35), often preceded by mucocutaneous pigmentations. The most common GI symptoms are obstruction of the small bowel, abdominal pain, and rectal bleeding, with obstruction occurring in over 50% of patients before adulthood (33, 60). Several papers have discussed the natural history (33, 34, 61) and surveillance programmes, especially the question of small bowel surveillance (see also section 1.5) (58, 62, 63). Efforts have also been made to clarify possible genotype-phenotype correlations, but the results have not been consistent (35, 64-66). The risk of cancer has been assessed in studies with larger study populations (64, 67, 68). These studies have demonstrated a high, age-dependent risk of not only GI cancer but also extraintestinal cancer, especially testicular cancer, gynaecological cancers, and breast cancer. Germline mutations can be found in *STK11*, a gene consisting of nine coding exons and one non-coding exon. *STK11* mutations are detected in more than 90% of patients fulfilling the clinical criteria (69). Approximately 50% of the patients are *de novo* cases (34). The high risk of cancer in different organs leads to a rationale of a somewhat extensive surveillance program. This should at least include surveillance of the breast, cervix, GI tract, and testes, while surveillance of the ovaries and pancreas is debated (58, 62).

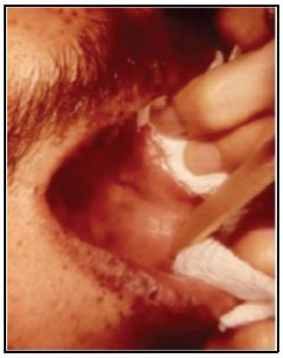

Figure 4*: Characteristic mucocutaneous pigmentations on the lips and the oral cavity of a PJS patient. With permission from Professor Flemming Skovby, Department of Paediatrics, Roskilde Hospital, Denmark.*

Juvenile Polyposis Syndrome

JPS (OMIM 174900) was first recognized as a clinical entity in the mid 1960es (70). The syndrome is characterised by multiple JPs throughout the GI tract, but mainly in the colon, rectum, and ventricle. The incidence is estimated to be approximately 1:100,000 (50). The widely used clinical criteria is based on those suggested by *Jass et al.:* (1) The findings of more than five JPs in the colon or rectum, and/or (2) multiple JPs throughout the GI tract, and/or (3) a JP together with a family history of JPS (71). Compared to PJS, the natural history and cancer risk estimates are less well investigated. The GI symptoms are mainly rectal bleeding, as with patients with a single JP, but can also be prolapse of the polyp, melena pain, diarrhoea, and/or anaemia (72). The risk of CRC and gastric cancer is reported to be high in several studies, yet the estimates vary: *Brosens et al.* calculated a cumulative life-time risk for CRC to be 38.7% (73), while *Howe et al*. found that 38% of a JPS kindred had CRC and 21% upper GI cancers. As with other inherited cancer syndromes the cancers seem to develop at a young age with a mean age reported to be around 40 years of age (74). Pancreatic cancer and cancer in the small bowel appear to be rare (74). Germline mutations are detected in *SMAD4* and *BMPR1A* in 20-30% of cases respectively, which leaves approximately 40-60% of JPS patients without a known genetic cause (75-78). Approximately 50% of affected patients have a positive family history (79).

Previous studies, before the era of molecular diagnostics, have reported JPS patients with symptoms of hereditary haemorrhagic telangiectasia (HHT), which include epistaxis, telangiectasias, and AV-malformations, mainly pulmonary (80, 81). Later studies have confirmed that patients with *SMAD4* mutations can have symptoms of both conditions (82-85), and the syndrome is now referred to as the JP-HHT syndrome (OMIM 175050). In addition, cases of aortic root dilatation have been described in patients with *SMAD4* mutations (86, 87). The AV-malformations in the lungs, GI tract, liver, and brain in JP-HHT patients can cause severe bleeding and potentially be life threatening. Moreover, gastric polyposis seems to be more frequent compared to *BMPR1A* mutations carriers (88-90).
The rationale for surveillance in JPS patients is based on the high risk of CRC and gastric cancer, and to avoid morbidity in relation to polyposis. As the clinical picture varies, so does the clinical approach: In some patients continuous endoscopic polypectomies will be sufficient, while others need a subtotal colectomy or gastrectomy. British guidelines for surveillance have been published in 2009-10 (91) and American guidelines in 2015 (62). *SMAD4* mutations carriers require additional follow-up for HHT and aortopathy. Guidelines for HHT surveillance have been described by *McDonald et al*. (92) and *Shovlin et al.* (93).

PTEN hamartoma tumour syndrome
PTEN hamartoma tumour syndrome (PHTS, OMIM 601728) includes Cowden Syndrome (CS, OMIM 158350), Bannayan-Riley-Ruvalcaba Syndrome (OMIM 153480), *PTEN*-related Proteus syndrome, and Proteus-like Syndrome. CS is the most common with a prevalence of approximately 1:200,000 individuals (94). The phenotypic spectrum of PHTS is wide and variably, and especially CS and Bannayan-Riley-Ruvalcaba Syndrome have a considerable phenotypic overlap. *PTEN*-related Proteus syndrome and Proteus-like syndrome are related to Proteus Syndrome, are very rare, and still rather undefined. The conditions are characterized by hamartomatous overgrowth of multiple tissues and diagnosis is usually based on the phenotype. The syndromes are not discussed further here.

Cowden Syndrome: CS is named after the patient Rachel Cowden, whose symptoms were described in a scientific paper in 1963 (95). She expressed several clinical features now recognized as typical of CS including mild mental retardation, multiple hyperkeratotic papillomata, as well as fibrocystic disease of the breast. In general, CS is characterized by a wide range of symptoms caused by multiple hamartomatous lesions of the skin and mucous membranes. Yet, some symptoms are considered to be pathognomonic, see Table 1. Furthermore, cancer in the thyroid, breast, endometrium, and brain characterize CS. More than 90% of individuals with germline *PTEN* mutations are believed to have symptoms by the age of 20 years, whereas nearly 100% have symptoms by the age of 30 years (96). Consensus diagnostic criteria for CS have been developed and are continuously updated (97), see Table 1. GI involvement, especially with polyps in the colon and rectum but also in the stomach, has been reported several times (98-101). The histology of the polyps is not solely HPs, but also adenomas and hyperplastic polyps as well as ganglioneurinomas. *Heald et al.* (99) found that GI polyps were reported in 51.2% of 127 individuals with *PTEN* mutations with 24 having both upper and lower GI polyps, and the authors argued for colonosocpy in the surveillance program (99). Studies including numerous patients with CS and/or *PTEN* mutations have demonstrated a high increased risk for cancer at various anatomical sites such as breast, thyroid, endometrial, colon, and renal carcinoma (102, 103). Germline mutations can be detected in *PTEN* with a mutation detection rate reported to be between 25-80% depending on the inclusion criteria (96, 104). The proportion of de novo cases is unknown, but the *de novo* frequency of *PTEN* mutations has been estimated to be 10-47% (105). Surveillance guidelines have been presented and discussed, but many follow the guideline from the National Comprehensive Cancer Network http://www.nccn.org/professionals/physician_gls/f_guidelines.asp.

Pathognomonic criteria

- Lhermitte-Duclos disease (adult) (cerebellar dysplastic gangliocytoma)
- Mucocutaneous lesions:
  - Facial trichilemmomas
  - Acral keratoses
  - Mucosal lesions
  - Papillomatous lesions

Major criteria

- Breast cancer
- Endometrial carcinoma
- Non-medullary epithelial thyroid cancer, especially follicular thyroid cancer
- Macrocephaly (≥97 percentile)

Minor criteria

- Other thyroid lesions (e.g, adenoma, multinodular goiter)
- Intellectual disability (IQ ≤75)
- Hamartomatous intestinal polyps
- Fibrocystic disease of the breast
- Lipomas
- Fibromas
- Genitourinary tumors (especially renal cell carcinoma)
- Genitourinary malformation
- Uterine fibroid

Operational diagnosis in an individual meeting any one of the following:

- Pathognomonic mucocutaneous lesions combined with one of the following:
  - Six or more facial papules, of which three or more must be trichilemmoma
  - Cutaneous facial papules and oral mucosal papillomatosis
  - Oral mucosal papillomatosis and acral keratoses
  - Six or more palmoplantar keratoses
- Two or more major criteria
- One major and three or more minor criteria
- Four or more minor criteria

In a family in which one individual meets the diagnostic criteria for CS as listed above, other relatives are considered to have a diagnosis of CS if they meet any one of the following criteria:

- The pathognomonic criteria
- Any one major criterion with or without minor criteria
- Two minor criteria
- History of Bannayan-Riley-Ruvalcaba Syndrome

Table 1: *The diagnostic criteria for Cowden Syndrome as reviewed by Eng. (106). Last updated February 2016.*

Bannayan-Riley-Ruvalcaba Syndrome
Bannayan-Riley-Ruvalcaba Syndrome is characterized by macrocephaly, GI polyposis with HPs, lipomatosis, and pigmented macules of the glans penis. HPs have been reported in up to 45% of cases (107). Recommendations of surveillance have not been established, but patients with *PTEN* mutations (~60% of patients with the syndrome) should undergo the same surveillance program as patients with CS (108).

Other syndromes with hamartomatous polyps
Hereditary Mixed Polyposis Syndrome (OMIM 601299): The syndrome is characterized by a mixed pattern of polyps in the large bowel, including HPs, hyperplastic polyps, and/or adenomas. CRC occurs in a high proportion of reported families (109). The syndrome has been mapped to the chromosomal region of 6q (110) as well as 10q23, which includes *BMPR1A,* and mutations in this gene have been found in a few families (111, 112). *Jeager et al.* mapped a causative gene to 15q13.3 and detected a duplication spanning from intron 2 in *SCG5* gene to just upstream of the *GREM1* locus (113).

Gorlin Syndrome (or Basal cell nevus syndrome, OMIM 109400) is characterized by multiple basal cell carcinomas, childhood medulloblastoma, macrocephaly, frontal bossing, and palmar and plantar pits, as well as odontogenic keratocysts. *Schwartz et al*. described

multiple gastric HPs in patients with Gorlin Syndrome (114), but GI polyps are not a major feature and GI surveillance is not recommended. Mutations are found in *PTCH1, PTCH2*, and *SUFU*. International guidelines for surveillance has been published by *Bree et al.* (115) and we discussed these in a Danish context in 2015 (116).

Neurofibromatosis type 1 (OMIM 162200) is characterized by multiple neurofibromas, multiple café au lait spots, iris Lisch nodules, as well as axillary and inguinal freckling. Most GI involvement is usually incidental and asymptomatic (48), but some suggest that approximately 25% of patients have GI stromal tumours (GISTs) (117). GI polyposis including ganglioneuromatosis has also been reported (118), but the risk of GI cancer does not seem to be increased (119). Neurofibromatosis type 1 has a prevalence of approximately 1:5000 and germline mutations are found in NF1 in ~95% of patients.

Multiple endocrine neoplasia type 2B (OMIM 162300) is one of three subtypes of the multiple endocrine neoplasia type 2 syndrome, the others being multiple endocrine neoplasia type 2A and familial medullary thyroid carcinoma. The syndrome is characterized by medullary carcinoma of the thyroid, pheochromocytoma, GI ganglioneuromatosis, and skeletal abnormalities. Diffuse GI ganglioneuromatosis is observed in up to 40% of patients (120). Mutations in *RET* are detected in ~95% of patients.

*Birt-Hogg-Dubé (*OMIM 135160): Cutaneous fibrofolliculomas, bilateral pulmonary cysts, spontaneous pneumothorax, and renal tumours characterize this syndrome. Early case reports linked colonic polyps and CRC with the syndrome (121), but the correlation is unclear. *Zbar et al.* did not find an increased risk for the development of colonic polyps or CRC in their study (122), whereas *Nahorski et al.* found an increased risk, though not significant, of CRC in patients compared to the general population (123). Mutations are detected in *FLCN* in ~90% of patients.

Cronkhite-Canada syndrome is characterized by GI polyposis with HPs, enteropathy, and skin-manifestations. The syndrome appears to be an autoimmune inflammatory condition (124).

The molecular functions of *STK11, PTEN, BMPR1A,* and *SMAD4*

STK11 encodes the enzyme serine/threonine kinase and is regarded as a tumour suppressor gene. It has numerous functions and controls the activity of AMP-activated protein kinase (AMPK) family members; it thereby plays a role in various processes such as cell metabolism, cell polarity, apoptosis, cell cycle arrest, and cell proliferation (125). Importantly, *STK11* downregulates the mammalian target of rapamycin (mTOR) pathway. The mTOR is a highly conserved kinase in all eukaryotes and is a central regulator of numerous cell activities. Numerous pathological conditions have been linked to the mTOR pathway, including both monogenetic conditions (Neurofibromatosis type 1 and Von Hippel-Lindau Syndrome) and multifactorial conditions such as obesity and type 2 diabetes. Specific targeted therapy, the mTOR inhibitors, has been developed, which have shown some benefits for patients with conditions related to this pathway such as Tuberous Sclerosis (126).

PTEN is widely expressed throughout the body and encodes the protein phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase. It is regarded as a tumour suppressor gene with multiple roles in cellular regulation. Thus *PTEN* is involved in protein synthesis, cell cycle, migration, growth, DNA repair, and survival signalling, and a defect or altered protein leads to deregulation of these processes (127). It has numerous functions, but notably it affects the mTOR pathway through downregulation of the PI3K/AKT pathway to inhibit cell survival, growth, and proliferation (128), see Figure 5.

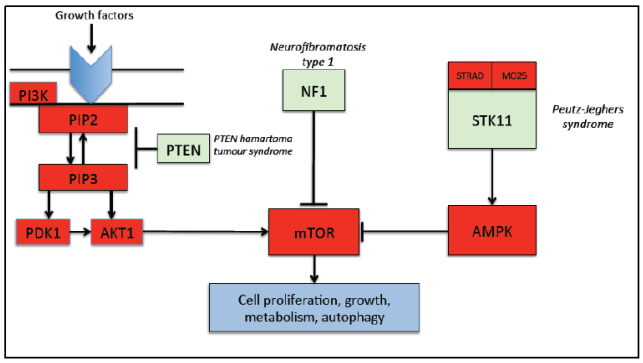

Figure 5: *A simplified illustration of the effect of PTEN and STK11 on the mTOR pathway. The protein encoded by STK11 is activated by binding to the pseudokinase STRAD and the protein MO25. The complex is an active unit, which phosphorylates AMPK, which activate the protein TSC2 in order to downregulate mTOR. The protein encoded by PTEN is a downstream regulator of the PIP3/AKT1 pathway: A receptor on the cell surface is activated by growth factors. This will activate PI3K, which phosphorylates PIP2 to PIP3. PTEN inhibits this reaction and thereby negatively regulates the AKT and PDK1 dependent processes. For details on the molecular function of STK11 see Fan et al. (125) and Shaw (129). For details on PTEN function see Hopkins et al. (127).*

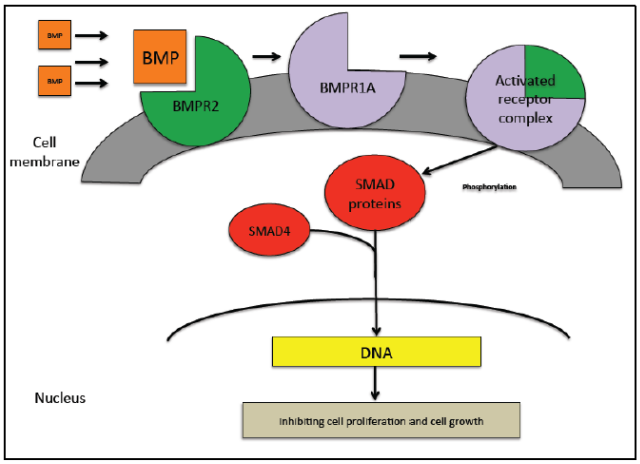

Figure 6: *A simplified illustration of the TGF-β pathway in which SMAD4 and BMPR1A are involved. The signalling process begins when a member of the TGF-β family (in this case a BMP (orange squares)) binds to a type II receptor in this case BMPR2 (green) in the cell membrane. This activates a type I receptor (in this case BMPR1A (purple)), which then form a complex (130). This complex then activates the SMAD proteins (SMAD1, SMAD5, and SMAD8) called the R-SMADS (receptor regulated SMADs), which then bind a co-SMAD, SMAD4. SMAD4 mediates the translocation of the R-SMADs into the nucleus, where it acts as a transcription factor (131). Thus the SMAD proteins are central signalling molecules acting downstream of the type I and type II receptors, not only the BMPR1A and BMPR2 receptor, but also other receptors in this pathway.*

Both *BMPR1A* and *SMAD4* encode proteins that work in the transforming growth factor beta (TGF-β) pathway. This pathway is involved in several cellular processes including cell growth, cell differentiation, apoptosis, and cellular homeostasis. The pathway is activated when a ligand from the TGF-β superfamily binds to a Type

II receptor on the cell surface resulting in a cascade involving several SMAD proteins, including SMAD4 (130), see Figure 6. *BMPR1A* encodes the protein bone morphogenetic protein receptor 1A, which is a Type I receptor. Other receptors in this pathway include BMPR2 (type II-receptor). The ligands of these receptors include bone morphogenetic proteins (BMPs), Activin, and other members of the TGF-β superfamily (131). Other genes encoding proteins working in the TGF-β pathway include *ENG*, which encode a membrane glycoprotein, and *ACVRL1*, which encodes a type I receptor. Germline mutations in *ENG* and *ACVRL1* are found in a majority of patients with HHT.

The pathophysiological mechanisms of cancer development in PJS and JPS

Both *SMAD4* and *STK11* are found to be somatically mutated in various types of sporadic cancer and are considered tumour suppressors: Thus both loss of heterozygosity (LOH) and somatic mutations of *SMAD4* have been found in mainly sporadic CRC and in pancreatic carcinomas (132, 133). Somatic mutations of *STK11* accompanied with LOH have been found in several types of sporadic cancers but mainly in non-small-cell lung cancer (134). Even though genes associated with HPS may play a role in sporadic cancer, what are the pathophysiological mechanisms of cancer development in patients with JPS and PJS, who are predisposed to cancer from birth? Does cancer develops by the same molecular sequences as in sporadic cancer? Does cancer develops through the HPs or coexisting adenomas? Although, the mechanisms still are largely unknown, some studies have tried to address these questions:

In 1999 *Bosman* published the idea of a hamartoma-adenoma-carcinoma sequence as a pendant to the known adenoma-carcinoma sequence (135). This theory has since been debated. *Bosman* based his theory on a study where polyps from JPS patients were found to have a somatic 10q22 deletion in the lamina propria but not in the epithelial cells (136). Thus *Bosman* hypothesised that factors secreted by the stroma could drive the epithelial proliferation and be responsible for the induction of malignancy (135). This is the so-called landscaper defect: that the microenvironment surrounding epithelial cells disturbs the epithelial architecture, differentiation, and proliferation. Some studies have investigated this hypothesis, but without consistent results: *Woodford-Richens et al.* studied JPs from *SMAD4* mutations carriers and found loss of SMAD4 in epithelial cells and some in the stromal cell (137), hence arguing against Bosmans theory of a landscaper effect. Other studies have also found LOH of both *SMAD4* and *BMPR1A* in JPs and carcinomas from mutation positive JPS patients (138, 139). These studies as well as *Woodford-Richens et al.'s* study speak in favour of the hypothesis that a second hit of the wild-type allele initiates growth and neoplastic progression of JPS polyps. Yet, this was not supported by *Blatter et al.* who found no LOH of *SMAD4* in 14 JPs in *SMAD4* mutation carriers (140). In search of evidence for the adenoma-carcinomas sequence in JPS patients, molecular alterations as observed in sporadic CRC, have been investigated in JPs and carcinomas from JPS patients: The results point towards that molecular alterations that is important in sporadic cancer development play a limited role in JPS patients, thus altered expression of β-catenin and p53, and mutations in *APC* and *KRAS*, have only been detected in a few cases (23, 138).

In PJPs, dysplastic, adenomatous, and carcinomatous changes have been observed but are relatively rare (59, 141-144), thus speaking against a hamartoma-adenoma-carcinoma sequence in PJS. *Jansen et al.* proposed that germline *STK11* mutations lead to dysregulation of cell polarity and mucosal prolapse, but the PJPs in themselves are not pre-malignant (145). This was somewhat supported by *Korsse et al.* who investigated PJPs and carcinomas from PJS patients and found LOH of *STK11* in only three out of six GI carcinomas, and in the dysplastic epithelium in three out of five PJPs, but not in the non-dysplastic epithelium of the same polyps (144). Other studies have also detected LOH of *STK11* in both PJS carcinomas and PJPs, but not in all (142, 146, 147). Concerning alterations as observed in sporadic CRC the results resemble what have been found in JPS: mutations affecting β-catenin in both PJS carcinomas and PJPs have been identified in a few cases (142, 144), whereas *KRAS* mutations, *APC* mutations, or 5q LOH are rarely detected (142, 144, 146, 148). Yet, *Entius et al.* dentified *APC* mutations in four of five PJS carcinomas (148). Altered p53 expression has also been reported in some cases (144, 146, 148).

In conclusion, based on the presented papers, it is not possible to determine the exact mechanisms or the role of the HPs in cancer development in JPS and PJS. The pathophysiological mechanisms underlying cancer development in PHTS are to be unravelled as well. *PTEN* is frequently somatically mutated in various types of sporadic cancer, and LOH has been found in carcinomas establishing *PTEN's* role as a tumour suppressor (149). But as this thesis mainly focus on JPS and PJS, the mechanisms of cancer development in PHTS are not discussed further here.

Next generation sequencing

Since the detection of various candidate genes in HPS, genetic analyses have been used to assist the clinical evaluation. Just a few years ago, mutation analyses and sequencing of relevant genes were performed with Sanger sequencing as method of choice. Though the method is accurate, it is limited by cost, speed, and sample size. But in 2005 DNA sequencing technology took a giant leap forward, when the first Next generation sequencing (NGS) instrument was introduced (150). NGS (massive parallel sequencing or high-throughput sequencing) have revolutionized the sequencing process. The analyses performed with NGS can be divided in three subgroups: *Whole genome sequencing*, where the whole genome, both the coding and non-coding regions, is sequenced, *Whole exome sequencing (WES)* where the entire coding region is sequenced, *and targeted next generation sequencing* (targeted NGS), where exons in selected genes e.g. in a 200 gene panel, are sequenced. Whether one uses Whole genome sequencing, WES, or targeted NGS depends on the purpose of the analysis as well ethical and financial considerations.

In the beginning of this research project, NGS was just being implemented in the genetics labs in Denmark, but it is now the method of choice when performing genetic sequencing in many cases, and it has been shown that targeted NGS equals the quality of Sanger sequencing (151). The advantages in NGS are numerous: whereas several strands of template DNA was needed in Sanger sequencing, in NGS, in principle, a sequence can be obtained from a single strand. NGS is also less time consuming, as is it massively parallel, allowing multiple base positions to be read in a single run. The reduced time, manpower, and reagents in NGS leads to much lower costs per base (152). In other words, had we used Sanger sequencing to investigate the 26 genes in 77 patients (Paper III), the project would have taken much longer to conduct.

The most challenging part of NGS is handling the huge amount of generated genetic data. Even targeted NGS, where "only" a panel of genes is sequenced, generates information on a large number of personal genetic variants, which have to be interpreted. The key question is how to separate non-clinical relevant variants, e.g. common variants, from those of importance. There are no golden standard to this bioinformatics approach and the method differs between labs and between research groups. The technical details

of NGS, the bioinformatics pipeline, and our approach to evaluating the detected variants in Paper III are discussed later.

Ethical considerations when performing Next generation sequencing

The increasing use of NGS in both research and in clinical settings has led to a passionate debate on several ethical questions, which arise from the possibility of sequencing the whole or larger part of the genome. When The Regional Scientific Ethical Committees for Southern Denmark approved our research protocol, NGS was still a rather new technique, and the committees did not yet have official guidelines on the specific ethical issues concerning NGS. Though some had discussed the ethical implications (153, 154), it was not until 2013 that the American College of Medical Genetics published their guidelines (155) – a year later than the approval of our study. We initially got the approval for doing both WES and targeted NGS on DNA from patients with one or more HPs (Paper III) and we were faced with the ethical considerations as presented in Figure 7. In the following I will describe one of them: the issue concerning incidental findings, which we choose to address in Paper IV.

Incidental findings: The "opting out" possibility

One of the most discussed issues has been the risk of detecting one or more *incidental findings (IFs),* defined as: *"A finding concerning an individual research participant that has potential health or reproductive importance and is discovered in the course of conducting research but is beyond the aims of the study"* by *Wolf et al.* (156). In other words during NGS, where several genes or the whole exome is sequenced at the same time, the researchers and clinicians may stumble upon genetic variants of significance not related to the clinical/research question i.e. the finding of a cancer predisposing mutation in a child evaluated for mental retardation. One of the most discussed aspects on IFs has been on whether the patients/participants should be offered the possibility of not being informed about IFs, the so-called "opting out" possibility. The guidelines from the American College of Medical Genetics in 2013 did not recommend that patients should have this option (155), which caused a response from several clinicians and researchers arguing that in respect for patient autonomy and to avoid medical paternalism the possibility should exist (157-160). The counterpart argued that the patients and relatives could have an interest in knowing of IFs as they can potentially lead to treatment or prevention of disease, and that the duty to prevent harm supersedes concerns about autonomy (161, 162). The American guidelines have since then been revised to agree that patients could opt out of receiving these types of results (163). The recommendation from the European Society of Human Genetics (ESHG) from 2013 also discusses the opting out possibility and states that a patients' right not to know, does not automatically override professional responsibilities when the patient's own health or that of his or her close relatives are at stake (164). We too faced the key question as how to approach the possibility of IFs in our patient group and whether they should have the possibility of opting out. As the research in this field was quite new at the time we conducted a small study on what the research participants actually wanted to know (Paper IV).

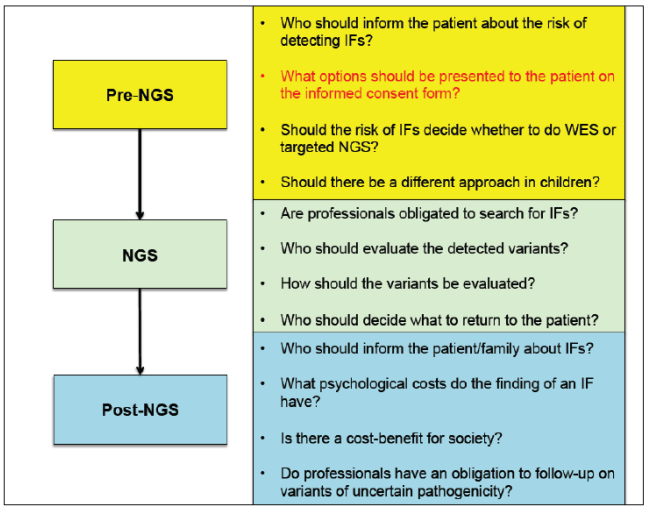

Figure 7: *Ethical questions arising when performing NGS. Highlighted in red is the question we choose to address in Paper IV.*

AIMS

The primary aim of the study was to expand the knowledge on clinical course and molecular genetics in patients with hamartomatous polyps and the Hamartomatous Polyposis Syndromes. In addition, we decided to investigate research participants' attitude towards the results of extensive genetic testing. Thus we designed six studies with the following aims:

1) To describe the occurrence of hamartomatous juvenile polyps in the Danish population.

2) To review the current literature on the Hamartomatous Polyposis Syndromes

3) To investigate whether patients with ≤5 hamartomatous polyps in the large bowel have a pathogenic germline mutation in the Hamartomatous Polyposis Syndrome-associated genes when using Next generation sequencing.

4) To investigate research participants' attitude towards disclosure of incidental genetic findings in Next generation sequencing-studies.

5) To identify Danish patients with a Hamartomatous Polyposis Syndromes, including 1) Peutz-Jeghers Syndrome and 2) patients with Juvenile Polyposis Syndrome and pathogenic SMAD4 mutations, and gather genetic and clinical information to further characterize the genotype and phenotype of these two patient groups.

METHODOLOGICAL CONSIDERATIONS

In the following sections I will describe the methodological considerations for Paper I-VI including strengths and limitations of the methodology.

Methodological considerations: Paper I

The aim of Paper I was to describe the occurrence of JPs in the Danish population from 1995-2014. For this purpose we used information on registered JPs in the Danish Pathology Data Bank (DPDB). The DPDB was also used as part of the methods in Paper III-VI.

Danish Pathology Data Bank: Quality of Data

As Paper I is solely based on data from DPDB the quality of this database is crucial: The DPDB contains detailed nationwide records of all pathology specimens analysed in Denmark. The register can be considered to be complete since 1997, but it also comprise records from several departments before that (the list can be seen on the website for DPDB: http://www.patobank.dk/index.php?ID=16&lang=da) (165). Searches in DPDB can be made with different modalities as the DPDB is build on a Danish version of Systemized Nomenclature of Medicine (SNOMED) codes (166). In our case the central administration office of DPDB provided the data (Search on SNOMED: "M75640 Juvenile polyp" from 1995-2014 with supplementary codes specifying the anatomic localization of the large bowel).

Why DPDB?
The DPDB provides an excellent opportunity to conduct research and has been reviewed in a few papers, which state that the coverage of the DPDB is high and nearly 100% (165, 166). When any evaluation at a Danish department of pathology is finished, the histopathological diagnoses and description are automatically sent online to the DPDB. Every patient in Denmark is provided with a social security number, which is noted together with the histopathological data; this allows for additional searches in other registers, such as medical files etc. And probably there is no other way of investigating the occurrence of JPs in Denmark, as the ICD-10 codes used by clinicians are often more broad i.e. "unspecified polyp" or "rectal polyp."

Limitations
As with all registers, miscoding cannot be ruled out, and in the beginning of the period the register was not entirely complete. Furthermore, we cannot be sure that the JPs in some cases were coded as HPs (SNOMED: M75630), but based on the results from Paper III we estimate this to be rare. In Paper III almost all polyps coded as HPs in the DPDB were sporadic gastric fundic gland polyps in the stomach. The histopathological difficulties in separating JPs from other types of polyps have been described in several studies, which report a significant interpathologist discrepancy in the diagnostic evaluation (32, 75, 167). In order to determine a more precise prevalence/incidence of JPs one would have to re-evaluate all JPs to confirm the diagnoses. Furthermore, a study like this only tells us about detected polyps, thus we cannot determine the exact prevalence of JPs as some polyps can be asymptomatic throughout life. Moreover, we do not know the manner of polyp removal (colonoscopy, sigmoideoscopy, surgery, or others) and the identified patients can have more polyps. Finally, the accuracy of the recurrence rate is limited by the study period.

Strengths
To our knowledge a study on Danish JPs in both children and adults has not been performed previously. The strength of our study is the quality of the data from DPDB, which allows us to assume that almost all removed JPs are recorded here.

Methodological considerations Paper II
The aim of Paper II was to conduct a review of the HPS based on the current literature.

Limitations
We used a systematic approach to identify relevant studies, yet we did not include all studies, or systematically evaluate the methods of the included studies. Most studies of HPS are small cohorts studies or case reports, which can be subjected to publication bias, ascertainment bias, and referral bias, and thus not give a accurate picture of the syndromes e.g. the phenotype, genotype, or the estimation of cancer risk. Thus, this review may not be as transparent as a completely systematic review or meta-analysis.

Strengths
Even though the approach was not completely systematic, the review can be useful as an overview, to form the basis for further studies, and to increase awareness of the syndromes.

Methodological considerations: Paper III
In Paper III we investigated whether patients with five or less HPs in the large bowel had a pathogenic germline mutation in HPS associated genes. If this hypothesis was correct one could use genetic testing to diagnose HPS when the first HP gives symptoms, and thereby be able to offer relevant surveillance to the patient and at at-risk family members. In the following I will elaborate on the enrolment of patients, the genetic technique, and evaluation of the detected genetic variants.

Identification and inclusion of patients
Patients with HPs were identified through the DPDB. We searched on the SNOMED codes: Hamartomatous polyp: M75630, Peutz-Jeghers Syndrome: S54320, and Juvenile polyp: M75640. The search was initially nationwide, but only patients whose polyps were evaluated in the Region of Southern Denmark were offered participation. Patient/parents/guardians consented in writing after written and oral information. We wanted to include patients of a wide age-span, as the age of diagnosis and presentation of HPS are reported to be wide. Thus we included patients aged 0-80 years. Yet, one could hypothesise that the risk of detecting yet undiagnosed HPS in children would be higher as they have not had many years of developing additional symptoms. We did not systematically ask for family history of cancer or other diseases at inclusion, so the first part of the study was solely based on the phenotype of the polyp(s). After genetic analysis, we were allowed to contact the involved families and ask for further clinical information or additional blood samples from family members if necessary.

Sporadic gastric fundic gland polyps
We initially choose to enrol patients with HPs in both the upper and lower GI tract. But when looking closer at the medical files of those with HPs in the stomach, it was revealed that they had sporadic gastric fundic gland polyps, which were coded as HPs in DPDB. Sporadic gastric fundic gland polyps are one of the most common types of gastric polyps and have been found in up to 2% of all endoscopic studies (168). Their association to CRC and gastric cancer has been studied: *Genta et al.* found an association to colonic adenomas, but only in women, and not to CRC, whereas Cimmino et al. did not find an association to adenomas (169, 170). Moreover, sporadic gastric fundic gland polyps are linked to the use of proton pump inhibitors (171). A large part of patients with Familial Adenomatous Polyposis has gastric fundic glands polyps, but no relation to the HPS has been described. Thus, these patients were excluded, and at the end only patients with HPs in the large bowel participated in the study.

Why targeted NGS?
The Regional Scientific Ethical Committees for Southern Denmark initially approved the use of both WES and targeted NGS in our study. Thus we enrolled patients with the purpose of doing extensive genetic analysis and the patients were informed accordingly.

In the end we choose to perform targeted NGS as it gives a much higher coverage of the genes of interest and reduces sequencing cost and time. Furthermore, the coverage pr. base is 200-1000x or even higher, which makes it possible also to analyse for larger deletions and duplications.

Creation of the gene panel
The design of the gene panel is described in Paper III. In addition to HPS-associated genes we included genes, which are not directly related to HPS, such as CDH1 and APC. This was partly because the NGS gene panel was to be integrated in the clinic, but also because we wanted to have a broad view of the genetic changes in genes related to GI cancer. Giving the rather broad gene panel there was still a small risk of detecting IFs, although the risk was significantly reduced compared to WES.

NGS step by step
In this section, I will describe, though not in complete details, the more technical aspects of the NGS analysis. This is to aid in the understanding of the strengths and limitations of the method. Several NGS platforms are available on the market, but as we used the Illumina Sequencing by Synthesis Chemistry on the Illumina HiSeq 1500 platform, this approach is described here, see also Figure 8, Picture 1-9:

1) Library preparation (Picture 1 in Figure 8): DNA is fragmented into fragments of approximately 200 bp with random breakpoints. The fragments are ligated with a 5' and 3' adapters and PCR-amplified. At this point, WES and targeted sequencing require an additional step of capturing where the desired regions of the genome are selected. Capture is performed by hybridization of target specific probes (or baids) to the adaptor-ligated fragments. The baids are short RNA biotinylated oligonucleotides. Due to the biotin label, hybridized fragments can be selected using magnetic streptavidin conjugated beads. To perform capturing different versions of capture kits have been developed. They vary by probe, design, and, accordingly, specificity of the target region. In this study we used a custom designed capturing method by Agilent SureDesign http://www.genomics.agilent.com/article.jsp?pageId=3083). For illustration of capturing, see this webside.

2) Cluster generation (Picture 2-6 in Figure 8): The library, now containing the genomic regions of interest, is then loaded into a flow cell (glass slide with lanes) where fragments are captured on a lawn with two types of surface-bound oligos complementary to the library adapters (Picture 2). The free end of a ligated fragment then folds to forms a bridge as it hybridizes to a complementary oligo on the surface (Picture 3). A DNA polymerase then produces the complementary strand, and thus creating a double stranded bridge, which is then denatured (Picture 4-5). The result is two copies of the original DNA-fragment, which are attached to the flowcell. This process is then repeated, and each fragment is amplified into distinct, clonal clusters through bridge amplification (Picture 6). When cluster generation is complete, the reverse strand is removed leaving several copies of the forward strand to be sequenced.

3) Sequencing (Picture 7-8 in Figure 8): Single fluorescent dNTPs are then incorporated into the DNA template strands with DNA polymerase (Picture 7). The first cycle consists of incorporation of a single fluorescent nucleotide followed by high-resolution imaging, where nucleotides are identified by fluorescent emission (Picture 8). This is repeated for the second base etc. Thus every position of the sequence is read. The critical difference to Sanger sequencing is that, instead of sequencing a single DNA fragment in one reaction, NGS extends this process across millions of fragments in a massively parallel fashion.
A skilled laboratory technician performed our library preparation, cluster generation, and sequencing.

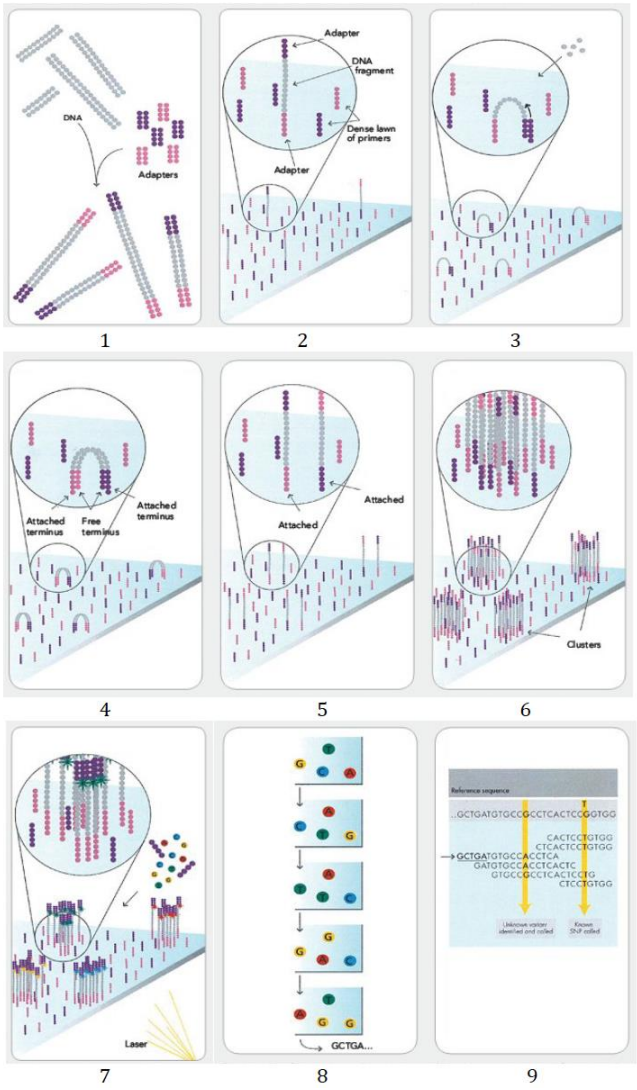

Figure 8: Illustration of NGS with Illuminas Sequencing by Synthesis Chemistry. Picture 1: Library preparation. Picture 2-6: Bridge amplification and cluster generation. Picture 7-8: Incorporation of fluorescent nucleotides and imaging of the sequence. Picture 9: Alignment to the reference genome. Pictures are Courtesy of Illumina, Inc. www.illumina.com

Data analysis
The result from NGS is several fragmented reads of sequences, which have to be "translated" into a file format, which lists the genetic variants of the patient, so we can interpret them. This bioinformatic stepwise analysis of data is crucial, yet no golden standard exist for this. Different software is used in the pipeline, and the choice and use of these will determine the output. The approach varies between labs and research group, and accordingly the output can differ and potentially lead to different clinical interpretations. In general, NGS data analysis involves the following steps:

1) *Alignment:* The sequence reads identified from the sequencing process are mapped to the reference genome (Figure 8, Picture 9).

Each sequence is mapped to the place on the reference genome of which it originates.

2) *Variant calling:* After alignment, the nucleotide differences between the patient and the reference genome are identified at a given position in the genome.

3) *Annotation of variants:* Once the variants are identified, each variant is *annotated*. This include that information on the functional effect of the gene, protein sequence, and other information from databases such as the Human Gene Mutation Database; ((HGMD) http://www.hgmd.org/) are listed together with the variant. Importantly, also information on minor allele frequency is annotated. In our case the allele frequency was annotated from The Exome Variant Server, which is retrieved from the Exome Sequencing project (ESP), http://evs.gs.washington.edu/EVS/), and the 1000 Genomes Project.

We used the same validated bioinformatics pipeline to handle the sequencing data as is used for clinical samples in the Department of Clinical Genetics, Odense University Hospital. For alignment to the human reference genome (build hg19) we used the software NovoAlign v.3.01 (NovoCraft). For variant calling we used GATK Best Practice pipeline c.2.7 (http://www.ncbi.nlm.nih.gov/pubmed/?term=25431634), and for annotation we used VEP (Variant Effect Predictor, http://www.ensembl.org/info/docs/tools/vep/index.html).

Filtering of a genetic variants

In our study hundreds of nucleotide differences (genetic variants) per patients were identified and annotated. A lot of these variants were non-synonymous: a single nucleotide substitution that potentially could affect protein function. In order to find a potentially clinically significant variant, some sort of filtering was needed. This approach was carefully considered, because at every step of this process there was a risk of filtering out variants of significance. The used filtering was described in Paper III, but was based on the assumptions that the causal variant would (1) alter the protein coding sequence and (2) would be extremely rare. Concerning (1) we began with filtering out all variants found in introns (except for the splice site consensus sequence) and furthermore all synonymous variants as they are not expected to change the protein coding sequence. Next, concerning (2), we compared the allele frequencies with estimated populations frequencies. Thus we filtered out all variants with a minor allele frequency occurring in >1% of the populations in the 1000 Genomes Project and The Exome Seqencing Project (ESP).

This filtering resulted in a much smaller list of rare, non-synonymous genetic variants, which were evaluated carefully. We classified each of these variants into pathogenicity classes to aid in the clinical interpretation. This scheme was inspired by the classification described in *Plon et al*. for the International Research on Cancer (IARC) Unclassified Variants Working Group (172). The evaluation of these variants does not particularly differ from those found with Sanger sequencing: Some variants will be assumed to be pathogenic based on previous reported findings or the nature of the mutation e.g. frameshift mutations leading to a premature stop codon. Nevertheless, missense variants, splice variants, and UTR variants can be difficult to evaluate and several factors must be taking into account. These include the phenotype of the patient, family history, segregation analysis in the family, functional studies, prediction tools, previous literature, and allele frequency databases (see Figure 9).

In silico prediction tools

To assist in the evaluation of missense variants, we used three public *in silico* prediction tools, which can predict the effect of non-synonymous variants: SIFT (sorting the intolerant from the tolerant) (http://sift.jcvi.org), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph), and AlignGVD (http://agvgd.iarc.fr/). Several tools are available, yet SIFT and PolyPhen-2 are two of the most commonly used. The prediction tools use different approaches to predict the effect of the variant, such as the difference in biochemical properties between the variant and wild-type amino acid, the evolutionary substitution frequencies between the wild-type and variant amino acid, and evolutionary conservation at the position of the variant. This is based on the assumption that biochemical changes of a variant are more likely to be disease-causing (or likely disease- causing), and that conserved amino acids across species are more likely to have an important structural or functional role. Furthermore, the prediction tools can take considerations on protein structure into account (173, 174).

We choose SIFT, PolyPhen-2, and AlignGVGD, as they have different approaches: SIFT combines the conservation of the sequence and physical properties as well as consider the amino acid change in the structural protein (175). PolyPhen-2 is based on evolutionary (phylogenetic) information as well as sequence, and structural features of the variant, which is feed to a probabilistic classifier (176). Align-GVGD uses multiple protein sequence alignments and the biophysical characteristics of amino acids (174). *In silico* tools may give a clue to the importance of the variant, but the sensitivity and specificity of the tools vary. Thus the evaluation of a missense variant must never be based on prediction tools alone. It is beyond the aims of this thesis to go in depth with comparison of the different prediction tools, but these have been reviewed in several papers (173, 177, 178).

Evaluation of splice variants

The precise recognition of splicing signals is critical and variants affecting splicing comprise a considerable part of pathogenic germline mutations. In order to evaluate a splicing variant, the most reliable method would be to analyse RNA samples of the patient, but this is not always possible and was beyond the aim of this project. As with missense mutations, several *in silico* tools that predict the effect of splicing variants, can give a hint on importance. In the evaluation of splice mutations we used the SpliceSiteFinder-like, MaxEntScan, NNSPLICE, GeneSplicer, and Human Splicing Finder. These prediction tools have been evaluated in different reviews (179, 180).

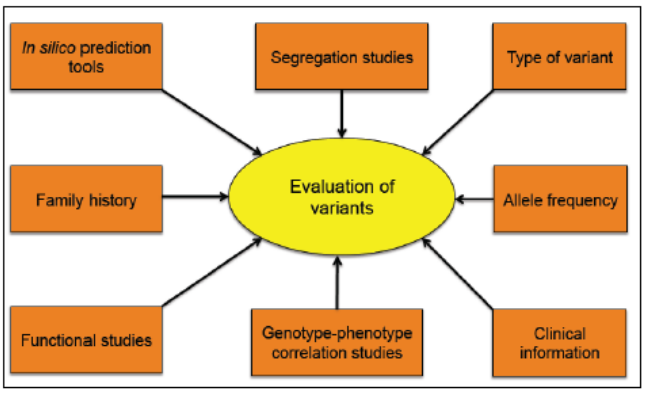

Figure 9: *Different strategies when evaluating genetic variants*

Variant frequency
As described, the annotated variants from NGS sequencing were filtered using the minor allele frequencies as reported in 1000 Genomes Project and ESP, which were available at the time of variant annotation. But soon after our analysis was finished, the Exome Aggregation Consortium (ExAC), Cambridge, MA (URL: http://exac.broadinstitute.org) became available. This database contains information on results from WES of approximately 60,0000 unrelated individuals, including the results from the 1000 Genomes Project and ESP. In ExAC, all data from these projects have been reanalysed with the same bioinformatics software and pipelines to increase consistency. We used the population frequency from ExAC to evaluate the rare variants that we had detected (Paper III, supplementary table) after initial filtration. It is, though, important to note that although ExAC have tried to eliminate the possibility of monogenetic diseases in their study-population, some variants may also have incomplete penetrance or potentially be associated with age dependent variable expressivity.

Copy number variation
The software GATK was used for variant calling, which enable detection of single nucleotide polymorphism and small deletions or small insertions. Yet, this software does not allow for detection of structural variants or copy number variants. Thus, to detect large deletions and duplications we used the software Contra (Copy Number Analysis for Targeted Resequencing, http://contra-cnv.sourceforge.net).

Quality control of NGS
As presented, NGS is a multiple step analysis and multiple quality control checkpoints exist throughout preparation of the library, the actual sequencing, the data analysis, and the interpretation. Despite its complexity it is relatively easy to identify samples that are of insufficient quality (181). A widely used quality score is the coverage of each base, that is how many times has one base been sequenced: the more times the less is the risk that a detected variant is a sequencing error. Coverage of over 30x per base is an acceptable quality control checkpoint. The target region has to be covered nearly 100%. Furthermore, the proportion of reads i.e. forward and reverse should be approximately equal.

Validation of findings
The variants of uncertain pathogenicity were validated with Sanger sequencing. Furthermore, a senior expert pathologist reviewed the histopathological diagnoses of polyps in enrolled patients.

Limitations
We gained sufficient quality of our NGS, yet, seen in a larger perspective, no genetic analysis is 100% complete. NGS is a complex technical analysis and the chosen software can filter out variants of importance. Bioinformatic tools may be useful, but this is still far from trivial. Segregation of the variant within a family can also be helpful in assessing pathogenicity, but factors such as penetrance, expressivity, and genetic mosaicism can still limit clear identification. The same holds true for frequently observed variants e.g. >1% and one cannot completely exclude such variations as being benign. The technique is constantly improving and so is the knowledge of our variants. Taking it all in consideration it comes down to the question of whether we actually can rule out that the patients have a HPS? This is probably not the case. A significant part of HPS patients are mutation negative. So whether one or more of our patients will develop more symptoms on HPS is unknown. In patients having more than one HP, only one HP was re-evaluated by our senior expert pathologist, which could be a problem because of the interpathologist difference in diagnosis. However, patients with more than one polyp comprised only five out of 77 patients.

Strengths
To date only a few studies have addressed the issue of HPS in patients with a low or moderate polyp burden (167, 182). We demonstrated that is was possible to design a gene panel of sufficient quality to be used in a clinical diagnostic laboratory. Finally, the histopathology of the polyps was also re-evaluated by a pathologist increasing the quality of the results.

Methodological considerations: Paper IV

Type of research
In Paper IV we reported the research participants' attitude towards the disclosure of IFs in NGS-studies. The method is qualitative in its nature, but the end-point was rather simple: Did the participant want to have (A) information on all IFs, (B) information on actionable IFs, or (C) no information on IFs at all.

Inclusion and exclusion of participants
The inclusion for this paper was based on all participants over the age of 18 years, with one or more HP, who initially responded to participate in the genetic studies as presented in Paper III. Thus all participants who were informed about the project and who gave consent were included, that was before the type of NGS to be performed (WES or targeted NGS) was decided, and before discovering that a part of the participants had sporadic gastric fundic gland polyps. Thus the participants' answers were not influenced on whether they were later excluded from the main project in Paper III. This part of the study was approved by additional protocol nr. 4 to the original protocol numbered S-201220057 by the The Regional Scientific Ethical Committees for Southern Denmark.

The semi-structured interview
In Paper IV we used the term "semi-structured interview," to describe the interviews with the research participants. The term can be broadly interpreted. In this case the term covered that the structure and information giving to the participants were framed beforehand: a list of questions and topics to be covered during the conversation was made e.g. the same examples of untreatable conditions (category A) and treatable conditions (category B) were given. A semi-structured interview allows for the interviewer to have a wide framework, and thus, when giving information in this study, the mode of conversation differed from participant to participant.

Limitations
The survey did not include participants less than 18 years of age, although this is very relevant in research of genetic diseases of which many present in childhood or adolescence. We decided only to include participants who were capable of answering for themselves, and thus only included participants over 18 years of age. Another limitation is that we do not know the reasons for the decisions made by the participants. The majority of our participants considered themselves healthy, as there is usually no follow-up when one or a few JPs are removed, but other research projects may include affected participants, who perhaps have other motivations for participating. Finally, we cannot exclude that a so-called "interviewer effect" may influence the answers of the participants as the Ph.D.-student conducted all but one of the interviews.

Strengths
The definite strength of the study is that, at the time of publication, the opting out possibly on IFs was very much discussed, as the guidelines of the American College of Medical Genetics did not have that option in their recommendations from 2013 (155). Our paper was meant to add to the discussion from a participant's perspective as a lot of surveys before that had been conducted on clinicians, geneticist, and professional's views. As discussed later, other surveys have since been published (183). The strength of the semi-structures interview is the possibility of going into depth and details with complex topics. Furthermore, the interviewers can explain views in their own terms.

Methodological considerations: Paper V-VI
The purpose of Paper V was to collect information on all Danish patients with PJS, and in Paper VI to collect information on patients with JPS and pathogenic *SMAD4* mutations. We aimed to describe the disease pattern, genotype, and phenotype. The methodology of the papers, that is the strategies for identification of patients and gathering of clinical information, was practically the same for the two papers, although the use of some specific registers differed.

Identifications of patients
The identification of patients was based upon the Danish registers and relevant departments. In Paper V we used the DPDB (search on PJPs), the Danish National Patient Register, Danish departments of clinical genetics, and the Polyposis Register, Hvidovre Hospital, Denmark, to identify patients. In Paper VI we used the Danish HHT-registry and Danish departments of clinical genetics to the identification of *SMAD4* mutations carriers. In both papers, we asked the Danish laboratories performing screening of *STK11* and *SMAD4* for lists of mutation carriers. All patients in the registers are listed with their social security number, which enable crosschecking between the registers, and enable gathering of further information from the patients' medical files.

The Danish National Patient Register
The Danish National Patient Register was established in 1977 and includes information on all patients admitted to a Danish hospital. The register collects information in the form of code (ICD-10) made by the clinical departments after a procedure and/or diagnosis. The register is very comprehensive but also very complex and its content have changed over time (184). The validity of this register has been investigated and reviewed with mixed conclusions: most agree that the register is very good and wide-ranging, but that the registered codes added by clinicians are not always
precise (185, 186). In Paper V we searched for patients with the ICD-10 diagnose code for Peutz-Jeghers Syndrome (DQ858B). With this search, we identified patients with their social security number, which we used for further collecting of information.

Collecting medical information
To collect medical information we used the medical files of the patients. The files were obtained from relevant departments (surgical, paediatric, or genetic) throughout the country.

Limitations
Retrospective studies are always limited by the available information on patients. Furthermore, the patients in both article V and VI followed different surveillance programs, which makes it difficult to obtain robust results and compare the patients with each another. In Paper V, concerning patients with PJS, some patients were born in the first half of the 20th century and it was difficult to obtain medical files from this period, thus information about initial presentation and early symptoms was not always available.

Strengths
The strength of both studies is that data based on the Danish registers offers relatively easy access to identifying patients and collect comprehensive medical information. Therefore it is possible to make nationwide studies of this type. The work also forms the basis for further studies on the patient groups.

RESULTS

Paper I: Juvenile polyps in Denmark from 1995-2014
We used the DPDB to collect information on all histopathologically examined JPs from 1995 to 2014. A total number of 2108 JPs in 1772 patients was examined in the period, of which approximately 25% were from children. We calculated the incidence of JPs to be between 1:45,000 to 1:65,000. Most patients (n=1666) in the study period had a single JP removed. The mean age at diagnosis was 37.9 years of age. JPs were detected in the rectosigmoid colon in 82.9% of adult cases and 94% of children cases. Approximately 1% of the patients fulfilled the diagnostic criteria of JPS having more than five JPs.

Paper II: Hamartomatous polyposis syndromes: A review
In this paper we conducted a review of the HPS based on the current literature.

Paper III: Germline variants in Hamartomatous Polyposis Syndrome-associated genes from patients with one or few hamartomatous polyps
We created a panel of 26 genes associated with HPS and other GI cancers, and analysed DNA from 77 included patients. The quality of the method was sufficient, as we obtained a mean coverage of the target region of 2222x (range: 459x - 4593x). For all patients 99.97% of the bases in the target region had a minimum coverage of 30x, which is acceptable. We detected several germline variants and among them were three in *ENG*, two in *BMPR1A*, one in *PTEN*, and one in *SMAD4*. None of the detected variants could be classified as definitively pathogenic (Class 5) or likely pathogenic (Class 4) according to our variant classification scheme. Furthermore, we observed a significant interpathologist difference in evaluation of polyp type, as 30% of the JP diagnoses in the enrolled patients could not be confirmed.

Follow-up in patients with variants of uncertain pathogenicity
During the genetic analysis we detected some variants of uncertain pathogenicity, which could have clinical implications for the patients. This was only in a few cases, but the families were contacted for additional clinical information, and, in some cases, additional analyses. The decision on which variants to follow up on, was based on the phenotype-genotype correlation of the gene in which the variant was detected, predictions tools, and the allele frequency. We have until now followed up on these detected variants:

ENG:c.374T>C, p.Val125Ala: The patient had one JP diagnosed with colonoscopy as an adult, and no family history of HHT symptoms, cancer, or polyps. The patient was offered clinical examination for symptoms of HHT. The examination was negative. We concluded that the variant was likely not pathogenic.

AKT1:c.719C>G, p.Ser240Cys: The patient had one JP diagnosed as a child. During the research period, the patient had been referred to genetic counselling for unspecific neurological symptoms. An expert neurologist could not confirm these. The phenotype-genotype correlation of *AKT1* is still unclear. We concluded, based on our current knowledge, that the variant had no clinical implications for the patient.

CDH1: c.2335C>T, p.Arg779Trp: The patient had one JP diagnosed in adolescence. The family was contacted for further clinical information and to obtain a family history of cancer. Family history revealed no history of breast cancer or ventricular cancer. Samples from the parents revealed that the variant was inherited from the father, who is healthy. The evaluation of the family is on going.

BMPR1A c.1327C>T, p.Arg443Cys: The family was contacted in writing to gather further information on family history, and to offer genetic testing of the parents for this variant of uncertain pathogenicity, but the family has not responded.

Paper IV: Research participants in NGS studies want to know about incidental findings

The 127 research participants were asked to decide whether he/she wanted to receive information on IFs in three categories: (A) To receive disclosure of all IFs, that might be found during the research period – even if the variant leads to risk of an untreatable or unpreventable disease. (B) To receive disclosure on IFs only if the variant leads to a condition that is treatable, preventable, or for which there can be offered surveillance. (C) Not to receive disclosure on IFs at all. The majority of participants wanted disclosure of all IFs (A) (n=78 (61%)). 45 participants wanted disclosure of actionable variants (B) (36%), Four participants (3%) did not want to receive information on IFs at all (C). There was no significant difference in the answers relating to sex or age.

Paper V: Disease pattern in Danish patients with Peutz-Jeghers Syndrome

We identified 43 Danish patients with PJS through Danish registers, relevant hospital departments, and laboratories. We included 43 patients of which 14 had deceased. The male:female ratio was 26:17. We estimated the prevalence of PJS to be approximately 1 in 195,000 individuals. The median age at diagnosis was 29 years (10 month to 67 years) with obstruction of the small bowel as the most frequent presenting symptom seen in 35% of PJS patients. Approximately 50% of the study population had a family history of PJS related symptoms. We noted 18 occurrences of cancer at various anatomical sites. The included patients showed great variability in phenotypic expression.

Paper VI: JP-HHT phenotype in Danish patients with SMAD4 mutations

We identified 14 Danish patients with pathogenic *SMAD4* mutations through the Danish departments of clinical genetics, the Danish HHT-registry, and Danish laboratories carrying out *SMAD4* analysis. All patients had polyps removed and 11 out of 14 fulfilled the diagnostic criteria for JPS. Eight patients were screened for HHT symptoms and seven of these fulfilled the HHT (Curaçao) criteria. One patient had aortic root dilation.

GENERAL DISCUSSION

The aim of the research was to expand the knowledge on clinical course and molecular genetics in patients with HPs and the HPS, as well as investigating research participants' attitude towards the results of extensive genetic testing. This was obtained by investigating the occurrence of JPs (Paper I), to perform a literature review (Paper II), to test the hypothesis that a subgroup of patients with one or few HPs may have HPS with the use of genetic testing (Paper III), to investigate research participants views towards opting out on information on incidental genetic findings in NGS studies (Paper IV), and finally to collect clinical and genetic information on all Danish patients with PJS and *SMAD4* mutations (Paper V-VI).

Management of juvenile polyps in the GI tract

The presented research, in addition to current literature, allows us to suggest how to manage the finding of JP(s) in the GI tract. In Figure 10 and Figure 11 we present a possible approach after detection of JP(s) in children and adults, respectively.

Children: In Paper I we estimated that approximately 20 Danish children per year are diagnosed with a JP. Because of the possibility of detecting further JPs proximal to the rectosigmoid, the child should always be offered colonoscopy if the JP(s) initially has been detected otherwise (14-16). Most children have a single JP (17, 18), and the first question is how to separate children with a single JP from those with JPS or another HPS. Although cancer in HPS develops in adulthood, the diagnosis of a HPS in early life establishes the possibility of surveillance and identification of at-risk members of the families. Most cases of HPS present in childhood or adolescence, and special caution should be taken in these age groups, but the phenotypic heterogeneity is wide and some present in infancy and some in (late) adulthood. As shown in Paper VI some patients with pathogenic *SMAD4* mutations have very few polyps and the phenotypic expression varies even within the same family. Thus the distinction between a patient with one or few JPs and JPS/HPS is blurred. To add further to the confusion some have used a working definition of three or more JPs (187, 188) in JPS and not five or more (the Jass criteria)(189). We did not find that genetic testing is relevant in children with a single JP (Paper III), but we suggest that the clinician should evaluate family history of polyps and cancer as well as look for and ask for extraintestinal manifestations such as dermatological manifestations, intellectual disability etc. At this point it is important to consider that approximately 50% of cases are *de novo* and family history may be negative. Other information such as the age of the child could also be taken into considerations as a single JP often present at a younger age than JPS and that anaemia is more common in patients with JPS than with a single JP (11, 18).

If nothing supports the diagnosis of HPS, the next question is whether the child should be offered clinical follow up with endoscopy in order to detect recurrent polyps. This is not a simple question and no evidence-based guidelines exist at the moment. Previous studies are small and sometimes include other polyp types than JPs. Thus the recurrence is hard to determine. *Fox et al*. investigated a cohort of 192 children with JPs, including 117 with a single JP, detected with colonoscopy. Eighteen children with a single JP were followed up with endoscopy, and three had recurrence (17). Based on this recurrence rate the authors argued that children who present with a single polyp should be followed up with endoscopy (17). The authors also found neoplasia in 3.9% of the investigated polyps in the whole cohort (17). Any recommended surveillance programme with colonoscopy in this group of patients has to be weighed against the drawbacks and risks in addition to the cost-effectiveness. The introduction of colonic video capsule endoscopy may change this in the future. But as such, at the moment, there is no strong evidence to support that clinical follow-up is necessary.

The finding of multiple JPs, 2-5 JPs, is a special challenge because of the difficult distinction from JPS. As NGS are implemented, the threshold for doing genetic testing can be lowered, and genetic testing could be considered. Further evaluation of the patient should at least include exploring family history and extraintestinal findings as presented in Figure 10.

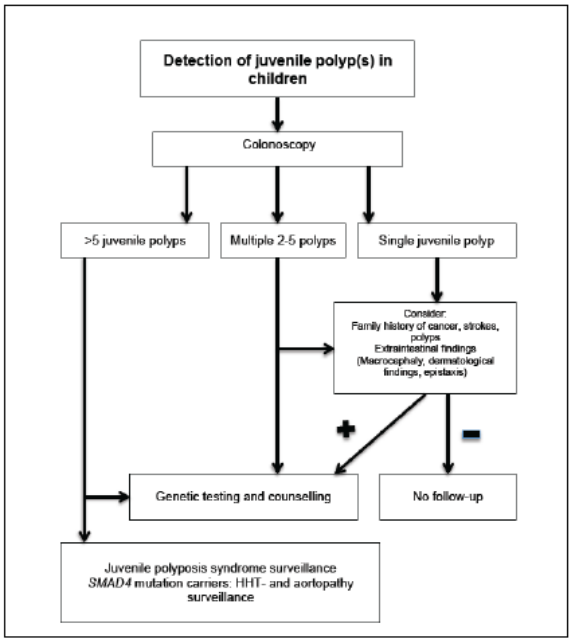

Figure 10*: Proposed algorithm when detecting one or more JP(s) in children*

Adults: In Paper I, we calculated an incidence of detected JPs of approximately 1:40,000 to 1:65,000 per year, with 75% being detected in adults. Though rarely, adults are still being diagnosed with JPs and the same questions as in children can be asked: Is there any signs of JPS/HPS? And should the patient be followed up with endoscopy? Even fewer studies of the natural history and recurrence exist of JPs in adults, but as HPS mainly manifest in childhood and adolescence the finding of a single JP should not necessarily lead to such extensive investigations as in children. The reason for referral should be considered: Is the JP an incidental finding? Family history, the age of the patient, previous history of polyps, or cancer should be considered. The finding of 2-5 polyps, perhaps in addition to polyps of another histopathological type, should lead to suspicion of a JPS or mixed polyposis syndrome. As in children genetic testing could be relevant at this point. The histopathological type of polyps should also be taken into consideration: Adenomas are frequent in adults; however, inflammatory polyps are rare and could be mistaken for a JP.

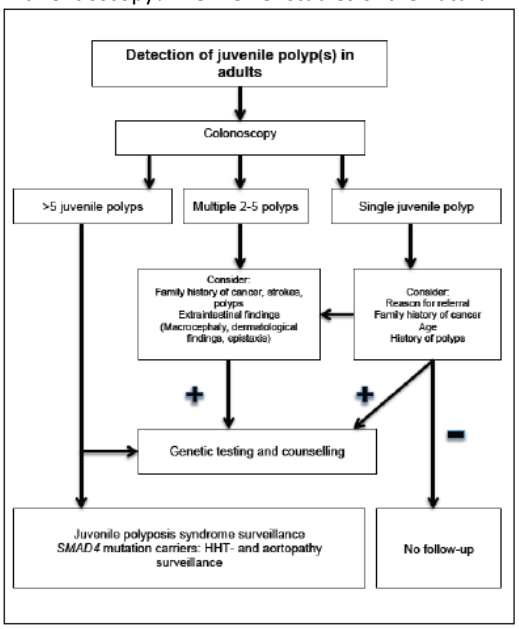

Figure 11: Proposed algorithm when detecting one or more JP(s) in adults

Management of Peutz-Jeghers polyps in the GI tract

The finding of a PJP at any age should always be followed up by further examinations, as cases of solitary PJPs are rare. Colonoscopy, gastroscopy, and small bowel screening with MR-enteroclysis, MRE, or VCE should be performed as well as genetic testing for *STK11* mutations. A thorough family history of polyps, cancer, rectal bleeding, and bowel obstruction should be obtained. History of mucocutaneous pigmentations, which importantly tend to fade after puberty, should be considered. Genetic analysis is a helpful tool in the diagnostic process as mutations in STK11 are found in over 90% of patients fulfilling the diagnostic criteria for PJS (69). The clinical significance of solitary PJPs without PJS is debated, though reports of such cases exist (19-21). Some authors have suggested that having a single PJP should be regarded as a separate clinical entity (21), while others disagree: *Burkart et al.* studied eight cases of solitary PJPs, and found that all patients had some indications of PJS when reviewing clinical information or family history (22). *Burkart et al.* then argued, that the occurrence of a single PJP presumably does not exist and at least the patients have the same risk of cancer as those with PJS (22). Furthermore, as shown in Paper V, the expressivity and age of diagnosis of PJS is very wide (10month-67y). If the patient after thorough examination does not have further polyps or indications of PJS, one should consider revising the histology of the polyp, and if the pathology is confirmed one should still consider clinical surveillance. A proposed algorithm for follow-up is presented in Figure 12.

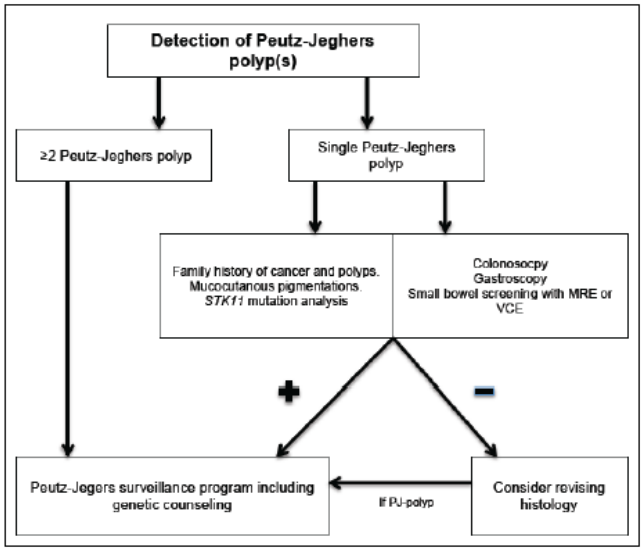

Figure 12*:* Proposed algrithm when finding one or more PJP(s). MRE=MR-enterography/MR-enteroclysis. VCE= video capsule endoscopy

Surveillance – does it help?

Although NGS may elucidate further genetic causes of HPS, one can ask: What follows diagnosis? What do we offer family-members at risk? What kind of surveillance is relevant and does it help? The answers are far from simple: The purpose of surveillance in JPS and PJS patients is to detect cancer in early stages and to avoid morbidity in relation to severe polyposis. Clinical guidelines have been proposed (58, 62, 91), but the evidence for these guidelines is sparse, and most are based on expert opinions (the lowest degree of evidence, level D) or evidence level C, low (62, 91) e.g. in PJS and JPS only a few papers have described the long-term outcome of GI surveillance including surgery in relatively small patient populations (44, 63, and 13 patients) (72, 190, 191). All three studies were retrospective in their design, and it is important to note that previous publications are subjected to the risk of referral bias, publication bias, and ascertainment bias, which must always be taken into consideration before making any conclusions based on the current knowledge. In order to establish stronger evidence more studies, with larger patient and control groups are needed. Studies of the underlying mechanisms of cancer development are also missing. In Denmark, no consensus guidelines for surveillance of PHTS, PJS, and JPS exist, although we discussed JPS guidelines in a Danish context some years ago (192).

The histopathological aspects of HPS

One significant finding in our studies (Paper VI) was that HPS patients seem to have a wide variety of histopathological types of GI polyps, and that the histopathological diagnosis is not always clear (Paper III). In Paper III the diagnosis of a HP could not be confirmed in 30% of cases by our experienced gastropathologist. Others have described this interpathologist discrepancy as well as the wide range of polyp types seen in JPS patients (32, 75, 167). When a diagnosis of e.g. PJS or JPS is based on only a few polyps a ”misclassification” of polyps may led to the wrong diagnosis and in some cases delay important follow-up for the patient and their families as found in *Aretz et al.* (75) Thus it is important to examine a large enough number of polyps in order to determine the predominant type (52), but if the patient only has few polyps (as we saw in some patients in Paper V and VI) this is not always possible and other information e.g. family history should be taken into account.

The role of genetic analysis in HPS diagnostics

As HPS, with the exception of Cronkhite-Canada Syndrome, is caused by germline mutations, genetic analysis can be used to aid diagnostics, but the finding of a pathogenic mutation is not (always) part of the criteria for HPS. This, in addition to the fact that mutations are not found in all patients with HPS, emphasizes the importance of the clinical evaluation. We did not, based on genetic analysis, confirm our hypothesis in Paper III: that patients with one or a few polyps may have HPS, but we did create a gene panel and performed NGS of sufficient quality to be used in a clinical diagnostic setting. It is indisputable that the increasing use of NGS has made genetic testing much cheaper and opens up possibilities in assisting the clinicians in the diagnostic evaluation to a larger extent than just a few years ago. Yet, one must still consider the implication of finding a pathogenic or a variant of uncertain pathogenicity for the patients and their relatives. Genetic counselling should be a cornerstone in the management of HPS patients, both in families with or without a known pathogenic mutation as well as in de novo patients.

Evaluation of genetic variants

The data analysis and evaluation of variants in Paper III highlight the difficulty in interpreting the significance of germline variants generated from genetic analysis, even in genes with a well-established role in HPS pathogenesis. All who perform NGS, which generates information on several variants of uncertain pathogenicity, faces this challenge. A particular problem with the evaluation of pathogenicity is the usage of previous results: Genetic studies based on Sanger sequencing were often based on single cohort studies of symptomatic populations and may be erroneously associated with disease due to small cohort sizes, limited validation studies, and unmatched control populations (193). This was illustrated by *Dorschner et al.* who investigated 114 genes in 1000 individuals and identified 239 variants listed as disease-causing in HGMD. The authors evaluated these variants with allele frequency and literature reviews, and found only 16 autosomal-dominant variants to be pathogenic or possibly pathogenic (194). This paper also illustrated that NGS with WES has offered a new dimension to variant evaluation, as it is now possible to investigate large healthy and non-healthy populations allowing us to compare the detected variants with the frequency in the general population. Thus projects such as the 1000 Genomes Project, ESP, and ExAC have indicated that germline variants including deleterious variants are more frequent in healthy individuals than earlier anticipated (193, 195). Another example is *Bodian et al*. who investigated 681 individuals of reproductive age with no history of cancer in the family. When performing NGS sequencing in 158 cancer related genes, the authors found that every individual carried multiple non-synonymous variants with an average of 68 variants per person (196). Although, we did not report any likely or definitively pathogenic variants in Paper III, we do not know whether the variants of uncertain pathogenicity are a contributing factor to polyp formation or whether we in the future, with further knowledge, may evaluate the variants differently.

Genotype-phenotype correlations

Genotype-phenotype correlations studies are important in elucidating the HPS further; these studies can shed new light on the pathophysiological mechanisms of the syndromes and aid in the development of more individualized surveillance programs. In Paper VI we showed that extra attention must be paid to patients carrying a *SMAD4* mutation as these patients may have potential severe manifestations of their disease: thus several had both a heavy polyp burden and HHT manifestations with potential life-threatening AV-malformations, and aortopathy was seen in one patient. The fact that *SMAD4* mutations carriers have a “broader” phenotype may be explained by the central position of *SMAD4* in the TGF-β pathway, see Figure 6. To add further to the picture of *SMAD4* it is interesting that patients with Myhre Syndrome, characterized by intellectual disability, dysmorphic facial features, and skeletal anomalies, also harbour a de novo mutations in *SMAD4* (197). Mutations in *SMAD4* have also been detected in patients with LAPS syndrome (characterized by laryngotracheal stenosis, arthropathy, prognathism, and short stature syndrome), suggesting that Myhre syndrome and LAPS are a clinical entity with variable expression (198). The effect of the altered protein function in patients with Myhre Syndrome, LAPS, and JP-HHT patients must be different, but this illustrates that we still have a lot to learn. Yet, the rarity of these syndromes makes it difficult to gather large patient populations. Nationwide studies, and preferably international co-operations, are necessary in the future.

Interestingly, in Paper III, we also detected variants in *ENG*. Pathogenic mutations in this gene are detected in approximately 40-50%

of HHT patients (199). ENG also works in the TGF-β signalling pathway, and previous studies have reported germline variants in *ENG* in polyposis patients (167, 182, 200). Whether these mutations are insignificant, causative, or perhaps a contributing factor to polyposis is unknown.

New candidate genes

With the available techniques and knowledge it is not possible to detect germline mutations in all HPS patients: 20-40% of JPS patients, 5-10% of PJS patients, and at least 50% of patients with CS are mutation negative. One can hypothesise that this is either because of limitations in our technique or that the conditions show genetic heterogeneity. Some patients can also be mosaic for the causative mutation and thus the mutation may not be detected in a blood sample. In the last years it has been shown that other mutations than *PTEN* is causative of CS or CS-like phenotype: germline mutations in *SDHB* and *SDHD* in patients with CS and a CS-like syndrome have been reported (201) as well as a germline hypermethylation of *KLLN* (202). Another 8.8% of unrelated CS patients without germline PTEN mutations were found to have germline *PIK3CA* mutations and 2.2% had germline *AKT1* mutations (203). The development of WES, although there are still limitations to this method, offers a great possibility of investigating mutation negative patients. This approach was used in *Ngeow et al.* who recently identified a new susceptibility locus for HPS as they detected a germline missense mutation in *SMAD9* in a patient with JPS associated with GI ganglioneuromas (204).

HPS pathogenesis and influencing factors

The underlying molecular mechanisms in cancer development in HPS are largely unknown. As addressed in the background and in this discussion we need a far better understanding of the molecular details in order to understand these conditions. A somatic second hit theory seems intriguing as an elegant explanation of polyp formation or cancer development, and though some studies have supported this (137), the results are far from conclusive. Studies on this matter are also limited by sample size and inconsistencies in the used techniques e.g. some have used mutation analysis, microsatellite markers, hypermethylation analysis, and immunohistochemistry for investigations of the molecular changes. Furthermore, we still need an explanation for the extraintestinal manifestations as seen in e.g. JPS or CS. These specific anatomical sites are subjected to tissue specific genetic factors, and several environmental factors or epigenetic factors may also play a role. NGS opens up the possibility of studying somatic mutations in polyps and carcinomas to a greater extent than previous.

Ethics of NGS

The development in genetics has been explosive: the first human genome required 13 years to sequence and did cost nearly 3 billion dollars. In contrast, today, several genomes can be sequenced in one day for approximately 1000 dollars each. However, the knowledge of what the genes actually do and how they interact with each other and other proteins is still lagging. We generate a lot of data, that we do not understand and concurrently we face ethical questions as how to manage the data and what to tell the patient and the families. The ethical discussion, which has followed the integration of NGS into clinical practise, has raised several questions. Ethical views are founded in culture and on religious beliefs, personal experience, and legal as well as moral obligations, and the questions are hard to agree upon. Yet, the debate in itself also supports the idea of genetic exceptionalism: that genetic results somehow should be treated differently from other clinical information. But is genetic testing any different than performing e.g. an MR scan of the brain in the case of headache? The risk of IFs is still present. *Green et al.* concluded that at least in the case of predictive testing no clear, significant distinctions between genetic and non genetic tests justify a different approach than with other clinical testing, and that predictive testing does not alter the obligations of physicians e.g. not to harm unnecessarily (205). But still it is important to be aware of other aspects of genetic testing such as predictive testing of minors, implications of the test result for other family members, and theoretical risks of insurance or employment discrimination. In Paper IV we addressed a specific issue of the opting out possibility when performing NGS and showed that at least most research participants are "not afraid" of information. Almost simultaneously with our study a similar, though much larger study, was published. In a web-based survey *Middleton et al.* investigated the views of the public as well as genetic researches and professionals and found the same tendency as us: That members of the public were positive towards gaining information on genetic results (183). The study also demonstrated that genetic health professionals had a significantly more conservative view. *Middleton et al.* conclude that their finding illustrates a disconnection between the views of those handling the findings of research and those participating in research (183). Whether or not, and how to decide what information to return to the patient/participant is difficult. The ethical discussion will continue and somehow must be modified by reality; in a few years we will have more experience in how to handle information and have an idea of the extent of the issues.

CONCLUSIONS

This thesis presents some of our research over the last 3-4 years on HPs and HPS. In the six papers we obtained several results to fulfil our aims, which was to expand the knowledge on clinical course and molecular genetics in patients with HPs and HPS, and to investigate research participants' attitude towards the results of extensive genetic research.

In Paper I we investigated the occurrence of JPs in a 20-year period. Based on the registered histologically examined JPs, we found a total number of 2108 JPs in 1772 patients, of which approximately 25% were in children. Most patients had a single JP and the mean age of diagnosis was 37.9 years of age, in children 5.7 years. Though the risk of cancer in HPS is well documented, the risk of cancer when having one or few HPs is unknown, and the clinicians are faced with the clinical question of how to separate patients with few HPs from patients with HPS. In cases of only one or few HP(s), we did not find evidence to support the use of additional genetic testing in order to diagnose a HPS (Paper III). But in Paper VI, where we collected clinical information of SMAD4 mutation carriers, we observed that at least some patients with one or a few polyps have a pathogenic mutation associated with HPS. Diagnosis of HPS is essential, as patients should be offered surveillance of the GI tract, but also at other extraintestinal sites, and genetic counselling. Hence, family history and considerations of extraintestinal symptoms are essential in evaluating patients with one or few HPs. The inter- and intrafamiliary variability in expression was also reflected in Paper V where we studied Danish patients with PJS. We identified 43 patients, who fulfilled the diagnostic criteria. The median age at diagnosis was 29 years, with some being diagnosed in infancy and early childhood, and some in the sixth decade of life. Small bowel obstruction was the most frequent presenting symp-

tom seen in 35% of JPS patients. We noted 18 occurrences of cancer at various anatomical sites and thereby showing that PJS patients are predisposed to not only cancer in the GI tract but also at extraintestinal sites.
Although the use of genetic testing is increasing as new technologies develop, we did underline the difficulty in integrating NGS in a clinical setting in Paper III and Paper IV: interpretation of genetic variants is difficult and furthermore there are several ethical issues to consider. Nevertheless, as we concluded from Paper IV, it seems that at least research participants are not afraid of genetic information: The majority of participants (61%) wanted disclosure of all incidental genetic findings and 36% wanted disclosure on actionable incidental findings.

FUTURE PROJECTS AND PERSPECTIVES

The presented studies investigated research questions and hypotheses on HPs and HPS. There are still a lot of unanswered questions, and the future will show how the rapid development in genetic techniques can assist in answering some of these. Our research and those of others form the basis for future projects and perspectives of which I will mention some here:

Cancer development in HPS

One of the most intriguing questions is the uncovering of the underlying pathophysiology in cancer development in HPS. NGS opens the possibility of studying somatic mutations in both polyps and cancer to a larger extend than so far. And although such investigations may be problematized by the possibility of multiple different cell lines in both polyps and cancer, we may gain evidence for or against a hamartoma-adenoma-carcinoma sequence by such studies. In the long perspective knowledge of molecular alterations in cancers from HPS patients may help in individualizing cancer treatment.

Evidence for surveillance

Surveillance programs for HPS are based on low or limited quality of evidence and often on expert opinions. To gain evidence larger groups of patients and controls are needed. Because of the rarity of the syndromes international cooperation seems rational. In addition, the basic information on the phenotype of the HPS is still sparse, and further phenotypic description and studies on genotype-phenotype correlations are valuable in order to describe the clinical course, also in patients who are less affected.

Medical treatment?

The management of HPS has so far been symptomatic. As described, *STK11* mutations cause dysregulation of the mTOR pathway resulting in a missing inhibition of the pathway. A drug, Rapamycin, has been developed that works by regaining inhibition of the pathway. Rapamycin has been tested in *STK11* knock-out mice and was found to decrease tumour burden, polyp size, and vascularization (206-208). As we gain more knowledge on the molecular mechanisms and cancer development, we might be able to develop targeted therapies for the treatment of HPS in the future.

SUMMARY

Hamartomatous polyps (HPs) in the gastrointestinal tract are rare compared to other types of gastrointestinal polyps, yet they are the most common type of polyp in children. The symptoms are usually rectal bleeding, abdominal pain, obstipation, anaemia, and/or small bowel obstruction. The polyps are typically removed concurrently with endoscopy when located in the colon, rectum, or stomach, whereas polyps in the small bowel are removed during push-enteroscopy, Device-assisted enteroscopy, or by surgery. HPs can be classified as juvenile polyps or Peutz-Jeghers polyps based on their histopathological appearance. Patients with one or a few juvenile polyps are usually not offered clinical follow-up as the polyp(s) are considered not to harbour any malignant potential. Nevertheless, it is important to note that juvenile polyps and HPs are also found in patients with hereditary Hamartomatous Polyposis Syndromes (HPS). Patients with HPS have an increased risk of cancer, recurrences of polyps, and extraintestinal complications. The syndromes are important to diagnose, as patients should be offered surveillance from childhood or early adolescence. The syndromes include Juvenile Polyposis Syndrome, Peutz-Jeghers Syndrome, and the *PTEN* hamartoma tumour syndrome. Currently, the HPS diagnoses are based on clinical criteria and are often assisted with genetic testing as candidate genes have been described for each syndrome.

This thesis is based on six scientific papers. The overall aim of the studies was to expand the knowledge on clinical course and molecular genetics in patients with HPs and HPS, and to investigate research participants' attitude towards the results of extensive genetic testing

Paper I: In the first paper we investigated the occurrence, anatomic distribution, and other demographics of juvenile polyps in the colon and rectum in Denmark from 1995-2014. Based on the Danish Pathology Data Bank we found that 1772 patients had 2108 JPs examined in the period, and we calculated the incidence of juvenile polyps to be between 1:45,000 and 1:65,000. The majority of patients with juvenile polyps were adults and 1% fulfilled to diagnostic criteria of JPS. The majority of patients had a single juvenile polyp.

Paper II: In this paper we conducted a review of the HPS based on the current literature.

Paper III: We investigated the hypothesis that patients with one or few HPs may have a HPS based on genetic screening. We designed a panel of 26 genes associated with HPS and used targeted Next generation sequencing in 77 patients with mainly one juvenile polyp. We detected several germline variants, among them three in *ENG*, two in *BMPR1A*, one in *PTEN*, and one in *SMAD4*. Although some of the detected variants have been reported previously none could be classified as definitely pathogenic or likely pathogenic according to our variant classification scheme and thus we concluded that genetic screening of patients with one or few JPs are not indicated.

Paper IV: In Paper IV we investigated one of the ethical aspects of Next generation sequencing: the issue whether research participants in NGS studies should be offered the possibility of not receiving information on incidental genetic findings (the "opting out possibility"). We conducted semi-structures interviews in 127 research participants, and found that the majority (61%) wanted information on all incidentals findings, while 36% wanted information on actionable incidental findings. Only 3% did not want information on incidental findings at all.

Paper V: In this paper we wanted to gather information on all Danish patients with Peutz-Jeghers Syndrome in order to investigate the phenotype and genotype. Through Danish registers we detected 43 patients of which 14 had deceased. We calculated the prevalence of Peutz-Jeghers Syndrome to be approximately 1 in

195,000 individuals. The median age at diagnosis was 29 years with obstruction of the small bowel as the most frequent presenting symptom. We noted 18 cancer occurrences in the population in both the GI tract and at extraintestinal sites, demonstrating that these patients are predisposed to cancer at various anatomical sites. The study also underlined the wide phenotypic expression of the syndrome.

Paper VI: In the last paper we identified patients with Juvenile Polyposis Syndrome, who carry a *SMAD4* mutation, and described their genotype and phenotype. We especially investigated whether these patients have symptoms of both Juvenile Polyposis Syndrome and Hereditary hemorrhagic telangiectasia. We identified 14 Danish patients. Most of these had symptoms of both conditions and one had aortic root dilatation. Thus this group of patients requires a multidisciplinary follow-up program.

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