Susanne E. Korsse

Peutz-Jeghers syndrome and cancer

Linked by LKB1

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Peutz-Jeghers Syndrome and Cancer
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Verbonden door LKB1

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CONTENTS

Chapter 1

General introduction and outline of the thesis

PEUTZ-JEGHERS SYNDROME

Peutz-Jeghers syndrome (PJS) is an autosomal dominant inherited disorder, characterized by mucocutaneous pigmentations, hamartomas presenting as gastrointestinal polyps, and an increased cancer risk.

Historical background

The first notification of the typical skin findings of PJS goes back to 1896, when the London physician Sir Jonathan Hutchinson illustrated a case of twin sisters with 'black pigmented spots on the lips, and inside of the mouth' (Figure 1) [1]. These pigmentations were different from the typical brown freckles located on the wings of the nose and cheeks. A follow-up of the so-called 'Hutchinson twins' noted that one sister died of intestinal blockage at the age of 20 years, and the second twin died of breast cancer at age 52 [2,3].

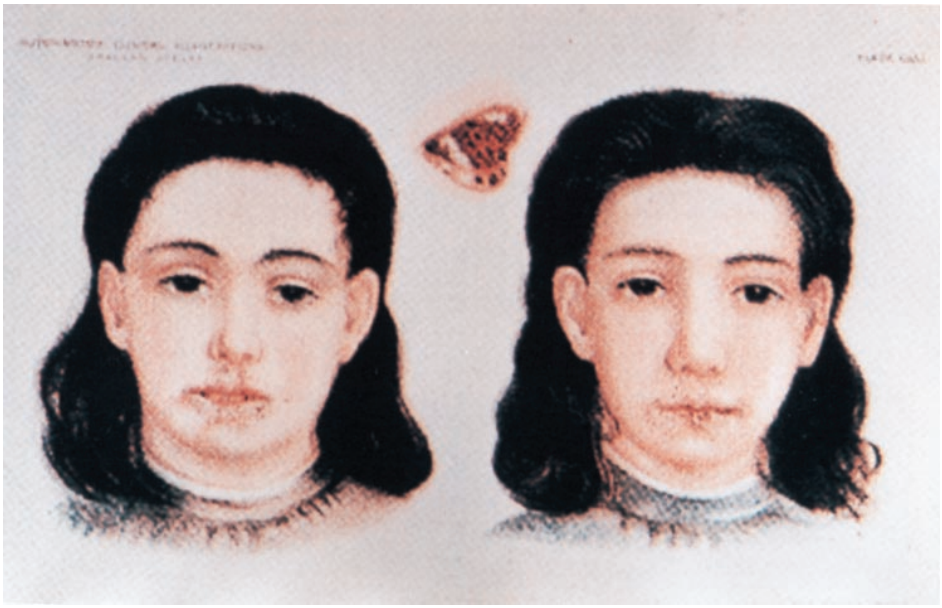

Figure 1. *Illustration of mucocutaneous pigmentation in 9-yr-old twin girls by Sir J. Hutchinson (adapted from: McGarrity et al. [8]).*

In 1921, the Dutch physician Jan Peutz (Figure 2, left) described the combination of gastrointestinal polyps and mucocutaneous pigmentations in three young siblings in the Netherlands Monthly Journal of Medicine ("Nederlandsch Maandschrift voor Geneeskunde") [4]. More than 20 years later, the American physician Harold Jeghers (Figure 2, right) described two patients with the same symptoms [3]. These observations led to the description of the syndrome in 1949 by Jeghers, McKusick and Katz in the "New England Journal of Medicine". Finally, in 1954 the eponym Peutz-Jeghers syndrome was introduced by Bruwer and colleagues [5].

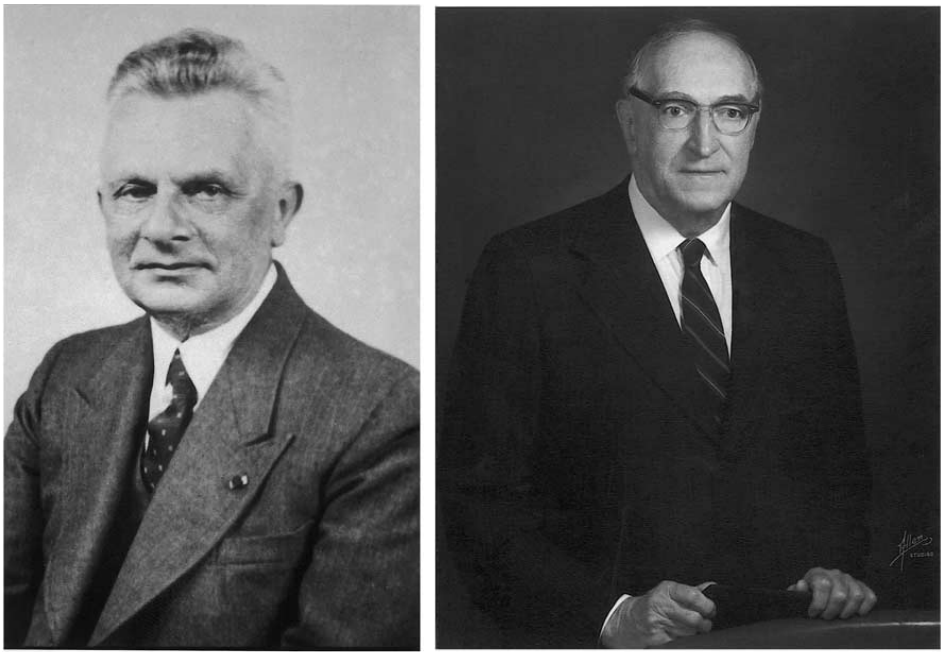

Figure 2. *Jan Peutz (1951) and Harold Jeghers (1988) (adapted from: Keller et al. [9]).*

Today, the clinical diagnosis of PJS is defined by diagnostic criteria of the World Health Organisation (WHO) (Box 1) [6]. The syndrome is rare, with an incidence estimated between 1 in 50,000 to 1 in 200,000 live births [7]. At present, in the Netherlands there are approximately 100 living PJS patients originating from over 60 families, including the original "Peutz-family".

Box 1. *Diagnostic criteria for Peutz-Jeghers syndrome (PJS) recommended by the World Health Organisation [6].*

A. Positive family history of PJS, and
1. Any number of histologically confirmed PJS polyps*, or
2. Characteristic, prominent, mucocutaneous pigmentation.
B. Negative family history of PJS, and
1. Three or more histologically confirmed PJS polyps, or
2. Any number of histologically confirmed PJS polyps and characteristic,prominent, mucocutaneous pigmentation.

**Histology of PJS polyps: a central core of smooth muscle that shows tree-like branching, covered with normal epithelium.*

Genetic background

PJS is caused by a germline mutation in the liver kinase B1 (*LKB1,* also known as serine/threonine kinase 11; *STK11*) tumour suppressor gene, located on chromosome 19p13.3 of the human genome [10,11]. The syndrome inherits in an autosomal dominant way, and in approximately 25% of cases a *de novo* mutation is present. With the currently available tech-

niques, a germline mutation is found in 80-94% of clinically affected patients, of which more than 60% is a point mutation [12-14]. Around 150 different germline *LKB1* mutations, mainly located in the kinase domain of the gene, have been associated with PJS [15]. The majority of the mutations carried by PJS patients result either in truncation or in abnormal splicing of the protein product, and cause inactivation of LKB1. The clinical features of PJS vary among patients and affected families. Despite several studies, no convincing genotype-phenotype correlation has been demonstrated [16-18].

Clinical features

Mucocutaneous pigmentations are seen in around 95% of PJS patients, and can be the first clinical sign before any gastrointestinal symptoms occur. The pigmentations develop already in infancy and fade during adolescence in most patients. They are mostly seen in the perioral region, especially on the vermilion border of the lips (Figure 3), but can also be found on the buccal mucosa, around the nostrils, at the hands and feet, and in the peri-anal region. The lesions are flat, have a blue-greyish colour, and vary in size between 1 to 5 mm. Although the pigmentations do not have malignant potential, the cosmetic effect can be burdensome.

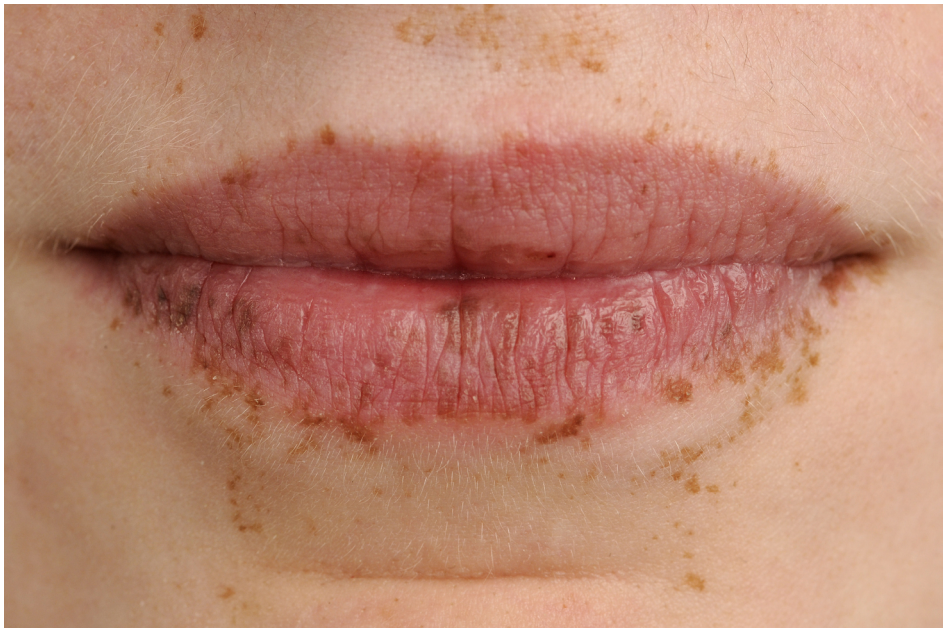

Figure 3. *Characteristic pigmentations at and around the lips of a Peutz-Jeghers syndrome patient.*

The polyps seen in PJS can develop throughout the gastrointestinal tract, but are mainly located in the proximal small bowel [19,20]. Number and size of gastrointestinal polyps differ per patient. PJS polyps have a typical macroscopic and microscopic appearance. They are referred to as hamartomas. They are often pedunculated and consist of a branched, tree-like smooth muscle core covered with normal epithelium (Figure 4) [21]. Hamartomas can develop already in the first decade of life and may cause complaints of abdominal pain, blood loss, or

acute intestinal obstruction, resulting from intussusception of a small bowel segment carrying a large hamartoma. The latter often requires acute surgical intervention. By the age of 10 years, one-third of patients experience polyp-related symptoms [22]. The cumulative risk of intussusception by the age of 20 years is 50% [20].

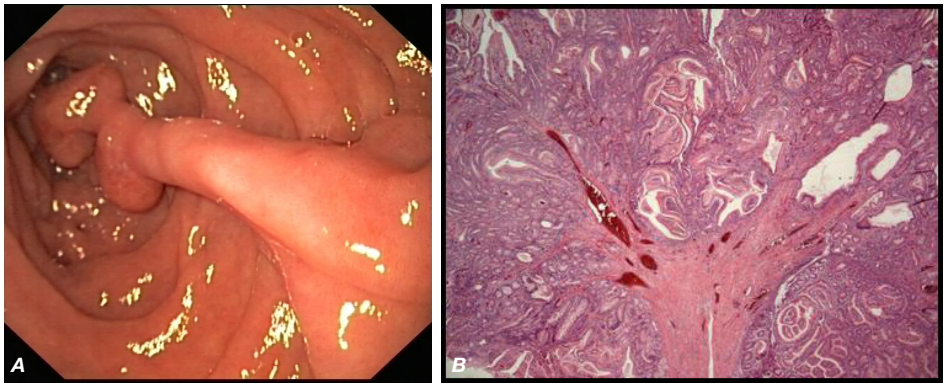

Figure 4. A. *Endoscopic picture of a pedunculated small bowel polyp of a Peutz-Jeghers syndrome patient.* ***B.*** *H&E stained tissue of a Peutz-Jeghers syndrome hamartoma, showing the branched smooth muscle core.*

PEUTZ-JEGHERS SYNDROME AND CANCER

During the second half of the 20th century, epidemiological and molecular genetic studies revealed that PJS is also associated with an increased cancer risk. A lifetime risk for any cancer between 37% and 93%, and relative risks ranging from 10 to 18 in comparison with the general population have been reported [23]. A substantial part of this elevated cancer risk is attributed to an increased risk for gastrointestinal tumours (mainly colorectal, small bowel, gastric and pancreatic cancer). A cohort study of 133 Dutch PJS patients showed a cumulative cancer risk of 76% at age 70 years, with a gastrointestinal cancer risk of 51% at this age [24]. Female patients are at even higher risk than male patients, because of the additional risk of breast cancer and gynaecological cancers in women.

Predominantly due to the elevated cancer risk, mortality in PJS patients is significantly increased compared to the general population. In the study of van Lier *et al*. 42 of the 133 patients had died at a median age of 45 years, including 28 cancer related deaths (67%) [24].

In addition to malignant gynaecological tumours, female PJS patients of reproductive age can also develop small, asymptomatic, bilateral ovarian tumours, known as “sex-cord” tumours with annular tubules (SCTATs) at a young age [25]. PJS-associated SCTATs have a low malignant potential and a good prognosis. These tumours are often associated with signs of hyperestrogenism, causing precocious puberty. Male PJS patients have an increased incidence of Sertoli cell testicular tumours [26,27]. These lesions are often hormonally active and patients can present with testicular enlargement or gynaecomastia.

Surveillance and treatment

Because of the risk of hamartoma-related complications and the increased cancer risk in PJS and due to the lack of effective methods for chemoprophylaxis to prevent the formation of hamartomas and malignancies, patient management should focus on prevention by surveillance. Over the years, several surveillance recommendations have been proposed, including the recommendations of the Dutch PJS working group in 2010 (Box 2) [7,23,28-30]. It should be noted that all these recommendations are based on clinical experience and expert opinion. In fact, no evidence-based surveillance strategy for PJS is available since no controlled trials on the effectiveness of such a program have been published.

In view of the morbidity caused by the hamartomas, it is generally accepted that surveillance of the gastrointestinal tract should start already at a young age (8-10 years). At later age, surveillance of the gastrointestinal tract should also address detection of precursor lesions or malignancies at an early, asymptomatic stage. Furthermore, female PJS patients should undergo regular surveillance of the breasts and genital tract.

Currently, PJS patients with cancer are treated according to standard protocols. More insight in the pathophysiology of PJS-associated cancer might lead to personalized treatment for patients with PJS. This topic is further discussed in chapter 2 of this thesis.

Box 2. *Dutch surveillance recommendations for Peutz-Jeghers syndrome patients [23].*

Examination[1]	Starting age	Interval
History, physical examination (including testicular palpation), and hemoglobin analysis	10 years	1 year (paediatrician)
VCE and/or MRI enteroclysis[2]	10 years	2-3 years
Gastroduodenoscopy	20 years	2-5 years (depending on findings)
Colonoscopy	25-30 years	2-5 years (depending on findings)
MRI and EUS pancreas	30 years	1 year, only in a prospective ongoing trial
Breast exam and breast MRI	25 years	1 year
Mammography and breast MRI	30 years	1 year[3]
Pelvic exam, cervical smear, transvaginal ultrasonography, and CA-125	25-30 years	1 year

EUS: endoscopic ultrasound; MRI: magnetic resonance imaging; VCE: video capsule endoscopy.
[1]Earlier and/or more frequently in symptomatic patients or if clinically indicated.
[2]If VCE shows polyps, it is recommended to perform an MRI enteroclysis to determine the exact localization and size of the polyps. Polyps >10-15 mm in diameter are an indication for double-balloon enteroscopy with polypectomy. In addition, we recommend intra-operative enteroscopy with polyp removal in each case that a laparotomy is indicated, to avoid re-laparotomies. If surgery is indicated a laparoscopic approach is preferred when possible.
[3]Mammography and MRI alternately performed every 6 months.

LKB1 AND CANCER

The *LKB1* tumour suppressor gene codes for the LKB1-kinase. The function of this kinase is complex and not completely unravelled yet. It is considered a "master-kinase", involved in a range of cellular processes, including energy metabolism, cell polarity and cell growth. LKB1 regulates these processes partly via the AMP-activated protein kinase/mammalian target of rapamycin (AMPK/mTOR) signalling pathway. Inactivation of LKB1 can lead to disruption of this cascade, resulting in upregulation of mTOR activity. This oncogenic protein triggers multiple cellular responses involved in uncontrolled proliferation and tumour growth [31-33].

Altered LKB1/AMPK/mTOR signalling is associated with a wide variety of cancers and hereditary hamartoma syndromes including PJS. Yet, the exact tumour suppressor functions of LKB1, both in sporadic as in PJS-associated carcinogenesis, remain elusive. The lack of a genotype-phenotype correlation in PJS patients reflects the involvement of additional genetic modifiers participating in carcinogenesis. Loss of heterozygosity of *LKB1* is found in hamartomas, but it is more frequently detected in carcinomas of PJS patients [34,35]. This might suggest that *LKB1* acts as a haplo-insufficient tumour suppressor gene. Current knowledge of the LKB1/AMPK/mTOR signalling pathway and its role in disease will be further discussed in chapter 2.

AIM OF THIS THESIS

As pointed out in this introduction, the link between PJS and cancer is indisputable, but questions remain. The aim of this thesis is to investigate 1) the role of LKB1 signalling in cancer development and treatment, and 2) the prevalence and prevention of cancer in PJS.

OUTLINE OF THIS THESIS

In continuation of the general introduction, in **chapter 2** the function and regulation of the LKB1/AMPK/mTOR signalling pathway is further explained. In addition, we describe how aberrant signalling of this cascade can cause disease, and in particular cancer. Finally, we reflect on the design and use of rational treatment targeting LKB1/AMPK/mTOR-associated disease.

Despite the strong association between germline *LKB1* mutations and an increased risk for colorectal cancer (CRC), the role of altered LKB1/AMPK/mTOR signalling in sporadic CRC is unclear. Somatic *LKB1* mutations have been rarely detected, but loss of heterozygosity of the *LKB1* locus is found in 15-53% of CRC cases. Previous studies have shown that loss of LKB1 increases cell migration and invasion in breast, lung and oesophageal cancer *in vitro.* In **chapter 3**, we aimed to identify the role of LKB1 signalling in sporadic gastrointestinal cancer by investigating the effect of reduced LKB1 expression on human colon cancer cells.

The pathogenesis and molecular mechanisms underlying PJS-associated gastrointestinal cancer development are still poorly understood. Although a hamartoma-carcinoma sequence has been described by some, this is still debated. If *LKB1* acts as a haplo-insufficient tumour suppressor gene, which additional oncogenic triggers are involved in carcinogenesis in PJS? In **chapter 4 and 5** we analysed tissue of both gastrointestinal carcinomas and dysplastic hamartomas of PJS patients to identify molecular alterations underlying PJS-associated gastrointestinal carcinogenesis.

To prevent complications of hamartomas and, at a later age, to detect (pre)malignant lesions, surveillance of the gastrointestinal tract of PJS patients, including the small bowel, is

indicated. However, visualization of the small bowel is technically challenging and the optimal strategy for surveillance has not been determined yet. **Chapter 6** of this thesis gives an overview of the currently available techniques to examine the small bowel in PJS patients, and proposes a step-up approach for surveillance.

Although PJS is known to be associated with an increased cancer risk, risk estimates and data about median age of onset differ widely in literature. This hampers counselling and care of patients and interferes with the implementation of surveillance strategies. Given the poor prognosis of pancreatic cancer, surveillance of the pancreas could be beneficial for high-risk populations. Do PJS patients belong to such a high-risk population? We address this question in **chapter 7**, by determining a reliable risk estimate for pancreatic cancer in a large cohort of Dutch PJS patients. Uncertainty also remains around breast cancer surveillance in PJS. It is believed that breast cancer risk in PJS patients approaches that of patients with BRCA1/2 mutations, but no solid studies have been performed to confirm this. In **chapter 8**, we assess the breast cancer risk and clinicopathological features of breast cancer cases in the Dutch PJS cohort. Based on these data, we aim to provide a breast cancer surveillance recommendation for female PJS patients.

Both the physical and psychological disease burden associated with PJS might influence family planning of patients. Genetic testing before birth could be considered as an extreme prevention method. In case of a known *LKB1* mutation, two methods for genetic testing of an unborn child are available in the Netherlands, i.e. prenatal diagnosis and pre-implantation genetic diagnosis. In **chapter 9**, we investigated the desire to have children in PJS patients and their attitudes towards antenatal genetic testing.

Finally, in **chapter 10**, the results and conclusions of the studies reported in this thesis are summarized and discussed.

Chapter 2

Targeting LKB1 signalling in hereditary and sporadic cancer

Adapted from:

The long and winding road to rational treatment of cancer associated with LKB1/AMPK/TSC/mTORC1 signaling

Wendy van Veelen, Susanne E. Korsse, Lianne van de Laar, Maikel P. Peppelenbosch

Oncogene. 2011;30(20):2289-303.

Targeting LKB1 signaling in cancer

S.E. Korsse, M.P. Peppelenbosch, W. van Veelen

Biochim Biophys Acta. 2013;1835(2):194-210

ABSTRACT

The serine/threonine kinase LKB1 is a master kinase involved in cellular responses such as energy metabolism, cell polarity and cell growth. LKB1 regulates these crucial cellular responses mainly via AMPK/mTOR signalling. Alterations in this signalling pathway are associated with a wide variety of cancers and hereditary hamartoma syndromes. Germline mutations in *LKB1* cause Peutz-Jeghers syndrome, in which patients develop gastrointestinal hamartomas and have an enormously increased risk for developing gastrointestinal, breast and gynaecological cancers. In addition, somatic inactivation of *LKB1* has been associated with sporadic cancers such as lung cancer. The exact mechanisms of LKB1-mediated tumour suppression remain so far unidentified, however, the inability to activate AMPK and the resulting mTOR hyperactivation have been detected in PJS-associated lesions. Therefore, targeting LKB1 in cancer is now mainly focusing on the activation of AMPK and inactivation of mTOR. Preclinical *in vitro* and *in vivo* studies show encouraging results regarding these approaches, which have even progressed to the initiation of a few clinical trials. In this review, we describe the functions, regulation and downstream signalling of LKB1, and its role in hereditary and sporadic cancers. In addition, we provide an overview of several AMPK activators, mTOR inhibitors and additional mechanisms to target LKB1 signalling, and describe the effect of these compounds on cancer cells. Overall, we will explain the current strategies attempting to find a way of treating LKB1-associated cancer.

LKB1 SIGNALLING AND FUNCTION

The LKB1 protein

The liver kinase B1 (*LKB1,* also known as serine/threonine kinase 11, *STK11*) gene encodes a 48kDa protein, LKB1, which is ubiquitously expressed in adult as well as foetal tissues, particularly in pancreas, liver, testes and skeletal muscle [10,11]. It contains an evolutionary conserved serine/threonine kinase domain and a C-terminal region, where some posttranslational modification sites have been identified (Figure 1) [11,36-38]. First, four autophosphorylation sites have been described, i.e. threonine (Thr) 185, Thr189, Thr336 and Thr402 [39,40]. Autophosphorylation of Thr336 seems not to affect its catalytic activity nor its cellular localization, however, it may inhibit the cell growth suppressive capacity of LKB1 [40]. In addition, four phosphorylation sites have been identified, i.e. serine (Ser) 31, Ser325, Thr363 (mouse Thr366) and Ser428 (mouse Ser431) [40-42]. Thr363 is phosphorylated by ATM in response to ionizing radiation [43]. Phosphorylation of Ser428 by cAMP-dependent kinase (PKA), p90RSK, and protein kinase Czeta (PKCζ) is not affecting its catalytic activity, but is essential for LKB1 to inhibit cell growth [41,42,44]. The kinases for Ser31 and Ser325 have so far not been identified. Finally, a prenylation site, i.e. Cys430 (mouse Cys433), has been identified [42]. Since it has been shown that farnesylation at Cys430 is not essential for LKB1 to suppress cell growth, the functional relevance of this prenylation is not yet understood [41].

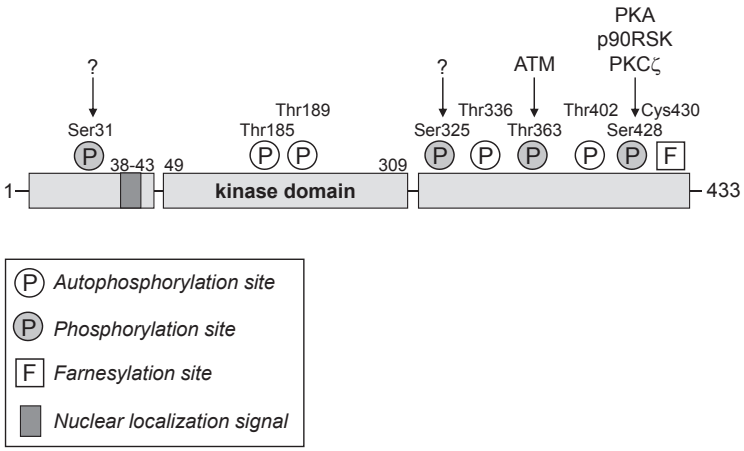

Figure 1. *Schematic diagram of human LKB1.*
The human LKB1 protein constitutes 433 amino acids. Amino acids 38-43 form a nuclear localization signal domain. Amino acids 49-309 form the serine/threonine kinase domain. Four autophosphorylation sites have been identified, i.e. Thr185, Thr189, Thr336 and Thr402. Four phosphorylation sites for other kinases have been identified, i.e. Ser31, Ser325, Thr363 and Ser428. Cys430 is a farnesylation site.

LKB1 activation

LKB1 contains a nuclear localization signal domain which is likely to be the reason that LKB1 is normally localized in the nucleus (Figure 1 and 2) [45]. Because LKB1 lacks a nuclear export domain of its own, it needs interaction with other proteins in order to be actively exported out of the nucleus. Activation of LKB1 is therefore associated with its translocation to the cytoplasm which is induced upon formation of a heterotrimer with the STE20-related adaptor

(STRADα) and scaffolding mouse 25 (MO25) proteins (Figure 2) [36,39]. By facilitating the binding of exportins to LKB1 and acting as a competitor for importin-α/b, STRADα prevents nuclear re-localization of LKB1. MO25 merely serves as a stabilizer of the LKB1-STRAD interaction [36]. When in complex with STRADα and MO25 and located in the cytoplasm, LKB1 phosphorylates and activates kinases of the AMP-activated kinase (AMPK) family (Figure 2) [46-48]. Via AMPK and several other downstream mediators, LKB1 regulates crucial cellular processes involved in cell polarity, energy metabolism and cell growth (Figure 3).

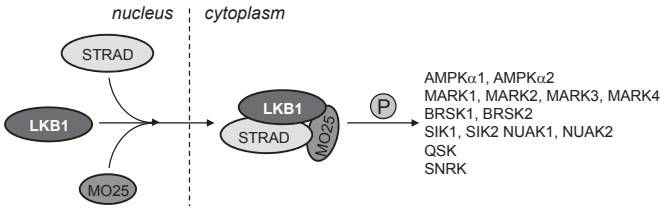

Figure 2. *LKB1 activation.*
Lacking a nuclear export signal of its own, LKB1 resides in the nucleus. Upon binding to STRADα and MO25, LKB1 is exported to the cytoplasm and becomes catalytically active. In the cytoplasm, LKB1 has been shown to phosphorylate the 14 serine/threonine kinases of the AMPK family.

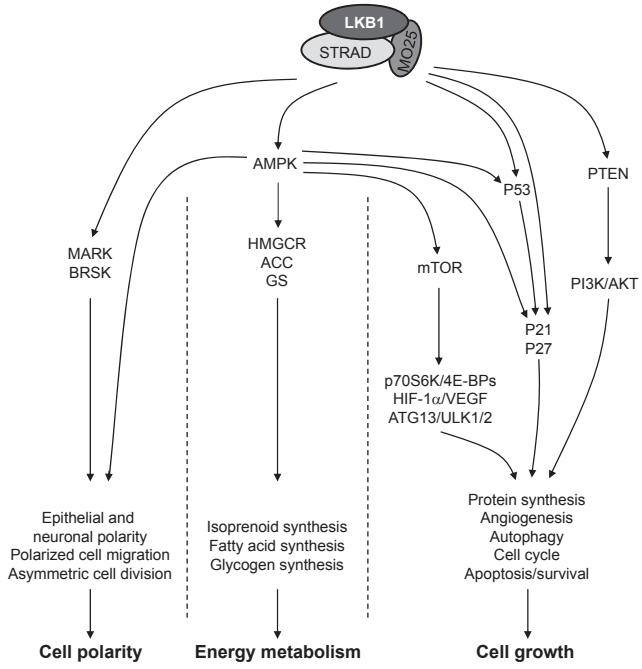

Figure 3. *LKB1 signalling and function.*
Via several downstream mediators, LKB1 regulates crucial cellular processes involved in cell polarity, energy metabolism and cell growth. In addition, LKB1 regulates hematopoietic stem cell homeostasis. Since this seems to be independent of AMPK/mTOR signalling, the underlying molecular mechanisms are yet to be identified.

LKB1 in cell polarity

Originally, the *Caenorhabditi elegans* and *Drosophila melanogaster* counterparts of *LKB1*, *Par-4* and *dLkb1* respectively, have been shown to play major roles in cellular polarity [49,50]. LKB1 induces epithelial-cell polarization through sorting of apical and basolateral membrane proteins, formation of epithelial junctions and reorganisation of the actin cytoskeleton, which is at least in part mediated via activation of AMPKα [51-56]. In addition to activation of AMPKα, LKB1 regulates polarization through the activation of MARK isoforms (mammalian counterparts of *C. Elegans* and *D. melanogaster* Par-1), which play a remarkable role in microtubule skeleton organisation [57,58]. Through activation of BRSK (or SAD) proteins, LKB1 regulates neuronal migration and axonal outgrowth [59,60]. A role for *C. elegans* and *D. melanogaster* Par-4/dLkb1 in asymmetric positioning of the mitotic spindle and cytoplasmic determinants during mitosis has been established [61]. In mammalian cells, LKB1 has recently been shown to be involved in epithelial polarization by controlling Rho GTPases, primary cilium, phosphoinositide and Wnt/GSK3β signalling [62-65]. More specifically, LKB1 is involved in centrosome positioning, lumen initiation and brush border formation during epithelial morphogenesis [63,64]. Together, this shows that LKB1 acts as a master kinase regulating neuronal and epithelial polarization, polarized cell migration and asymmetric cell division (Figure 3). Given the role of LKB1 in these attributions, normal LKB1 function is required for proper function and proliferation of epithelial tissues.

LKB1 in energy metabolism

In addition to the regulation of cellular polarity, AMPK signalling controls lipid and glucose metabolism (Figure 3). In circumstances of energy stress due to either excessive ATP consumption, or reduced aerobic ATP production, e.g. in the case of hypoxia, cellular AMP/ATP ratios increase. This is sensed by AMPKγ which binds AMP, leading to the complex formation of AMPKα, -β and –γ subunits [66-68]. Threonine residue 172 in the activation loop of AMPKα is now accessible to be phosphorylated by LKB1 [69-71]. Activated AMPK controls metabolic processes such as isoprenoid, fatty acid and glycogen synthesis via regulation of downstream targets such as HMG-CoA reductase, acetyl CoA carboxylase (ACC) and glycogen synthase [72-74]. Thus, by suppressing energy-consuming processes on the one hand, and enhancing energy gaining pathways on the other, AMPK activation by LKB1 aids in restoration of the cellular energy status.

LKB1 in cell growth

Moreover, LKB1 has been associated with cell growth control via multiple different signalling pathways (Figure 3). One such major downstream pathway of LKB1/AMPK is TSC/mTOR signalling [75]. Activated AMPK phosphorylates TSC2, thereby activating the TSC1:TSC2 complex [76], which in turn regulates the activity of the mTORC1, a complex consisting of mTOR, raptor and mLST8. The activated TSC1:TSC2 complex, which expresses GTPase activity towards Rheb, a small G-protein that promotes mTORC1 activity when GTP-bound, induces conversion of active GTP-bound Rheb to inactive GDP-bound Rheb which subsequently results in inhibition of mTORC1 [77]. In addition to inhibiting mTORC1 via phosphorylation of TSC2, AMPK directly phosphorylates raptor resulting in the inhibition of mTORC1 [78]. mTORC1 plays a key role in protein translation by phosphorylating and activating the ribosomal protein S6 kinase (S6K), and through the inhibition of eukaryotic initiation factor 4E

binding proteins (4E-BPs) [79]. In addition, mTORC1 activation stimulates angiogenesis by stabilizing hypoxia-inducible factor 1α (HIF-1α) in conditions of hypoxia [80].

Furthermore, mTORC1 activity inhibits autophagy via phosphorylation of ATG13 and ULK1/2 [81,82]. Thus, LKB1 regulates mTOR signalling to control cell growth via different pathways and cellular responses. However, recently it has been shown that LKB1 is necessary to maintain hematopoietic stem cell (HSC) homeostasis mainly independent of AMPK and mTOR [83-85], indicating that also other molecular pathways are involved in LKB1-regulated cell growth (Figure 3).

LKB1 is also involved in cell cycle regulation and apoptosis. Reintroduction of LKB1 expression in cells lacking LKB1 induces cell cycle arrest in G1 [86-92], while knocking down LKB1 expression triggers cell cycle progression from G1 to S phase [93]. Notably, reintroduction of LKB1 catalytic deficient mutants in colorectal cancer (CRC) cells could not induce cell cycle arrest, but caused even upregulated expression of the cell cycle inducer cyclin D [92]. LKB1-induced cell cycle arrest is mediated by upregulation of the cell cycle inhibitors p21 and/or p27 [86-90,92,94]. In addition, *Lkb1*-mediated tumour promotion has been shown to be mediated by suppression of p21-dependent growth arrest *in vivo* [95].

LKB1-mediated cell cycle regulation has been shown to be regulated via p53-dependent and p53-independent mechanisms [86,87,91,92,94,96]. Additionally, LKB1 has been shown to interact with and phosphorylate p53, and mediate p53-induced apoptosis [86,97,98], while in *Drosophila* dLkb1 induces p53-independent apoptosis via activation of the JNK pathway [99]. In mice, compound loss of p53 and Lkb1 accelerates onset and increased incidence of polyps, indicating that Lkb1 and p53 cooperate in tumour development [100,101]. LKB1 mutants have been shown to diminish p53 activity [102]. Thus, LKB1 suppresses cell cycle progression and induces apoptosis most likely via suppression of p21, though the role of p53 in these processes remains contradictory (Figure 3).

Several studies in cells and in mice have shown that LKB1 acts upstream of PTEN (Figure 3). In fact, LKB1 has been shown to interact with and phosphorylate PTEN, which was disrupted by introducing mutations in LKB1 [103,104]. In addition, LKB1 induces expression and nuclear export of PTEN resulting in reduced PI3K/AKT signalling and apoptosis [89,104,105]. This LKB1-mediated nuclear export of PTEN has been shown to be independent of AMPK/mTOR signalling [105]. *In vivo*, loss of *Lkb1* cooperates with *Pten* loss to accelerate tumourigenesis [106,107]. The tumourigenesis in these compound mutant mice was shown to be, at least in part, mediated by mTOR signalling since treatment with mTOR inhibitors reduced tumour formation [107,108].

These studies reveal a pleiotropic role for LKB1 in cell polarity, energy metabolism, and cell growth, processes that are deregulated in cancer. Most of these processes are mediated via AMPK/mTOR signalling, suggesting that this major downstream pathway is a suitable candidate to target for therapy against LKB1-associated cancer. However, other responses induced by loss of LKB1 have been shown to be independent of AMPK and/or mTOR signalling, suggesting that the tumour suppression functions of LKB1 are, at least in part, also mediated via other downstream effectors.

LKB1 IN DISEASE

Through specific genetic alterations in different components of the LKB1/AMPK/mTORC1 signalling pathway, this metabolic pathway is associated with disease (Figure 4). Germline inactivation of the tumour suppressor genes *PTEN*, *NF1*, *LKB1*, *TSC1* and *TSC2* predisposes

to a group of rare autosomal dominant inherited hamartoma syndromes, including Peutz-Jeghers syndrome (PJS). These hereditary disorders are characterized by the development of hamartomas in multiple tissues (Table 1). Hamartomatous polyps have a relatively benign appearance, but with a markedly disturbed architecture of cells present in the area in which they normally occur, i.e. mesenchymal, stromal, endodermal, and ectodermal [109]. Despite the fact that these hamartomas follow a relatively benign course, they can cause e.g. bowel obstruction, seizures or haemorrhage which may lead to severe complications and even death [109]. In addition to the development of multiple hamartomas, these polyposis syndromes are associated with the development of a variety of cancers as well.

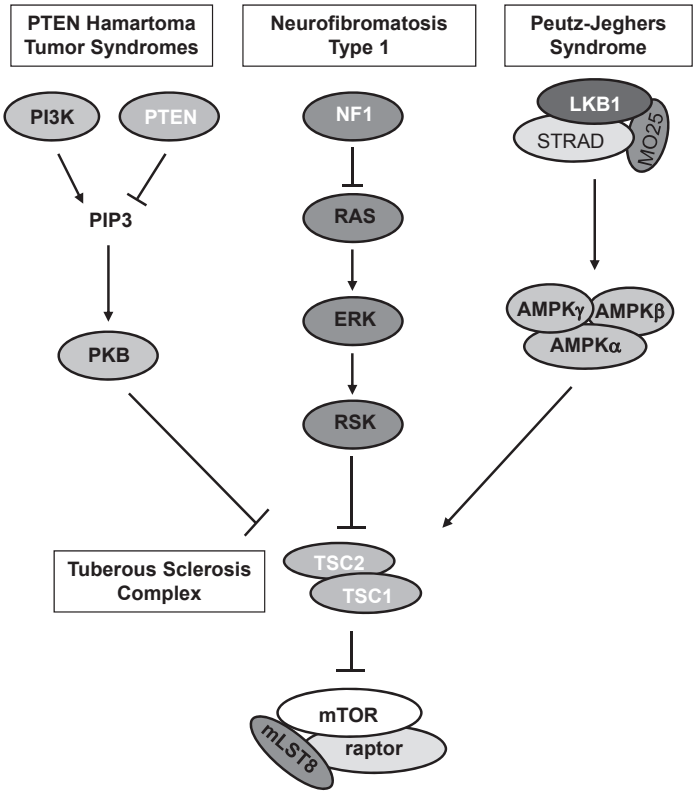

Figure 4. *LKB1/AMPK/mTOR signalling in hamartomas sydromes.*
Genetic alterations in the LKB1/AMPK/mTOR pathway are involved in several hereditary hamartomas syndromes. Germline inactivation of the tumour suppressor PTEN, which normally inhibits PI3K/PKB signalling, predisposes to a variety of hamartomas syndromes grouped as PTEN hamartomas tumour syndromes. Germline inactivation of the tumour suppressor NF1, an inhibitor of the RAS/ERK pathway, predisposes to NF1. Germline inactivation of the tumour suppressor LKB1, the activator of AMPK, predisposes to PJS. Germline inactivation of the tumour suppressors TSC1 or TSC2 both predispose to TSC. Tumour suppressors are indicated in white.

Table 1. *Autosomal dominant inherited hamartoma syndromes associated with the metabolic LKB1/AMPK/TSC/mTOR signalling pathway.*

Disorder	Prevalence	Gene (chromosome)	Hamartomatous lesions	Neoplastic lesions	Additional manifestations
Tuberous Sclerosis Complex (TSC)	1:5.800	*TSC1* (9q34) *TSC2* (16p13.3)	(Sub)cortical tubers (glial harmartomas), subependymal glial nodules, retinal hamartomas, facial angiofibromas, (peri) ungual fibromas, renal angiomyolipomas, cardiac rhabdomyomas, and pulmonary lymphangiomyomatosis.	Brain cancer (subependymal giant cell astrocytomas) and kidney cancer (cysts, oncocytomas, clear cell, papillary, or chromophobe).	Hypomelanotic macules, forehead plagues, shagreen patches, developmental delays, epilepsy, autism.
Peutz-Jeghers syndrome (PJS)	1:150.000	*LKB1* (*STK11*) (19p13.3)	Mainly in gastrointestinal tract (small intestines > stomach > colon/rectum), also in nose, bronchi, renal pelvis, uterus, and bladder.	Mainly gastrointestinal cancer (colorectal, pancreatic, small intestinal, gastric, esophageal), also breast, lung, endometrial, ovarian (primarily granulosa cell subtype) and cervical (primarily highly malignant cervical adenoma malignum subtype) cancer. Rarely sex cord tumours with annular tubules.	Hyperpigmentation of lips, buccal mucosa, hands/feet, genitals, around nose/eyes.
PTEN Hamartoma Tumour Syndrome (PHTS)[1]	1:200.000	*PTEN* (10q22-23)	Mucocuteneous lesions (facial trichilemmomas, acral keratoses, papillomatous papules), and intestinal hamartomas.	Breast, thyroid (follicular), endometrial cancer.	Macrocephaly, developmental delays, mental retardation, scoliosis, hyperpigmentation genitals.
Neurofibromatosis type 1 (NF1)	1:3.500	*NF1* (17q11.2)	Plexiform and cutaneous neurofibromas, schwannomas, iris hamartomas (Lisch nodules).	Malignant peripheral nerve sheet tumours, gliomas, astrocytomas.	Café au lait spots, axillary freckles, scoliosis, cognitive and learning disabilities, skeletal abnormalities.

[1]*PHTS include Cowden syndrome, Bannayan-Riley-Ruvalcaba syndrome, Proteus syndrome, and Lhermitte-Duclos disease.*

Peutz-Jeghers syndrome

Peutz-Jeghers syndrome (PJS) is a rare autosomal dominant disorder. PJS is characterized by mucocutaneous hyperpigmentation, gastrointestinal hamartomatous polyposis and a highly increased cancer risk. All PJS patients develop early-onset hamartomatous polyposis throughout the entire gastrointestinal (GI) tract. PJS polyps are clearly distinct from the more common adenomatous polyps, which are premalignant lesions characterized by a dysplastic epithelium ('adenoma-to-carcinoma sequence'). In contrast, the overlying epithelium in hamartomatous polyps is usually well differentiated but can be hyperplastic [110]. Adenomatous and carcinomatous changes in PJS-hamartomas have been reported, though rarely [111-113]. Therefore, the gastrointestinal hamartomas and carcinomas developed in PJS patients are believed to be two distinct entities, though this is still debated. PJS patients are also at increased risk for developing cancer at a relatively young age, and at an age of 70 years a cumulative risk of 37-93% have been determined for these patients [24,114]. A wide spectrum of malignancies has been described, with the highest cumulative risks attributed to cancers of gastrointestinal (38-93%), breast (32-54%) or gynaecological (13-18%) origin [114]. The most frequently observed gastrointestinal cancers constitute CRC, but also small intestinal, gastric and pancreatic are observed more frequently compared to the general population [24,114,115]. In addition, rare tumours such as testicular or ovarian sex cord tumours, Sertoli-cell tumours and adenoma malignum of the uterine cervix have been associated with PJS.

The tumour suppressor gene *LKB1*

In 1997, the PJS susceptibility locus was mapped to chromosome 19p13 [116]. One year later, inactivating germline mutations in LKB1 were detected to cause predisposition for PJS [10,11]. With the currently available techniques, an *LKB1* germline mutation can be detected in approximately 80-94% of clinically affected PJS families. Around 25% of cases are sporadic, thought to be due to *de novo* germline *LKB1* mutations.

Around 150 different mutations without a hotspot in *LKB1* have been associated with PJS, without a clear genotype-phenotype correlation [117]. The majority of mutations result in truncation or abnormal splicing, although in approximately 20% of the cases a missense mutation in the kinase domain of *LKB1* is detected. It has been suggested that truncating mutations tend to associate with an earlier age of onset of disease as compared to PJS cases associated with missense mutations in *LKB1* [16]. The vast majority of PJS-associated missense mutations are located in the serine/threonine kinase domain, resulting in impaired kinase activity and cell growth suppressive capacity [11,36-38]. In addition, a substantial proportion of PJS-associated mutations are located in the C-terminus of LKB1. These mutations have been shown to impair cellular polarity but not LKB1 kinase activity or its ability to promote growth arrest [118]. For several PJS-associated mutations, it has been shown that the resulting LKB1 mutants are retained in the nucleus [11,36-38].

LKB1 is classified as a tumour suppressor gene, implying that both alleles need to be inactivated to contribute to tumour development. Loss of heterozygosity (LOH) of the remaining *LKB1* allele has been detected in PJS-hamartomas, but it is observed more frequently in carcinomas [119,120]. This suggests that bi-allelic loss of *LKB1* is not necessary for hamartomatous polyp development, but favours progression to carcinoma. Therefore, *LKB1* is suggested to act as a haplo-insufficient tumour suppressor gene. Because LOH does not

necessarily take place in the invading epithelial cancer cells, there is still some controversy about where LKB1 loss contributes to carcinogenesis in PJS patients.

To investigate the tumour suppressor function of LKB1, mouse models have been generated and characterized. The original studies characterizing Lkb1 function in mice showed that *Lkb1*-/- mice die at embryonic stage, highlighting a crucial role during early development [121]. Mice with a heterozygous deletion are tumour prone, showing an increased incidence of spontaneous tumour formation as well as increased susceptibility to toxicity-induced carcinogenesis [35,91,121,122]. Moreover, *Lkb1*+/- mice develop hamartomatous polyps in the stomach and intestines, but, similar to their human counterparts, these polyps appear to lack or have only low malignant potential [34,35,122]. Finally, conditional *Lkb1* loss in various tissues is also associated with the development of cancer [123-126]. The interesting conclusion from these studies is that LKB1 is not likely to be a driver mutation in cancer, but as a secondary mutation it might enhance tumourigenesis.

LKB1 haplo-insufficiency

LKB1 is classified as a tumour suppressor gene, implying that both alleles need to be inactivated to contribute to tumour development. Loss of heterozygosity (LOH) of the remaining *LKB1* allele has been detected in PJS-hamartomas, but it is observed more frequently in carcinomas [119,120]. This suggests that bi-allelic loss of *LKB1* is not necessary for hamartomatous polyp development, but favours progression to carcinoma. Therefore, *LKB1* is suggested to act as a haplo-insufficient tumour suppressor gene. The haplo-insufficiency of *Lkb1* has been confirmed in mice, where neither loss of the wild-type *Lkb1* allele nor loss of expression of Lkb1 could be detected in most gastrointestinal polyps of heterozygous *Lkb1* knockout mice [34,35,122]. These results suggest that partial loss of Lkb1 is sufficient for tumour development. Haplo-insufficient tumour suppressor genes usually evoke their role in tumourigenesis in the context of additional oncogenic triggers. In the case of *Lkb1*, it has been shown that additional loss of *Pten* or *p53*, or additional oncogenic activation of Kras synergizes with *Lkb1* loss in order to promote tumour formation [95,100,101,106]. In a subset of gastrointestinal hamartomas of heterozygous *Lkb1* knockout mice loss of the wildtype *Lkb1* allele or Lkb1 expression could be detected specifically in the epithelial compartment, suggesting that Lkb1 exerts its tumour suppressive functions mainly in the epithelium [34]. However, myofibroblast-specific loss of *Lkb1* in mice has been shown to be sufficient for gastrointestinal hamartoma development, indicating that Lkb1 suppresses tumour formation through signalling in mesenchymal cells [127]. No phenotypical differences were observed between polyps of mice with mono-allelic or bi-allelic *Lkb1* deletion, suggesting that also in stromal cells Lkb1 acts as a haplo-insufficient tumour suppressor [127]. Lkb1-deficient mesenchymal cells stop producing TGFβ, which is a crucial factor suppressing tumour initiation and progression. Interestingly, LKB1 has recently been shown to inhibit Smad4-mediated transcriptional activation of TGFβ targets in epithelial cells [128], which might indicate that LKB1 controls TGFβ signalling at both ends. However no strategies for the moment have been devised for targeting the TGFβ pathway, likely due to its more complex role in cancer late progression.

Table 2. *Frequencies of LKB1 mutations/deletions and of LOH of 19p13.3 reported in sporadic carcinomas.*

Type of sporadic cancer	Mutation/ Deletion	LOH 19p13.3	Reference
Gastrointestinal			
CRC	0/20 (0%)	13/50 (26%)	143
	0/72 (0%)		144
	7/13 (54%)	10/19 (53%)	131
	1/71 (1%)	10/52 (19%)	145
	1/80 (1%)		146
	0/50 (0%)		15
		5/38 (13%)	147
		5/23 (22%)	148
		3/8 (38%)[1]	149
Small intestinal	0/6 (0%)		146
Gastric	3/28 (11%)		150
	0/8 (0%)		151
	0/40 (0%)		146
Pancreatic	1/12 (8%)		151
	3/103 (3%)	8/23 (35%)	152
	1/20 (5%)[2]	5/20 (25%)[2]	153
	0/5 (0%)[3]		154
Breast and gynaecological			
Breast	0/62 (0%)	3/40 (8%)	155
		5/30 (17%)	148
		9/16 (56%)[1]	149
Ovarian	0/45 (0%)	12/49 (24%)	156
SCTAT	0/12 (0%)[4]		151
	1/12 (8%)[4]		156
	0/5 (0%)	0/2 (0%)	157
	0/12 (0%)	12/29 (41%)	158
Cervical	1/26 (4%)		151
	0/8 (0%)[5]	2/8 (25%)[5]	157
Testicular	1/28 (4%)		143

Table 2. *Frequencies of LKB1 mutations/deletions and of LOH of 19p13.3 reported in sporadic carcinomas.*

Type of sporadic cancer	Mutation/ Deletion	LOH 19p13.3	Reference
Lung			
		7/12 (58%)[1]	149
NSCLC adenocarcinoma	1/12 (8%)		151
	5/20 (25%)	21/30 (70%)	159
	7/155 (5%)		140
	27/80 (34%)		142
	3/81 (4%)		141
	13/207 (6%)		160
	0/51 (0%)		161
	4/7 (57%)[6]		162
NSCLC squamous cell	0/12 (0%)		151
	8/42 (19%)		142
	0/14 (0%)		141
	5/92 (5%)		160
	6/67 (9%)		161
NSCLC large cell	0/3 (0%)		151
	1/7 (14%)		142
	0/2 (0%)		141
	3/11 (27%)		161
SCLC	0/1 (0%)		151
	0/1 (0%)		141
Additional			
Melanoma	0/6 (0%)		151
	1/15 (7%)		163
	2/35 (6%)		164
		2/8 (25%)[1]	149
Soft tissue	0/24 (0%)		151
Renal	0/19 (0%)		151
		2/8 (25%)1	149
Brain		31/248 (13%)[7]	149

CRC: colorectal carcinoma; LOH: loss of heterozygosity; NSCLC: non-small cell lung cancer; SCLC: small cell lung cancer; SCTAT: sex-cord stromal tumours of the ovary.

[1]Brain metastases from this cancer type

[2]Intraductal papillary mucinous neoplasms

[3]Pancreatic acinar cell carcinomas

[4]Ovarian granulosa cell tumours

[5]Adenoma malignum of uterine cervix

[6]Mucinous bronchioloalveolar carcinomas

[7]Various histological subtypes of which LOH was most frequently observed in gliomas (17%) and pituitary adenomas (19%).

LKB1 IN SPORADIC CANCER

Despite the strong association between *LKB1* mutations and increased risk for carcinogenesis in PJS, *LKB1* is not commonly mutated somatically in sporadic cancers, except for lung cancer (Table 2). In contrast, allelic loss, *LKB1* promoter hypermethylation or reduced LKB1 expression is observed in a wide variety of sporadic cancers (reviewed in [129,130]) (Table 2). Because of the suggested haplo-insufficiency of LKB1, this could indicate that loss of LKB1, probably in combination with additional oncogenic events, is involved in the development and/or progression of these sporadic cancer types.

Colorectal cancer

Since PJS patients have an increased risk for developing CRC [24], *LKB1* mutation analyses have been set-up for sporadic CRC as well. However, somatic *LKB1* mutations have been detected sparsely in these carcinomas (Table 2). In contrast, one study reported a mutation frequency of 54% in carcinomas in the left-sided colon [131]. Therefore it was suggested that LKB1 plays an important role in left-sided colon cancer carcinogenesis, however, in a later study no somatic *LKB1* mutations could be detected in 50 left-sided colon carcinomas [15] (Table 2). LOH of 19p13.3 has been detected more frequently in sporadic CRC with frequencies ranging from 13% to 53% (Table 2). This suggests that reduced levels of LKB1 may contribute to the development of sporadic CRC, though not through mutational inactivation. *Lkb1*-deficient mice did not develop intestinal carcinomas, nor did the hamartomatous polyps progress to a more malignant histological phenotype when *p53* was additionally deleted [100,101]. Therefore, it remains elusive how loss of LKB1 contributes to colorectal carcinogenesis.

Pancreatic cancer

PJS patients have an increased risk for developing pancreatic cancer, but somatic *LKB1* mutations have been detected only in a small proportion of sporadic pancreatic carcinomas (Table 2). In addition, a low frequency of reduced LKB1 expression has been observed in these carcinomas [132]. However, reintroduction of LKB1 expression in LKB1-silenced pancreatic cancer cells induced apoptosis, suggesting that LKB1 suppresses pancreatic cancer cell survival *in vitro* [133]. Deletion of *Lkb1* in murine pancreatic epithelial cells of the islets, ducts, and acini resulted in the development of ductal metaplasia and cystadenomas, but no formation of pancreatic intraepithelial neoplasia (PanIN) or adenocarcinomas [95,124]. Introduction of an oncogenic *Kras* mutation in heterozygous *Lkb1* knockout mice resulted in formation of pancreatic ductal adenocarcinomas, indicating that Lkb1 is a haplo-insufficient tumour suppressor and cooperates with Kras in the development of pancreatic cancer in mice [95].

Breast cancer

Somatic mutations in *LKB1* and LOH of 19p13 is not observed frequently in primary sporadic breast carcinomas, however, LOH was frequently detected in brain metastases from breast carcinomas (Table 2). In addition, it has been shown that LOH of the *LKB1* locus in primary breast carcinomas increased significantly as the tumours progressed to poorer histological grade, and that low LKB1 expression in breast carcinomas is associated with poorer histological grade, presence of lymph node metastasis and a shorter overall survival [88,134,135]. In

line with these observations, it has been shown that LKB1 suppresses breast cancer cell migration and invasion *in vitro*, and tumour growth, microvessel density and lung metastasis *in vivo* [136,137]. Together, this suggests that loss of LKB1 is a late event in breast cancer and promotes breast cancer progression.

Lung cancer

Despite the fact that lung cancer is not clearly associated with PJS, *LKB1* is the third most frequently mutated gene in sporadic non-small cell lung carcinomas (NSCLC). Though the proportion of missense mutations seems to be higher among the somatic mutations in sporadic cancer (45%) than among the germline mutations in PJS patients (21%), the mutations detected in NSCLC comprise mainly nonsense mutations or a combination of indels or large intragenic deletions on one *LKB1* allele - resulting in truncation of the protein - plus large chromosomal deletions on the other allele [130,138]. Somatic *LKB1* mutations are most frequently detected in adenocarcinomas, but also in other histological subtypes of NSCLC (Table 2). Notably, *LKB1* mutations are more frequently observed in NSCLC of Caucasians compared to East Asian populations (reviewed in [139]). In addition, *LKB1* mutations frequently coincide with *KRAS* mutations. NSCLC patients with *LKB1;KRAS* compound mutations tend to have a poorer prognosis compared to patients with *KRAS*-mutated NSCLC without a concomitant *LKB1* mutation, suggesting that loss of LKB1 induces more aggressive tumour phenotypes [140,141]. This is also shown in mice where double mutant *Lkb1;Kras* mice develop more aggressive tumours with higher tumour multiplicity, multiple NSCLC histologies and more frequent metastasis [142].

Overall, loss of LKB1 is involved in cancers associated with PJS as well as with a variety of sporadic malignancies, where it is associated with tumour progression. Therefore, LKB1 and its downstream signalling pathways suit a perfect target for therapeutic intervention of these cancers.

TARGETING LKB1 SIGNALLING IN CANCER

Since various cancers have been associated with impaired AMPK activation and/or mTOR inhibition, whether or not triggered by loss of LKB1, AMPK/mTOR signalling has been suggested to serve a suitable target for cancer treatment. Both pharmacological AMPK activators (metformin) and mTOR inhibitors (rapamycin and its analogs sirolimus, everolimus and temsirolimus) are available and used in clinical settings. In addition to these clinically approved drugs, additional compounds affecting LKB1 signalling have been identified and are being tested in preclinical settings for their efficacy as anti-cancer agents. Since the discovery of LKB1 as the causative gene for PJS, and its signalling routes regulating cell growth, several studies have been focusing on targeting LKB1/AMPK/mTOR signalling in order to treat PJS-associated tumours.

Metformin

In the 1970s, metformin, a biguanide (Figure 5A), was approved by the Food and Drugs Administration (FDA) for the treatment of Diabetes Mellitus type 2 (DM2) in Europe, and in 1995 in the USA. Since then, millions of persons are using metformin, which has been shown to increase overall survival and prevent macrovascular complications in DM2 patients [165].

Metformin is also used successfully in polycystic ovarian syndrome (PCOS) and the management of the metabolic syndrome [166,167]. The efficacy of metformin in these metabolic disorders is attributed to the potential of metformin to reduce hepatic gluconeogenesis and improve insulin sensitivity [168]. Metformin has been shown to be an activator of AMPK, which is one of the master regulators of these metabolic processes (Figure 5C) [169]. The interest in metformin as an anti-cancer drug arose when population studies showed that metformin use is associated with a significant reduction of neoplasms in general, and of breast, pancreatic and prostate cancer in particular [168,170,171]. Therefore, metformin might also serve as an anti-tumour agent in cancer prevention and treatment.

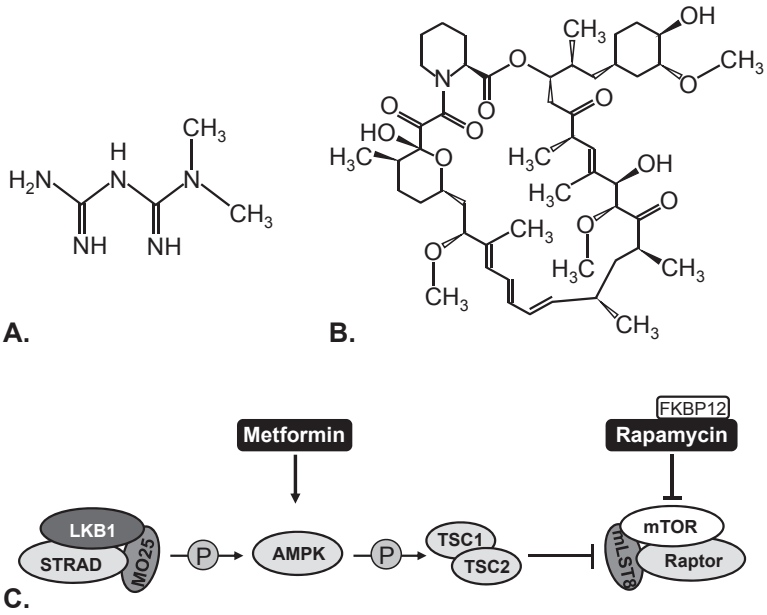

Figure 5. *Metformin and rapamycin target LKB1 signalling.*
A. *Chemical structure metformin.* ***B.*** *Chemical structure rapamycin (sirolimus).* ***C.*** *Both metformin and rapamycin target the LKB1/AMPK/mTOR pathway. Metformin induces activation of AMPK via upregulation of cellular AMP:ATP ratios, resulting in inhibition of mTOR. Rapamycin is a direct mTOR inhibitor. By binding to FKBP12 it induces dissociation of the mTOR complex c (mTORC1) consisting of mTOR, raptor, and mLST8.*

The anti-tumoural effect of metformin could partly be explained by its action in improving blood glucose and insulin levels [172]. However, metformin induces more direct anti-tumour responses in cancer cells as well. Several *in vitro* studies have shown that metformin treatment inhibits cell growth, induces apoptosis, and reduces migration and invasion in a variety of human cancer cell lines (Table 3). The anti-proliferative effects have been shown to be mediated via inhibition of insulin-like growth factor-1 (IGF-1) signalling [173-176], but also via suppression of genes inducing cell cycle S- and M-phases [177-179]. The apoptosis-inducing effects of metformin have been reported to be p53-dependent, mediated by downregulation of the Bcl-2 protein family, targeting ERK and STAT3 signalling, and through activation of the JNK/p38MAPK pathway [180-184].

Table 3. *Metformin treatment-induced cellular responses in cancer cell lines.*

Cancer cell type	Inhibiting cell growth	Inducing apoptosis	Inhibiting migration/invasion	Reference
Breast[1]	✓	✓	✓	174, 178, 179, 181, 184-188
Pancreas	✓	✓	✓	175, 176, 189, 190
Colon[2]	✓			191, 192
Stomach	✓			193
Lung[3]	✓	✓		173, 180
Ovary		✓		183
Prostate	✓	✓		182, 191
Endometrium			✓	194
Leukemia[4]	✓			195

[1]Part of the actions have been detected in triple-negative breast cancer cells.
[2]These actions have been detected in P53-deficient colon cancer cells specifically.
[3]Non-small cell lung cancer cells
[4]BCR-ABL-positive chronic myeloid leukemia cells

In addition to these anti-proliferative and pro-apoptotic effects of metformin *in vitro*, metformin treatment reduces growth of various tumour types and/or delays tumour onset in animal models (Table 4). Contradictory effects on angiogenesis and metastasis have been reported in xenograft mouse models (Table 4). Metformin treatment reduced tumour growth, microvessel density and metastasis in ovarian cancer cell tumours, while it promoted tumour growth and the angiogenic phenotype in ERα-negative breast cancer cell tumours (Table 4) [196,197]. In another high-energy diet fed xenograft mouse model, metformin did reduce growth of primary triple-negative breast cancer cell tumours, but did not affect metastasis (Table 4) [198]. These results suggest that the effects of metformin on tumour progression depend on the tumour type and/or additional circumstances such as hormonal and metabolic status. Notably, metformin potentiates the effects of chemotherapeutic agents, such as cisplatin, paclitaxel, doxorubicin and gemcitabine, suggesting that metformin serves a potential adjuvant in conventional chemotherapy [196,199-204].

Most of the *in vitro* and *in vivo* anti-tumoural actions of metformin have been associated with AMPK activation and/or reduction in mTOR activity [182,191,214-216]. However, other studies specifically report AMPK-independent mechanisms of action of metformin as well [213,217,218]. Even though AMPK is activated upon metformin treatment, AMPK is not the direct target of metformin. Metformin is suggested to directly target complex I of the mitochondrial respiratory chain which evokes a rise in cellular AMP:ATP ratios [219,220]. This subsequently potentiates AMPK to be phosphorylated and activated by LKB1. Interestingly, metformin has been shown to induce LKB1 cytosolic translocation [102,221]. Contradictory results are reported about the requirement of LKB1 in metformin-induced AMPK activation and subsequent effects on metabolism in cells [106,217,221-225]. However, in women with PCOS single nucleotide polymorphisms in *LKB1* were associated with a significantly reduced response to metformin treatment [226]. Moreover, metformin failed to inhibit cell growth in cells lacking LKB1, indicating that LKB1 is required for metformin-induced growth inhibitory effects [106,217]. Therefore, it is proposed that metformin is not a suitable drug for the treat-

Table 4. *Metformin treatment in animal models.*

Tumour type	Model	Effect of metformin treatment	Reference
Pancreas	Nu/nu mice xenograft MiaPaca-2 cells, Panc-1 cells	Reduced tumour growth	176
	Nu/nu mice xenograft LNCaP cells	Reduced tumour growth	205
	Syrian golden hamsters + BOP + high-fat diet	Reduced tumour incidence	206
Colon/Intestinal	Nu/nu mice xenograft HCT116 cells[1]	Reduced tumour growth	192
	C57/BL6 mice xenograft MC38 cells + high-energy diet	Reduced tumour growth	207
	Balb/c mice + AOM	Reduced tumour growth (mild)	208
	$Apc^{(Min/+)}$ mice	Decreased incidence of large (>2mm) adenomas	209
Breast	Nu/nu mice xenograft MDA-MB-231 cells[2]	Reduced tumour growth	185
	Nu/nu mice xenograft MDA-MB-435 cells[3]	Increased tumour growth, increased angiogenesis	197
	Balb/c mice xenograft 66cl4 cells[2] + high-energy diet	Reduced primary tumour growth, no effect on metastasis	198
	Sprague-Dawley rats + NMU	Delayed tumour onset	210
	HER2/neu transgenic mice	Decreased tumour incidence and size, delayed tumour onset	211
Ovarian	Nu/nu mice xenograft A2780 cells	Reduced tumour growth, and microvessel density, and metastatic nodules	196
Gastric	Nu/nu mice xenograft MKN74 cells	Reduced tumour growth	193
Lung	A/J mice + NKK	Reduced tumour growth	212
Melanoma	C57/BL6 mice xenograft B16 cells	Reduced tumour growth	213
Various[4]	$Lkb1^{fl/+}$;$Pten^{+/-}$ mice	Delayed tumour onset	106

AOM: azoxymethane; BOP: N-nitrosobis-(2-oxopropyl)amine; NKK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (tobacco carcinogen); NMU: N-methyl-N-nitrosourea.

[1]P53-deficient

[2]Triple-negative

[3]ERα-negative

[4]Intestinal polyps, lymphomas, pheochromocytomas, prostate -, breast - , pancreatic carcinomas

ment of tumours showing bi-allelic loss of *LKB1* and/or loss of LKB1 expression, as is often the case in PJS patients. In these patients, metformin might, however, be a useful drug to prevent hamartoma and carcinoma development and outgrowth, although studies testing this in *Lkb1*-mouse models and in PJS patients have not yet been performed.

Although scarce, clinical evidence for anti-cancer effects of metformin have been reported for sporadic cancer. Metformin use suppressed colonic epithelial proliferation and formation of rectal aberrant crypt foci, an early feature of CRC, in non-diabetic patients [214]. More re-

cently, anti-proliferative effects of metformin use in non-diabetic women with operable invasive breast cancer have been described [227]. All epidemiologic, preclinical and clinical evidence has led to the design of a number of prospective clinical trials investigating metformin therapy for cancer in the neoadjuvant and adjuvant (in combination with standard chemotherapy or hormone therapy) settings (www.clinicaltrials.gov). The majority of studies concern breast, prostate, and pancreatic cancer, in line with previous observations of population studies. Two phase II trials investigate the use of metformin as chemopreventive agent in pre-malignant conditions, i.e. Barrett oesophagus (NCT01447927) and colorectal adenomas (NCT01312467). In addition, one phase III trial is currently recruiting participants (NCT01101438), evaluating the effects of metformin on early-stage breast cancer outcomes, including recurrence and death. Results of these trials are awaited.

In addition to metformin, other compounds such as TZDs [228], statins [229] and D942 [230] have been shown to activate AMPK indirectly by inhibiting mitochondrial ATP production, thereby increasing the cellular AMP/ATP ratio. Also natural polyphenols such as berberine and resveratrol have been identified as indirect activators of AMPK [231,232]. AICAR is another indirect AMPK activator which, after uptake by the cells, is converted to ZMP, an AMP mimetic that binds to the AMPKγ subunit [233,234]. Novel direct AMPK activators such as A-769662 [235] and PT1 [236] have been identified, of which A-769662 has been shown to target AMPK subunit β1 [237] while PT1 activates both α1 and α2 subunits by reducing its auto-inhibition. Preclinical studies have shown that these AMPK activators can inhibit tumour cell growth [106,238-244].

Rapamycin

In 1999, rapamycin (sirolimus), a macrolide (Figure 5B) was approved as an immunosuppressant by the FDA, and used in organ transplantation to prevent allograft rejection [245]. Rapamycin specifically inhibits mTOR signalling by binding to the cytosolic FK-binding protein-12 (FKBP-12). This rapamycin-FKBP-12 complex binds to the mTOR protein resulting in the dissociation of mTORC1 (Figure 5C) [246]. Because of the discovery of hyperactivated mTOR signalling in a variety of human cancers, rapamycin got attention for its putative efficacy in inhibiting cancer cell growth. Numerous studies have revealed that rapamycin inhibits growth of several cancer cell types such as breast, pancreas, prostate, kidney and lung *in vitro*, and reduces tumour growth and formation of metastases in tumour xenograft animal models [247-251]. Notably, rapamycin has been shown to increase the sensitivity for chemotherapy (gemcitabine) and radiotherapy *in vitro* and *in vivo* [252-255]. Additional *in vivo* studies demonstrated that treatment with rapamycin reduces tumour growth in different genetically engineered mouse models that spontaneously develop tumours (Table 5). In these models, mTOR signalling was activated by deletion of e.g. *Lkb1*, *Tsc1* or *Tsc2*, *Nf1*, or *Pten*, but also by overexpression of *Akt* or *Neu*/*ErbB2* (Table 5). Since these genetic events are common events in sporadic cancers as well as in cancers associated with hereditary hamartoma syndromes such as PJS, tuberous sclerosis complex (TSC), neurofibromatosis (NF) and Cowden's Disease, rapamycin has been suggested to serve an effective anti-tumour drug in those disorders [256].

Table 5. *Genetically engineered tumour mouse models in which rapamycin treatment reduced tumour growth.*

Model	Tumour type	Reference
Lkb1$^{+/-}$ mice	PJS-like gastrointestinal polyps	257-260
ApcΔ^{716} mice	Intestinal polyps	261
Apc$^{(Min/+)}$ mice	Intestinal polyps	262
Basal colonic crypt cell-specific Apc$^{-/-}$ mice (Adeno-Cre)[1]	Distal colon tumours	263
cis-Nf1$^{+/-}$:p53$^{-/-}$ mice	Malignant peripheral nerve sheath tumours	264
Tsc2$^{+/-}$ mice	Renal cystadenomas and liver hemangiomas	265, 266
ENU-treated Tsc2$^{+/-}$ mice	Renal cystadenomas	267
Pten$^{+/-}$ mice	Pheochromocytomas and endometrial hyperplasia	268
Endometrial-specific Lkb1$^{-/-}$ mice (Sprr2f-Cre)	Invasive endometrial carcinomas	269
MISIIR-TAg transgenic mice	Ovarian tumours	270
Ovarian-specific Apc$^{-/-}$;Pten$^{-/-}$ mice (Adeno-Cre)[2]	Ovarian endometrioid adenocarcinomas	271
Pten$^{+/-}$;TR$\beta^{PV/PV}$ mice	Follicular thyroid carcinomas	272
Bladder-specific Pten$^{-/-}$;p53$^{-/-}$ mice (Adeno-Cre)[3]	Invasive bladder carcinomas	273
Prostate-specific Pten$^{-/-}$ mice (Pb-Cre4)	Prostate tumours	274
Pb-Akt1 transgenic mice	Prostate tumours	275
MMTV-NeuYD transgenic mice	Mammary tumours	276
MMTV-NeuYD;VEGF transgenic mice	Mammary tumours	276
MMTV-c-Neu/ErbB2 transgenic mice	Mammary tumours	277
MMTV-PyMT transgenic mice	Mammary tumours	278
BK5-ErbB2 transgenic mice	Gallbladder tumours	279

ENU: N-ethyl-N-nitrosourea; PJS: Peutz-Jeghers syndrome.
[1]Adeno-Cre delivery in distal colon
[2]Adeno-Cre delivery in ovarian bursae
[3]Adeno-Cre delivery in bladder lumen

Clinical trials have been set-up to test the efficacy of rapamycin in cancer patients, and in particular for patients with renal cell carcinoma (RCC), TSC or pancreatic neuroendocrine tumours (NETs). In an international randomized phase III trial, the efficacy of temsirolimus to treat RCC has been compared to conventional interferon (IFN)α treatment (NCT00065468). Temsirolimus superiorly improved progression-free and overall survival in these patients [280]. A randomized placebo-controlled phase III trial documented that the use of everolimus in patients with advanced RCC after progression on sunitinib and/or sorafenib stabilized tumour progression, and improved progression-free survival with acceptable tolerability (RECORD-1, NCT00410124) [281,282]. In two non-randomized, open-label trials (NCT00457808 and NCT00490789), it has been shown that sirolimus treatment of patients with TSC or sporadic lymphangioleiomyomatosis (LAM) resulted in regression of angiomyolipomas, however, these tumours tended to increase in volume after the therapy was stopped [283-285]. In a prospective, open-label phase I/II trial (NCT00411619), everolimus treatment of patients with subep-

endymal giant cell astrocytomas (SEGA) was associated with marked reduction in tumour volume and seizure frequency [286]. A multicentre phase II trial (NCT00126672), showed that sirolimus treatment of TSC patients induced regression of kidney and liver angiomyolipomas, and SEGAs [287]. Two phase II trials showed that everolimus, both alone and combined with octreotide long-acting release (LAR), improved disease control in patients with advanced NETs [288,289]. Two placebo-controlled phase III trials have been published investigating everolimus plus octreotide LAR versus octreotide LAR alone in patients with advanced carcinoids (RADIANT-2, NCT00412061) and everolimus monotherapy for advanced pancreatic NET (RADIANT-3, NCT00510068). In both studies everolimus improved progression-free survival with a low rate of severe adverse events [290,291]. Both temsirolimus (in May 2007) and everolimus (in March 2009) have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of advanced RCC after failure of treatment with sunitinib or sorafenib. In October 2010, the FDA approved the use of everolimus to treat subependymal giant cell astrocytomas in individuals with TSC who require treatment but are not candidates for surgery. In May 2011, everolimus has also been approved to treat patients with progressive NETs of the pancreas that are unresectable, locally advanced or metastatic.

Clinical studies to test the efficacy of mTOR inhibiting drugs in other cancers such as lung, breast, and CRC are currently ongoing. Besides the relatively high frequency of LKB1 mutations in sporadic NSCLC, mutations in EGFR and activation of AKT are frequently detected in these tumours as well [292,293]. Though all these events result in increased mTOR activity, the results of clinically inhibiting mTOR in NSCLC are so far not promising. Combined everolimus/gefitinib therapy in patients with advanced NSCLC led to a partial response in only 13% of the patients (8/62, of which two patients had mutations in *KRAS)* [294]. The *LKB1* mutation status in these patients was not reported. As described in this review, loss of LKB1 is detected in breast carcinomas, however, loss of PTEN and mutations in *PIK3CA* has been detected more frequently. These genetic events all result in aberrant mTOR signalling, and are associated with resistance to hormonal therapy [295,296]. In a randomized placebo-controlled phase III trial (BOLERO-2, NCT00863655), postmenopausal women with hormone-receptor positive advanced breast tumours, resistant to endocrine therapy alone, were treated with everolimus and an aromatase inhibitor, which improved their progression-free survival [297]. This study indicates that inhibiting mTOR signalling sensitizes breast carcinomas to hormonal therapy in human patients. Also for advanced CRC, in which loss of LKB1 occurs infrequently but mTOR hyperactivation has been reported more commonly [298,299], inhibition of mTOR with rapamycin analogs has been proposed for reversion of chemotherapy resistance. Several phase I and phase II trials are currently investigating this hypothesis by adding everolimus or temsirolimus to chemotherapy treatment regimens (www.clinicaltrials.gov). Furthermore, results of a phase II trial for the treatment of refractory metastatic CRC in 50 patients with a combination of bevacizumab and sirolimus were published last year [300]. Unfortunately, the combination of drugs showed modest activity on the disease and considerable side effects.

In addition to the approved rapalogs sirolimus, temsirolimus and everolimus, the efficacy of a fourth rapalog, i.e. ridaforolimus, is currently being tested in phase III clinical trials. Besides these mTORC1 inhibitors, dual mTORC1 and mTORC2 inhibitors such as AZD8055 [108,301,302], PP242 [303]; OSI-027 [291]; WAY-600; WYE-687; WYE-354 [304]; WYE-132 [305]; KU-0063794 [306]; X-387 [307] have been developed and are currently being tested for their efficacy as anti-tumour agent.

Combining mTOR inhibitors with other specific pathway inhibitors

Rapamycin has been shown to be most effective in cells that rely on AKT signalling for their proliferation and tumour growth, whereas cells which depend on other signalling pathways for their growth are resistant to rapamycin [248,308,309]. Tumours that are responsive to rapamycin may develop resistance when alternative survival pathways, such as the mitogen-activated extracellular kinase (MAPK/ERK) signalling pathway, become activated [263,308]. Combination of rapamycin with other specific pathway inhibitors could solve this problem of resistance. Notably, rapamycin has been shown to synergize with specific inhibitors of e.g. the EGFR/ErbB2, MEK/ERK and Hedgehog signalling *in vitro* and *in vivo* [310-319].

In rapamycin-sensitive as well as rapamycin-resistant tumours, inhibition of mTOR by rapamycin treatment induces a feedback loop resulting in upregulation of PI3K/AKT signalling [320,321]. Therefore, blocking of both PI3K/AKT signalling and mTOR signalling by combining rapamycin with specific PI3K inhibitors (LY294002, Wortmannin, ZSTK474) has been shown to be more effective in tumour-growth inhibition than rapamycin alone [321-324]. Recently, a new inhibitor has been identified targeting both mTOR and PI3K, i.e. NVP-BEZ235 [325]. NVP-BEZ235 is an imidazo-quinoline derivative, which can bind to the ATP-binding cleft of both PI3K and mTOR [325]. Several *in vitro* and *in vivo* studies have shown that this dual inhibitor induces apoptosis and cell cycle arrest in a wide variety of tumour-cell types, and in most of these cases, the anti-tumour efficacy of NVP-BEZ235 was greater compared to that of rapamycin (e.g. [322,325-332]). Even in chemo-resistant tumour cells, or tumour cells which have been shown difficult to treat, dual PI3K/mTOR inhibition by NVP-BEZ235 efficiently inhibits tumour-cell growth, e.g. in KRAS-mutant NSCLC and in breast cancers resistant to ErbB2 inhibitors [333-338]. In addition, NVP-BEZ235 enhances sensitivity to chemo- and radiotherapy [339-342]. In some cases, combining NVP-BEZ235 with MEK or RAF inhibitors showed an increased efficacy in inhibiting tumour-cell growth, suggesting that some cancers require inhibition of both PI3K/mTOR and MEK/ERK signalling pathways for efficient anti-cancer treatment [320,343-346]. NVP-BEZ235 has entered phase I/II clinical studies for patients with advanced breast, renal, endometrial and other solid cancers (www.clinicaltrials.gov).

In addition to the NVP-BEZ235 inhibitor, novel dual PI3K/mTOR inhibitors such as PI-103 [347-349], PF-04691502 [350,351], GDC-0980 [352,353], NVP-BGT226 [354-356], GSK2126458 [357], and PKI-402 [358,359] have been developed and are currently under investigation for their anti-tumour activities.

Other options to treat PJS-associated tumours

Since germline *LKB1* mutations predispose to PJS, LKB1/AMPK/mTOR signalling has been proposed as a suitable target for treatment of PJS-associated hamartomas and carcinomas. In addition to the preclinical studies using metformin and rapamycin to inhibit tumour cell growth *in vitro* and *in vivo* as described in this review, other options for targeted treatment have been suggested. In tumours of Lkb1+/- mice as well as in tumours of PJS patients, elevated levels of cyclooxygenase-2 (COX-2) have been detected [360,361]. Inhibition of COX-2 with celecoxib, a non-steroidal anti-inflammatory drug, in *Lkb1*+/- mice reduced tumour burden and was associated with decreased vascularity [362]. In addition, 2 of 6 PJS patients responded well to celecoxib treatment as they showed reduced gastric polyposis [362], suggesting that inhibition of COX-2 serves a suitable strategy to treat PJS-associated lesions.

Recently, the SRC protein has been identified as a target of LKB1 in an integrative genomic and proteomic approach [363]. In this study, Src was shown to be activated in Lkb1-deficient *Kras*-mutated murine primary and metastatic lung tumours, which was validated in human lung carcinoma samples. Inhibition of Src by Dasatinib did not affect tumour growth, but restored the sensitivity to PI3K/mTOR/MEK inhibition using NVP-BEZ235 and AZD2644 [363]. These results suggest that inhibition of SRC, whether or not in combination with other specific inhibitors, might be a suitable approach for treatment of PJS patients as well, although the activity status of SRC in PJS-associated lesions has to be determined yet.

CONCLUSION

As described in this review, aberrant LKB1 signalling is involved in a variety of human cancers, both in sporadic cancers as well as in cancers associated with hamartoma syndromes like PJS. LKB1 signalling has been attributed to a wide diversity of biological processes, involving a multitude of downstream mediators. These biological processes are known to be essential and deregulated in tumours, indicating the relevance of LKB1 loss in cancer. However, since only little is known about the biological consequences of LKB1 loss in carcinomas and the molecular mechanisms underlying these biological consequences, the exact tumour suppressor functions of LKB1 are yet to be elucidated. So far, most efforts to target LKB1 signalling in cancer have focused on activating AMPK and inhibiting mTOR.

However, since activation of AMPK might require intact LKB1, and since it has been shown that some molecular and cellular responses of LKB1 loss are independent of AMPK and/or mTOR activity, targeting these two mediators might not be efficient in treating LKB1-associated cancer. More insight into the tumour suppressor functions and mediating molecular mechanisms of LKB1 will likely uncover additional and/or novel proteins and pathways to target, in order to treat LKB1-associated cancer. Nevertheless, hyperactivation of mTOR downstream of LKB1 is also mediated via other major signalling pathways such as the PI3K/AKT survival pathway, which is frequently aberrantly activated in cancer. More insights into the genes and pathways deregulated in tumours and essential for their growth, will provide novel strategies in order to develop therapeutic agents specifically targeting these pathways. In particular, a combination of these specific agents, and/or a combination of these agents with conventional hormonal, chemotherapy and radiotherapy are most likely to result in efficient anti-cancer regimens without inducing therapy resistance.

Chapter 3

The cell polarity kinase LKB1 counteracts colon cancer cell motility through cytoskeletal rearrangement

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ABSTRACT

Cytoskeleton-stabilizing drugs, such as paclitaxel, have become an important component in the pharmacological battle against gastrointestinal cancer. However, no examples of genetic tumour suppression through cytoskeletal stabilization have been identified. The cell polarity kinase LKB1 is an important tumour suppressor in the gastrointestinal tract as evident from the high incidence of neoplastic lesions in patients harbouring germline haploinsufficiency of the encoding gene. The mechanisms by which LKB1 counteracts malignant disease remain however largely obscure. In an effort to obtain insight, we created a panel of LKB1 knock-down clones from an established LKB1-proficient colon cancer cell line, i.e. HT29. Loss of LKB1 did not affect cell growth, but the LKB1-deficient cells displayed increased motility in migration assays. The increased cell migration coincided with downregulation of the epithelial marker E-cadherin, and upregulation of the cancer stem cell marker CD44. In addition, LKB1-knockdown cells showed increased levels of phosphorylated PAK, an important effector of the Cdc42/Rac motility GTPases, which coincided with a shift in the actin cytoskeleton of LKB1-inhibited cells. We conclude that *LKB1* constitutes a tumour suppressor gene that acts on the cancerous process of cell migration through cytoskeletal rearrangement by PAK signalling.

INTRODUCTION

The treatment of gastrointestinal cancers has been revolutionized by the introduction of cytoskeleton stabilizing drugs, such as paclitaxel. Pharmacological stabilization of cytoskeletal dynamics is generally considered an important rational avenue for gastrointestinal cancer treatment [364]. This has led to the notion that humans likely harbour similar genetic mechanisms limiting cancer progression through cytoskeletal stabilization.

The hereditary Peutz-Jeghers syndrome (PJS) is characterized by the development of mucocutaneous hyperpigmentation and gastrointestinal hamartomatous polyposis [28]. Patients afflicted with PJS are also at strongly increased risk for developing gastrointestinal cancer, in particular colorectal cancer (CRC) [24]. Predisposition for PJS is a result of inactivating germline mutations in the serine/threonine kinase 11 (*STK11*) gene - from hereon referred to as the liver kinase B1 (*LKB1*) gene [28]. LKB1 contains an evolutionary conserved serine/threonine kinase domain. Activation of LKB1 is associated with its translocation to the cytoplasm upon trimerisation of LKB1 with the STE20-related adaptor (STRADα) and scaffolding mouse 25 (MO25) proteins [36,39]. In the cytoplasm, complexed LKB1 phosphorylates and activates AMP-activated kinase (AMPK)α resulting in inhibition of mTOR. Via this canonical pathway, as well as via AMPK/mTOR-independent routes, LKB1 acts as a master kinase in regulating cell polarity, energy (glucose and lipid) metabolism and cell growth [256,365]. Although the link between LKB1 and PJS is undebated, the role of LKB1 in the pathogenesis of PJS-associated carcinogenesis is still unclear.

In addition to PJS, LKB1 is involved in sporadic cancers. For instance, *LKB1* is the third most frequently mutated gene in sporadic non-small cell lung carcinomas (NSCLC) [141,160]. Notably, *LKB1* mutations in NSCLC frequently coincide with *KRAS* mutations. NSCLC patients with *LKB1;KRAS* compound mutations tend to have a poorer prognosis compared to patients with *KRAS*-mutated NSCLC without a concomitant *LKB1* mutation, suggesting that loss of LKB1 induces more aggressive tumour phenotypes [140,141]. This is also shown in mice, where lung-specific double mutant *Lkb1;Kras* mice develop more aggressive tumours with higher tumour multiplicity, multiple NSCLC histologies and more frequent metastasis [142].

Sporadic CRC development is usually initiated by activation of the Wnt signalling pathway due to mutations in either the Adenomatous Polyposis Coli (*APC*) gene or the gene encoding β-catenin (*CTNNB1*) [366]. Despite the strong association between germline *LKB1* mutations and increased risk for developing CRC, *LKB1* is rarely mutated somatically in sporadic CRC [143,145]. However, LOH of 19p13.3 has been detected in up to 50% of cases, suggesting that reduced levels of LKB1 might contribute to the development of sporadic CRC [143,145]. Thus, the exact tumour suppressor functions of LKB1 in sporadic and PJS-associated colorectal cancer development remain elusive. LKB1-null mouse embryonic fibroblasts escape passage-induced senescence, suggesting a role for LKB1 in limitless replication [34]. In addition, in epithelial breast, lung and oesophageal cancer cells, loss of LKB1 increases migration and invasion, indicating that LKB1 plays a major role in epithelial cancer cell metastasis [136,364,367-369].

We therefore studied the role of LKB1 in colon cancer by knocking down LKB1 expression in human colon cancer cells. We show that LKB1 suppresses colon cancer cell migration, as a result of cytoskeletal rearrangement.

MATERIALS AND METHODS

Cell culture and treatments

The human colon cancer cell line HT29 was cultured in DMEM (Lonza), supplemented with 5% foetal bovine serum (FBS, Sigma Aldrich) and 1% penicillin/streptomycin (p/s) (Gibco) at 37°C;5% CO_2. Stable human LKB1-knockdown (iLKB1) and non-targeting control (iNT) HT29 cells were generated using Sigma Mission shRNA pLKO-puro lentiviral vectors; *LKB1* shRNA GCCAACGTGAAGAAGGAAATT and control non-targeting shRNA CAACAAGATGAAGAGCACCAA. Cells were selected and maintained in DMEM 5% FBS;1% p/s and 2µg/ml puromycin (Sigma) at 37°C;5% CO_2.

Quantitative PCR and microarray analysis

Total RNA was isolated from cultured cells using TRIzol (Sigma-Aldrich). RNA (1µg) was reversely transcribed using an iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Quantitative PCRs (qPCRs) were performed using Sensimix™ SYBR & Fluorescein Kit (Quantace, London, UK) and an IQ5 iCycler PCR machine (Bio-Rad). Expression levels were corrected for expression of *GAPDH,* averaged and presented as fold changes. Assay was performed three times in duplicate and *p* values were calculated using the Student t-test. The primers used are described in Supplementary Table 1.

For microarray analysis, RNA was isolated from four independent monoclonal HT29-iLKB1 and four independent monoclonal HT29-iNT lines. RNA quality and quantity controls and gene expression profiling (Affymetrix, GeneAtlas 2.0/U133) was performed at the department of Biomics (Erasmus MC, Rotterdam, the Netherlands). Raw intensity values of all samples were normalized by Robust Multichip Analysis normalization (background correction and quantile normalization) using Partek version 6.5 (Partek Inc., St. Louis,MO). The normalized datafile was transposed and imported into OmniViz version 6.0.1 (BioWisdom Ltd., Cambridge, UK) for further analysis. For each probe set, the geometric mean of the hybridization intensity of all samples was calculated. The level of expression of each probe set was determined relative to this geometric mean and 2log-transformed. The geometric mean of the hybridization signal of all samples was used to ascribe equal weight to gene expression levels with similar relative distances to the geometric mean. Differentially expressed genes were identified using statistical analysis of microarrays. Cutoff values for significantly expressed genes were a false discovery rate (FDR) of 0.05 or less and a fold change of 1.5. Functional annotation of the statistical analysis of microarrays results was done using Ingenuity Pathway Analysis (Ingenuity, Mountain View, CA).

Quantitative Western blot analysis

Subconfluent cells were lysed in Laemmli sample buffer containing 0.1M DTT, and incubated 5 min at 95°C. Immunoblotting was performed using fluorescent Odyssey immunoblotting (LI-COR Biosciences, Lincoln, NE, USA) [370]. Quantification was performed using Odyssey LI-COR software. Primary antibodies employed for immunoblotting are depicted in Supplementary Table 2.

PepChip analysis

Recombinant active LKB1 (LKB1/STRADα/MO25 complex; Upstate Millipore) was incubated with 2µl of filter-cleared activation mix (50% glycerol, 70mM $MgCl_2$, 70mM $MnCl_2$, 400µg/ml BSA, 400µg/ml PEG800 [-^{33}P]ATP (1000kBq)) on PepChip arrays (PepScan, Lelystad, The Netherlands) for 90 min in a humidified incubator at 37°C, and washed in 1% Tween-20;PBS (PBST). Subsequent washes in 2M NaCl;1% Tween-20 followed by PBST were conducted under continuous agitation at 50°C. After air-drying, chips were exposed to a phosphor-imager screen for 72 h and the intensity of the spots was analysed by ScanAlyze software.

The PepChip array contains 1024 sequences, spotted in triplicate. PepChip analyses were performed twice. The mean intensity of each of the 6 spots was calculated for each sequence, and spots deviating more than two times standard-deviation were excluded. Peptides of which the average phosphorylation minus 1.96 times the standard deviation was higher than the value expected from describing the background distribution were considered to represent true phosphorylation events. A list of peptides was generated by ranking-ordering the spots and curve-fitting analysis, resulting in an “On” or “Off”-call for each peptide. PepChip peptides were annotated in the following signalling pathways/functions: PI3K-PKB-mTOR, Mitogenic, G-protein, Nutrient, Cytoskeletal, Mitosis/DNA damage, Immunity and Stemness.

MTT cell viability assay

HT29 cells were seeded 6*10^4 cells per M24 well and measured at indicated time-points by adding 100µl 5mg/ml MTT per well, 30 min incubation at 37°C and replacement of the medium by 100µl DMSO. Absorbance (550nm) was measured and averaged. Assays were performed three times in duplicate.

Scratch wound-healing assay

Cells were seeded (5*10^5 cells per M6 well) and incubated at 37°C;5% CO_2. At 100% confluence, a scratch was made across the plates using a pipette’s tip. Cells were cultured in complete medium and images were taken every 24 h to monitor the wound healing process.

Transwell migration assay

Cells (2*10^4) were seeded in DMEM;1% FBS;1% p/s in 24-well Transwell inserts (8µm pore size; BD Falcon). The inserts were placed in M24 wells containing DMEM;10% FBS;1% p/s. After incubation for 72 h at 37°C;5% CO_2, the cells remaining at the top of the insert membrane were physically removed by cloth-swaps. The migrated cells attached at the underside of the insert membranes were fixed in methanol and stained using 5% Giemsa/PBS (Merck). Migrated cells were counted using a light microscope.

Collagen adhesion assay

Cells (2*10^4) were seeded in M96 wells coated with 0.02% collagen (Collagen R Solution, Serva Electrophoresis). After incubation for 30 min at 37°C; 5% CO_2, adherent cells were fixed in methanol and stained using 5% Giemsa/PBS (Merck). The adherent cells were counted using a light microscope.

β-catenin reporter assay

β-catenin reporter assays were performed as previously described [370]. Luciferase activities were measured using the dual-luciferase reporter assay system (Promega). Assays were performed three times in triplicate.

Immunofluorescence microscopy

Cells were cultured on sterile glass coverslips at 80% confluence and fixed in 2% PFA for 10 min. The cells were incubated in 1% BSA;0.1% Triton-X100/PBS (PBSAT) for 10 min, and subsequently with the primary antibody E-cadherin or β-catenin, or rhodamine conjugated phalloidin (#PHDR1 Cytoskeleton, Inc.) diluted in PBST for 1.5 h at room temperature. The cells were washed and incubated with the secondary antibody Alexa Fluor 488 Goat anti-mouse (A11296, Invitrogen) or Alexa Fluor 594 Goat anti-rabbit (A11037, Invitrogen), for 1 h at room temperature. The nuclei were visualized by incubating the cells in 67ug/ml DAPI (Sigma-Aldrich) in PBS for 5min. After washing, the cells were mounted with Vectashield (Vector Labs). The stainings were analyzed using the 63x oil objective of the Leica TCS SP5 Confocal microscrope.

Statistical analysis

All statistical analyses were performed with the use of Microsoft Office Excel 2003 (Microsoft Corporation, USA) and GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Data were presented as means ± standard deviation (SD), unless otherwise stated in the figure legend. Before statistical analysis, values were normalized to the control iNT-A6. For comparison to the control group the two-tailed independent student *t* test was used. Two-sided *P* values < 0.05 were considered statistically significant.

RESULTS

Reduced LKB1 expression does not affect cell growth in colon cancer cells

To dissect the mechanisms through which loss of LKB1 might contribute to colon cancer, we generated stable LKB1 knockdown HT29 colon cancer cells. Monoclonal LKB1-knockdown cell lines (iLKB1-E4 and E5) and monoclonal non-targeting control cell lines (iNT-A6 and D8) were selected. Significant knockdown of endogenous LKB1 was verified on protein and RNA level in these two independent clones (Figure 1A). LKB1 knockdown resulted in reduced phosphorylated levels of its direct target AMPK, whereas phosphorylated S6K (a target of mTOR) levels were unaltered (Figure 1A).

Colon cancer cell growth is largely dependent on its Wnt/β-catenin signalling activity, and LKB1 has been suggested to inhibit the canonical Wnt signalling pathway [371]. Therefore, the effect of LKB1-knockdown on β-catenin transcriptional activity was determined. HT29 cells harbour an *APC* mutation that activates β-catenin signalling. Transcriptional levels of *AXIN2*, a β-catenin target gene and negative modulator of β-catenin signalling, were reduced in LKB1-knockdown cells (Figure 1B). However, constitutive as well as Wnt3A-stimulated β-catenin transcriptional activity as measured in a luciferase reporter assay, were slightly, but not significantly elevated in LKB1-knockdown cells (Figure 1B). These data suggest that

knockdown of LKB1 does not strongly alter intrinsic Wnt/β-catenin signalling of HT29 colon cancer cells.

In line with this, cell viability as determined by MTT assays revealed no differences in the growth curves of LKB1-expressing and LKB1-knockdown HT29 cells (Figure 1C). Together, these data suggest that LKB1 does not greatly influence growth-related properties of *APC*-mutant colon cancer cells.

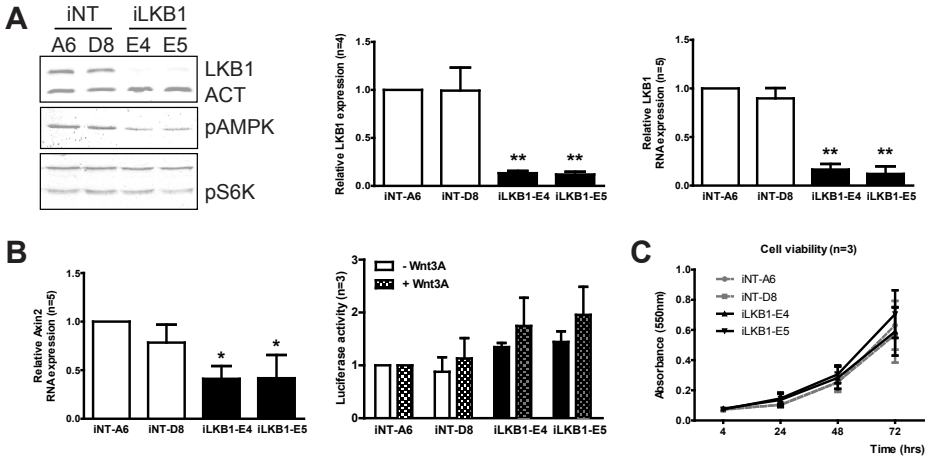

Figure 1. *Reduced LKB1 expression does not affect growth-related properties of APC-mutant colon cancer cells.*

A. *Generation of stable LKB1 knockdown in HT29 colon cancer cells (iLKB1) and non-targeting control cell lines (iNT). Significant knockdown of endogenous LKB1 on protein (left, centre) and RNA level (right) in iLKB1 cells compared to iNT cells. LKB1 knockdown results in reduced phosphorylation of its direct target AMPK, whereas phosphorylated levels of the further downstream target S6K are not affected.* ***B.*** *Transcriptional AXIN2 levels are significantly reduced in LKB1 knockdown cells (left). Luciferase reporter assays show that β-catenin-driven transcription is not significantly elevated in LKB1 knockdown cells, with or without stimulation with Wnt3A (right).* ***C.*** *MTT assays showing that LKB1 knockdown does not significantly affect HT29 cell viability. Error bars equal standard error of the mean. * and ** indicate p values of < 0.05 and < 0.005, respectively.*

Reduced LKB1 expression induces migration of colon cancer cells

To investigate which cellular functions of colon cancer cells were affected by loss of LKB1 expression, a genome-wide expression analysis approach was chosen using the high-throughput Affymetrix GeneAtlas 2.0 platform. We compared the transcriptome signatures of four independent monoclonal iLKB1 lines to four independent monoclonal iNT lines using SAM analysis. This revealed 196 records, corresponding to a set of 142 genes, being differentially expressed between these two groups (Figure 2A). Functional data analysis in Ingenuity identified ‘cellular movement’ and ‘lipid metabolism’ as the two most significantly affected cellular functions in HT29 cells lacking LKB1 (Figure 2A). Involvement of LKB1 signalling in lipid metabolism has already been shown and extensively studied [365]. However, a role for LKB1 in cellular movement of colon cancer cells has so far not been described. To investigate the effect of LKB1 knockdown on cellular movement of colon cancer cells, the migration

capacity of iLKB1 and iNT HT29 cell lines was determined using two different assays. Both the scratch wound-healing assay and transwell migration assay showed significantly increased migration of iLKB1 cells compared to iNT cells (Figure 2B,C). These functional assays confirmed the hypothesis obtained from the microarray analysis, indicating that LKB1 is involved in colon cancer cell migration. Interestingly, performing a pathway explore analysis with the 26 molecules of the 'cellular movement'-profile in Ingenuity, revealed the canonical pathway 'CRC metastasis signalling' being most strongly associated with reduced LKB1 expression (not shown).

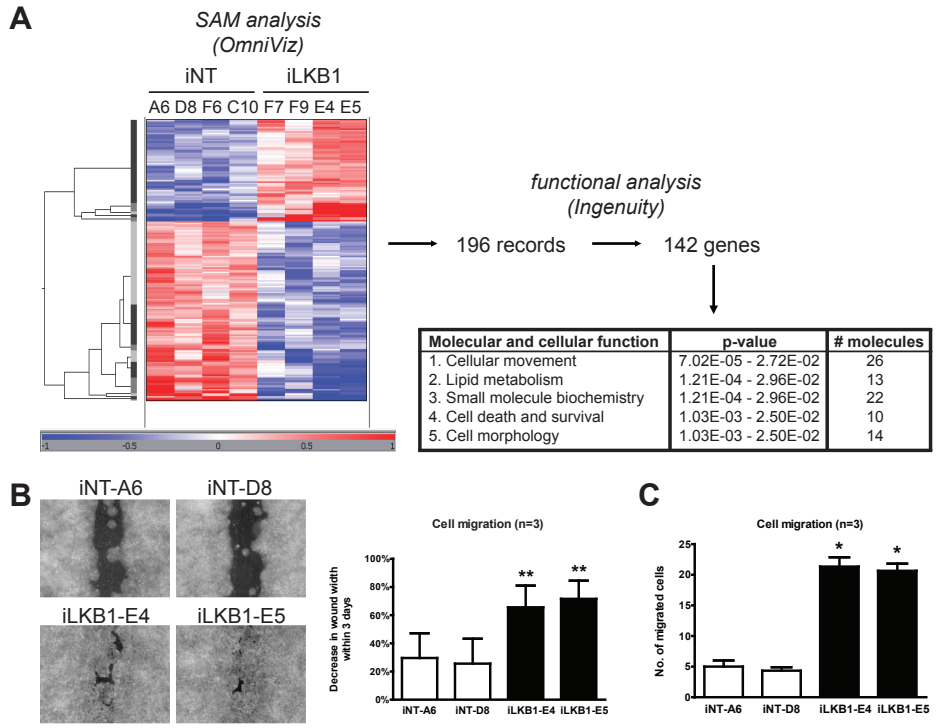

Figure 2. *Reduced LKB1 expression induces migration of colon cancer cells.*
A. *Genome-wide expression analysis, comparing four independent LKB1 knockdown cell lines (iLKB1) to four independent control cell lines (iNT), identify 'cellular movement' and 'lipid metabolism' as the two most significantly affected cellular functions in colon cancer cells lacking LKB1. Gene expression levels: red, upregulated genes compared with the geometric mean; blue, down-regulated genes compared with the geometric mean. The colour intensity correlates with the degree of change.* ***B. and C.*** *Both the scratch wound-healing assay (B.) and the transwell migration assay (C.) show significantly increased migration of iLKB1 cells compared to iNT cells. The error bars in figure 2B equal standard error of the mean. * and ** indicate p values of < 0.05 and < 0.005, respectively.*

Effects on CRTC1 signalling

A recent microarray study in LKB1 knockout mice showed that loss of LKB1 in lung tumours enhances metastasis through enhancement of *NEDD9* expression, as a result of increased activity of the transcriptional co-activator CRTC1 [364]. We questioned whether this mechanism

was also active in human colorectal cancer cells with reduced LKB1 expression. However, in our microarray, these genes were not found to be upregulated in iLKB1 cells compared to iNT cells (not shown). In contrast, despite slightly increased protein levels of CRTC1 in iLKB1 HT29 cells, *NEDD9* mRNA levels were significantly reduced in LKB1-deficient colon cancer cells (Figure 3A,B). These data suggest that in human colon cancer cells, LKB1 suppresses migration through an alternative mechanism.

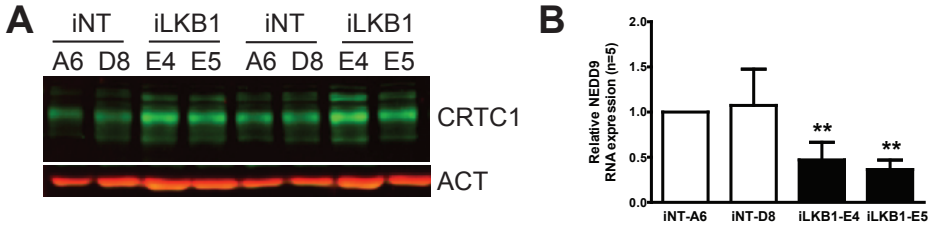

Figure 3. *Effects of LKB1 knockdown CRTC1 signalling in colon cancer cells.*
A. *Protein CRTC1 levels were slightly increased in LKB1 knockdown cells.* ***B.*** *Transcriptional expression of NEDD9 was significantly reduced in LKB1 knockdown cells. ** indicates p values of < 0.005.*

Reduced LKB1 expression affects the epithelial phenotype and adhesion of colon cancer cells

The establishment of malignant behaviour requires tumour cells to acquire novel adhesion and migration properties, leading them to detach from their original sites and to localize to distant organs. Migration of epithelial cells usually involves epithelial-to-mesenchymal transition (EMT). In the LKB1 knockdown cells, expression of the epithelial marker E-cadherin was significantly reduced (Figure 4A,B). However, expression of the mesenchymal markers Vimentin and N-cadherin could not be detected in LKB1-knockdown cells (Figure 4B). Despite the lack of mesenchymal induction, the cancer stem cell marker CD44, involved in EMT, cytoskeletal rearrangement and migration [372], was significantly increased (Figure 4A,B).

In epithelial cells, E-cadherin is typically located at the cellular membrane where it binds β-catenin to form adhesion junctions. In iLKB1 cells, reduced E-cadherin expression was associated with significantly reduced levels of β-catenin (Figure 4B). Immunofluorescence analysis showed diffuse expression of both E-cadherin and β-catenin at the site of the plasma membrane and cytoplasm of iLKB1 cells (Figure 4C). These data indicate that although a full EMT is not induced, the changes in epithelial phenotype of HT29 cells by reduced LKB1 expression are indicative of impaired cell adhesion.

To confirm this hypothesis, we compared the adherence propensity of LKB1-knockdown cells and control cells. iLKB1 cells showed significantly reduced adherence to the extracellular matrix (ECM) component collagen, compared to iNT controls (Figure 4D), indicating that loss of LKB1 in colon cancer cells impairs the interaction with the ECM.

Indirectly, this was also suggested by the observation that detaching cells from tissue culture plates using trypsin took longer for iNT cells than for iLKB1 cells (data not shown). These data suggest that reduced LKB1 expression induces colon cancer cell migration by inducing a partial EMT, and by altering the capacity of cells to adhere to the ECM.

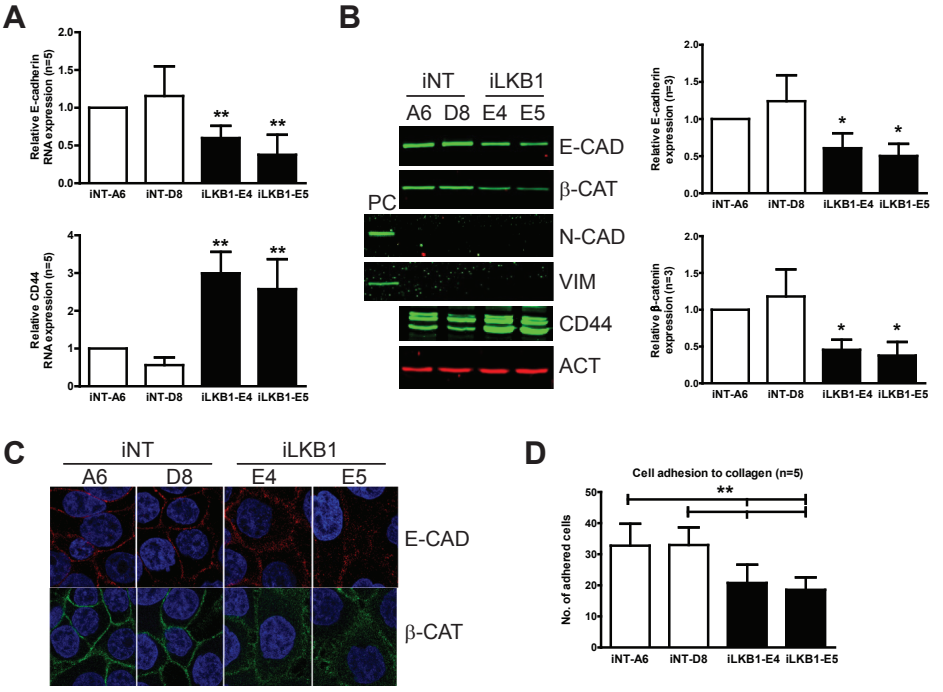

Figure 4. *Reduced LKB1 expression affects the epithelial phenotype and adhesion of colon cancer cells.* ***A.*** *Transcriptional levels of the epithelial marker E-cadherin are reduced in LKB1 knockdown cells, whereas transcriptional levels of the cancer stem cell marker CD44 are induced.* ***B.*** *In colon cancer cells lacking LKB1, E-cadherin and β-catenin protein levels are significantly reduced. Expression of the mesenchymal markers vimentin and N-cadherin are not detected in iNT cells, and are not induced in LKB1 knockdown cells. CD44 protein levels are induced.* ***C.*** *Immunofluorescence analysis demonstrates loss of both E-cadherin and β-catenin localization at cell-cell junctions in iLKB1 cells.* ***D.*** *LKB1 knockdown cells show significantly reduced adherence to the extra-cellular matrix component collagen compared to control cell lines. * and ** indicate p values of < 0.05 and < 0.005, respectively. PC, positive control (mesenchymal cells).*

Reduced LKB1 expression induces cytoskeletal rearrangements in colon cancer cells

Having observed a clear effect of LKB1 downregulation on cellular migration, we set out to further elucidate the molecular mechanisms through which this effect may be brought about. To this aim, we employed a kinome profiling approach, incubating recombinant active LKB1 on PepChip kinome arrays to identify its peptide substrates. Using the top 10% of the identified LKB1 target peptide substrates, a pathway annotation analysis was performed which showed that proteins of the LKB1, AMPK and mTOR/S6K pathways are substrates for LKB1, thereby validating this technique and analysis (Figure 5A). Interestingly, in addition to these known downstream pathways of LKB1, the cytoskeletal RAC/PAK pathway was also shown to be within the substrate pathways of LKB1. To validate these results, we analysed PAK phosphorylation in iNT and iLKB1 cells. As shown in Figure 5B, PAK phosphorylation was significantly enhanced in LKB1-deficient cells. The main function of the PAK pathway is regu-

lation of the actin cytoskeleton in order to promote migration. Indeed, immunofluorescence analysis showed rearranged actin filaments in LKB1-knockdown cells (Figure 5C). Actin fiber formation is carefully regulated by several actin binding proteins, amongst which cofilin. In line with enhanced migration and altered cytoskeletal rearrangement, we detected decreased protein levels of cofilin in iLKB1 cells (Figure 5B). In contrast, no differences in levels and protein phosphorylation of other cytoskeletal/migratory proteins (e.g. VASP, FAK, PAX) were observed (data not shown), indicating that LKB1-effects on the migratory phenotype of colon cancer cells is specifically regulated through PAK and cofilin. Together, these data suggest that loss of LKB1 in the colon cancer cell line HT29 induces changes in the cytoskeletal actin filaments, which enables cellular movement.

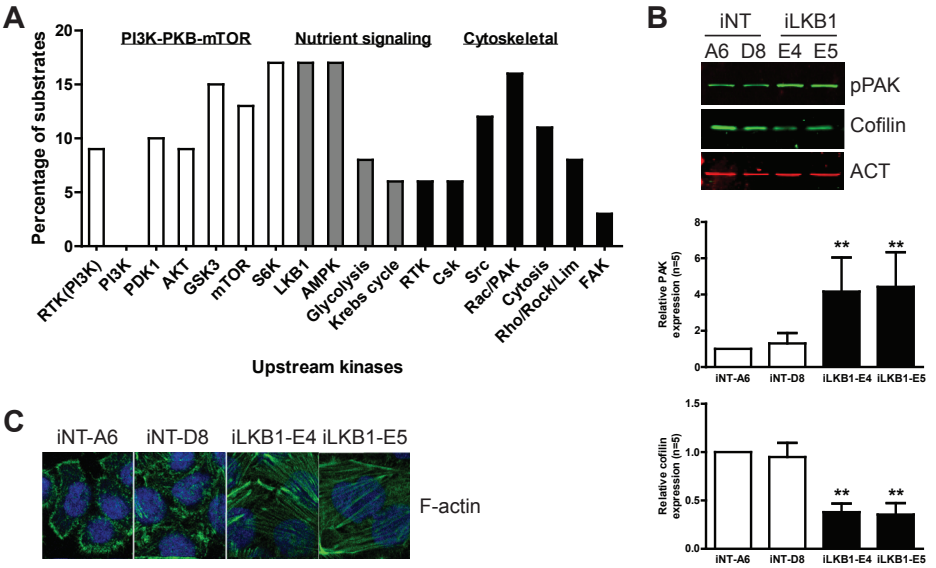

Figure 5. *Reduced LKB1 expression induces cytoskeletal rearrangements in colon cancer cells.* ***A.*** *Results of the pathway annotation analysis of the PepChip array, using the top 10% of the identified LKB1 peptide substrates. In addition to the known downstream pathways of LKB1, e.g. LKB1, AMPK and mTOR/S6K pathways, the cytoskeletal RAC/PAK pathway is also shown to be within the substrate pathways of LKB1.* ***B.*** *Phosphorylated PAK protein levels are significantly increased in LKB1 knockdown cells, while cofilin expression was significantly reduced.* ***C.*** *Immunofluorescence analysis demonstrates rearranged actin filaments in LKB1 knockdown cells, suggesting changes in the cytoskeleton in these colon cancer cells. * and ** indicate p values of < 0.05 and < 0.005, respectively.*

DISCUSSION

Germline mutations in *LKB1* predispose PJS patients to the development of CRC and other gastrointestinal and extra- gastrointestinal cancers [24]. In sporadic CRC however, LKB1 is rarely somatically mutated [143,145]. This has led to the suggestion that LKB1 is not involved in the development of sporadic CRC. However, allelic loss has been observed frequently, suggesting that reduced levels of LKB1 do contribute to colorectal carcinogenesis. LKB1 is considered a master serine/threonine kinase regulating cell polarity, energy metabolism and cell growth [365]. Though the functions of LKB1 have been the subject of several investiga-

tions over the past years, its tumour suppressor mechanisms in colorectal carcinogenesis remain largely obscure. This study shows that reduced levels of LKB1 enhance motility in human colon cancer HT29 cells by rearrangement of the actin cytoskeleton, suggesting that LKB1 suppresses sporadic CRC progression.

In sporadic lung and breast cancer, loss of LKB1 has been shown to associate with more aggressive tumour phenotypes and poor prognosis [88,134,135,140,141]. In addition, previous *in vitro* and *in vivo* studies showed that loss of LKB1 increases cell migration and invasion in epithelial breast, lung and oesophageal cancer cells [136,137,364,373]. Our results are in line with these studies, providing evidence that loss of LKB1 induces a migratory phenotype in colon cancer cells, and suggesting that LKB1 suppresses sporadic epithelial cancer progression in general.

In breast cancer cells, LKB1 was required for adiponectin-mediated modulation of the AMPK-S6K axis to suppress migration and invasion [136]. Activation of CRTC1-mediated signalling was demonstrated to underlie LKB1-induced migration in epithelial lung and oesophageal cancer cells [364,373]. In our study, we showed that these mechanisms are not involved in LKB1-induced colon cancer cell migration.

The effect of LKB1 knockdown on Wnt/β-catenin signalling, the major pathway underlying CRC initiation, seems to be complex. LKB1 is able to inhibit Wnt/β-catenin signalling through activation of GSK3, and the non-canonical Wnt5a is increased in *Lkb1*+/- mice as well as in PJS polyps [371,374]. Our results show that loss of LKB1 was not sufficient to significantly raise Wnt/β-catenin signalling in the context of an *APC* mutation. This is in line with our observations that LKB1 suppresses colon cancer cell migration rather than proliferation.

We identified reduced expression of the epithelial marker E-cadherin, though the 'classical' mesenchymal markers were not induced upon loss of LKB1. This indicates that loss of LKB1 in colon cancer cells does not induce a full EMT, as it has previously been shown for epithelial lung cancer cells [369]. Interestingly though, in our LKB1 knockdown cells we showed enhanced expression of CD44, a transmembrane glycoprotein associated with EMT, cytoskeletal rearrangement and migration [372]. CD44 is overexpressed and associated with metastases in several cancers including CRC [375-377]. The correlation between LKB1 and CD44 expression and the association with clinicopathological parameters in sporadic CRC needs further investigation.

Colon cancer is one of the most frequently observed cancers in the Western World and about half of the patients die due to metastatic disease. Tumour metastasis involves migration and invasion of primary tumour cells through tissue and through the ECM to distant sites, a process that requires cytoskeleton remodelling. PAKs are considered prime regulators of actin cytoskeleton dynamics and motility [378], and their expression has been associated with progression of CRC to metastasis [379]. Our data indicate that LKB1 suppresses cellular movement in colon cancer cells through actin cytoskeleton rearrangement mediated by PAK signalling. This important observation may indicate that recent pharmacologic cytoskeleton-stabilizing drugs, such as paclitaxel may counteract the tumour cell responses induced by loss of LKB1.

ACKNOWLEDGEMENTS

We thank interns Anja ten Hoeve-van Bergeijk and Corina Voorsluijs for technical assistance. We thank prof. dr. P.J. van der Spek (Dept. of Bioinformatics, Erasmus MC) for scientific input.

SUPPLEMENTARY MATERIAL

Supplementary table 1. *Primers quantitative PCR*

Gene	Forward sequence	Reverse sequence
LKB1	5'-GCTCTTACGGCAAGGTGAAG	5'-TTTTGTGCCGTAACCTCCTC
Axin2	5'-tatccagtgatgcgctgacg	5'-ttactgcccacacgataagg
CD44	5'-TGGCACCCGCTATGTCCAG	5'-GTAGCAGGGATTCTGTCTG
E-cadherin	5'-AATTCCTGCCATTCTGGGGA	5'-TCTTCTCCGCCTCCTTCTTC
NEDD9	5'-GATTACGTCCACCTACAGGGTA	5'-TGTCATTCTCCACGGGCTTT
GAPDH	5'-AAGGTCGGAGTCAACGGATTT	5'-ACCAGAGTTAAAAGCAGCCCTG

Supplementary table 2. *Antibodies immunoblotting and immunofluorescence*

Antibody	Dilution	Company	Catalog number
Primary antibodies			
Rabbit-anti-LKB1	1:2000	Cell Signaling Technology	3047
Rabbit-anti-phospho-AMPK	1:1500	Cell Signaling Technology	2535
Rabbit-anti-phospho-S6K	1:1000	Cell Signaling Technology	5364
Rabbit-anti-E-cadherin	1:1000	Cell Signaling Technology	3195
Rabbit-anti-β-catenin	1:2000	Epitomics	1247-1
Mouse-anti-β-catenin	1:2000	BD Transduction Lab.	610154
Rabbit-anti-N-cadherin	1:1000	Cell Signaling Technology	4061
Rabbit-anti-vimentin	1:1000	Cell signaling Technology	5741
Rabbit-anti-cofilin	1:500	Signal Way	21164-1
Rabbit-anti-phospho-PAK	1:1000	Cell Signaling Technology	2607
Rabbit-anti-MECT1 (CRTC1)	1:1000	Rockland	600-401-936
Rabbit-anti-CD44	1:1000	Abcam	ab41478
Mouse-anti-β-actin	1:2500	Santa Cruz	sc-47778
Secundary antibodies			
Goat-anti-mouse IgG IRDye 680LT	1:5000	LI-COR	926-68020
Goat-anti-rabbit IgG IRDye 800CW	1:5000	LI-COR	926-32211

Chapter 4

Identification of molecular alterations in gastrointestinal carcinomas and dysplastic hamartomas in Peutz-Jeghers syndrome

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ABSTRACT

Peutz-Jeghers syndrome (PJS) is caused by mutations in the *LKB1* gene. It is characterized by gastrointestinal polyposis and an increased cancer risk, mainly in the gastrointestinal tract. Mechanisms of PJS-associated carcinogenesis are unclear. We investigated the involvement of candidate genes and molecular pathways in PJS-associated gastrointestinal cancers and dysplastic hamartomas. Cases were selected from the Dutch PJS cohort. Available tissue was immunostained for phospho-S6, β-catenin, P53 and SMAD4. DNA was isolated from carcinoma tissue and dysplastic and non-dysplastic areas of hamartomas specifically. Mutation analyses were done for *BRAF*, *KRAS*, and *P53*, and LOH analyses for *LKB1* and *P53.* Twenty-four of 144 patients (17%) developed 26 gastrointestinal malignancies at a median age of 49 years (IQR 35-60). 11/792 hamartomas (1.4%) of nine patients were classified as dysplastic. LOH of *LKB1* was detected in 3/6 (50%) carcinomas and in the dysplastic part of 3/5 (60%) hamartomas. Aberrant P53 expression was observed in 8/15 (53%) carcinomas. Six carcinomas with P53 overexpression harboured a *P53* mutation, with loss of the remaining wildtype allele in four. Two hamartomas showing P53 overexpression in high-grade dysplastic foci harboured a *P53* mutation with LOH. Loss of nuclear SMAD4 was observed in high-grade dysplastic foci of 2/4 (50%) hamartomas, in contrast to low-grade dysplastic foci (0/4) and non-dysplastic epithelium. Our findings suggest a role for mutant *P53* in PJS-associated gastrointestinal carcinogenesis. Inactivation of TGF-ß/BMP signalling and complete loss of *LKB1* might be involved in dysplastic transformation of gastrointestinal hamartomas specifically.

INTRODUCTION

Peutz-Jeghers syndrome (PJS) is a rare, autosomal dominant inherited disorder. Incidence is estimated between 1 in 50,000 to 1 in 200,000 live births [7]. The syndrome is caused by an inactivating germline mutation in the *LKB1* tumour suppressor gene (also known as *STK11*), located on chromosome 19p13.3 of the human genome [10,11]. A pathogenic germline mutation in *LKB1* is detected in 80-94% of families affected with PJS [13,14].

The typical PJS phenotype consists of mucocutaneous pigmentations and gastrointestinal polyposis. These polyps, histologically defined as hamartomas, can develop already in the first decade of life and may cause anaemia, bleeding, abdominal pain, and intestinal obstruction [20]. Furthermore, PJS patients are at increased risk for developing cancer at a young age [24,380]. Although a wide spectrum of malignancies has been described, patients are mostly affected by cancers in the gastrointestinal tract, including colorectal, pancreatic and gastric cancer. A cumulative risk of up to 57% at the age of 70 years has been described for gastrointestinal cancers [24].

At present, the pathogenesis and molecular mechanisms underlying PJS-associated gastrointestinal cancer development are unclear. Though adenomatous and carcinomatous changes are rarely observed in PJS hamartomas [111,112,381], a hamartoma-carcinoma sequence is debated. In addition to the initial germline mutation, several mechanisms for inactivation of the remaining wildtype *LKB1* allele, such as loss of heterozygosity (LOH) and promoter hypermethylation, have been described of which LOH is observed most frequently. Interestingly however, although LOH is detected more frequently in carcinomas than in hamartomas, it is not detected in 100% of these carcinomas [119,120,382], suggesting that complete loss of functional LKB1 might not be required for the development of carcinomas in PJS. Coincidence of the proper secondary oncogenic hits in other genes in addition to this haploinsufficiency of LKB1 might be sufficient for carcinoma development in this syndrome [34,35].

The aim of this study was to get insight into the molecular pathways underlying PJS-associated gastrointestinal carcinogenesis. To this aim, we collected a unique panel of 15 gastrointestinal carcinomas from a large cohort of Dutch PJS patients. The expression, activity and mutation status of genes and signalling pathways associated with sporadic gastrointestinal carcinogenesis as well as with gastrointestinal cancer related to other inherited disorders were studied by immunohistochemistry and DNA mutation analysis. To investigate whether there is an overlap between the molecular alterations in these carcinomas and in PJS hamartomas, specifically in dysplastic lesions of these hamartomas, we also analysed the affected genes and pathways in a unique set of nine dysplastic hamartomas from our patient cohort.

MATERIALS AND METHODS

Case selection and data collection

Cases were selected from the Dutch PJS cohort, initiated by two Dutch academic hospitals. PJS patients throughout the Netherlands with a definite diagnosis of PJS – defined by diagnostic criteria recommended by the WHO (see chapter 1, Box 1, page 12), a proven *LKB1* mutation, or both – were included in this cohort. Informed consent was obtained of all patients or of their next of kin if patients had passed away, and the study was approved by the Institutional Review Board of both participating hospitals. In total, 144 PJS patients from 62 families were included in this cohort. Baseline characteristics of the cohort are shown in

Supplementary Table 1. Patients with a diagnosis of a primary malignancy of the gastrointestinal tract (i.e. oesophagus, stomach, small intestine (including duodenum), colorectum, pancreas, liver and biliary tract) were selected. Data were collected on the following variables: date of birth, date of death, gender, *LKB1* germline mutation, date of diagnosis, tumour type and origin, tumour invasion, data on confirmation (medical record or histology) and treatment.

To select hamartomas with dysplastic changes, all available electronic pathology reports of hamartomas removed during endoscopy, intraoperative enteroscopy or surgery were screened. Two expert pathologists independently reviewed all haematoxylin and eosin-stained slides of hamartomas with some degree of dysplasia according to the original pathology report. In case of disagreement, consensus was reached by consultation. Only cases with both hamartomatous characteristics and dysplastic changes were included in this study. Dysplasia was classified as low-grade dysplasia (LGD) or high-grade dysplasia (HGD).

Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded (FFPE) tissue of both carcinoma and dysplastic hamartoma cases was collected from pathology archives for further analysis.

Sections (4-5 µm) were cut and deparaffinised. Antigen retrieval was performed by boiling in 10mM Tris/1mM EDTA buffer pH9 (for β-catenin and SMAD4) or 10mM citric buffer pH6 (for αSMA and pS6) for 10min. Non-specific binding sites were blocked in 3% H_2O_2 for 10min and 5% non-fat dry milk for 30min and in PBS with 10% normal goat serum for SMAD4. Primary antibodies used for IHC analysis included monoclonal mouse anti-human alpha smooth muscle actin (1:1000, DAKO clone 1A4), monoclonal mouse anti-β-catenin (1:1500, BD Biosciences 610154), monoclonal rabbit anti-phospho-S6 Ribosomal protein (1:250, Cell Signalling Technology #5364), p53 (1:400, DAKO clone DO-7), Mib-1 (1:100, DAKO clone MIB-1) and SMAD4 (1:400, Santa Cruz Biotechnology, Inc.). Incubations with primary antibodies were carried out overnight at 4°C and for 1 hour at room temperature for SMAD4. Sections were washed 3 times with PBS containing 0.05% Tween-20 (PBST), and incubated with HRP-labeled goat anti-rabbit IgG or goat anti-mouse IgG (EnVision™+, DAKO) for 30min. Sections were washed 3 times with PBST. After DAB precipitation, a haematoxylin counterstaining was performed. Slides were scored by two authors independently and final consensus was reached by consultation. αSMA was stained for confirmation of hamartomas. Proliferation rates were determined by the average percentage of Mib-1 positive cells in three microscopic fields (20x). Nuclear β-catenin and SMAD4 staining was considered positive when one or more positive nuclei were observed in a microscopic field (20x). P53 staining was scored on a scale ranging from - to +++ (-, negative; +, positive/normal; ++, moderate overexpression; +++, strong overexpression). Epithelial pS6 staining was scored on a scale ranging from - to ++ (-, negative; +, positive; ++, overexpression).

DNA isolation

Tissue of gastrointestinal carcinomas was carefully dissociated from surrounding normal tissue, resulting in samples containing >50% tumour cells. DNA of dysplastic hamartoma tissue was isolated from epithelial cells in the non-dysplastic area and epithelial cells in the dysplastic lesion specifically, using P.A.L.M. laser micro dissection. For DNA isolation, samples were incubated overnight at 56°C in lysis buffer (Cell Lysis Solution, Promega, cat # A7933)

containing 5% chelex (Chelex 100 resin (Bio-Rad) catalog # 143-2832) and 10% proteinase K. Samples were heated to 95°C for 10 minutes to inactivate the proteinase K and to denature the DNA.

DNA mutation analysis

DNA was amplified for mutational analysis. DNA of all tumours was sequenced and checked for mutations in *KRAS* codon 12 and 13 and *BRAF* codon 600. Mutation analysis of exon 3 of ß-catenin was performed if nuclear expression of ß-catenin was observed with immunohistochemistry. *P53* mutation analysis for exons 5-8 was performed for all gastrointestinal carcinomas. For the dysplastic hamartomas, *P53* mutation analysis was only performed if aberrant P53 expression (i.e. strong overexpression or absence) was observed with immunohistochemistry. In cases of a known germline *LKB1* mutation or the detection of a *P53* mutation, loss of the wild-type *LKB1* and *P53* alleles, indicative for LOH, was determined from the nucleotide sequence analyses. PCRs were performed at an annealing temperature of 58°C, for 34 cycles using GoTaq® DNA Polymerase with 5x Buffer (Promega, Madison, Wisconsin, USA). For most tumours a nested PCR was performed with 2 µL PCR product. DNA purification and sequencing reactions were performed by LGC Genomics (Berlin, Germany). Primers and nested primers used for amplification and sequences of the different genes are available upon request.

RESULTS

Clinical characteristics – Gastrointestinal carcinomas

Twenty-four of the 144 patients (17%, 67% male) from 18 families developed 26 gastrointestinal malignancies (Table 1). Two patients were diagnosed with two primary tumours. Median age at diagnosis of the first tumour was 49 years (IQR 35-60). Two carcinomas were detected during surveillance endoscopies, in all the other cases patients presented with symptoms. Median survival after gastrointestinal cancer diagnosis was eight months (IQR 3-49 months). Twenty-one of these patients (88%) deceased at a median age of 55 years (IQR 38-61); 20 of whom died as a direct cause of gastrointestinal cancer. Three patients are still alive at a median time of four years (IQR 3-15) after cancer diagnosis. These data suggest that PJS patients can develop aggressive gastrointestinal cancers at a relatively young age.

Clinical characteristics – Dysplastic hamartomas

A total of 792 hamartomas of 52 patients were histologically examined. Nineteen (2.4%) hamartomas of 15 patients were originally classified as dysplastic. Upon revision by two expert pathologists 11 (1.4%) hamartomas of nine patients (eights male) were classified as dysplastic and included in this study (Table 2), with a 100% consensus between the two pathologists. In the eight other cases the morphologic abnormalities were reclassified as reactive proliferation instead of dysplastic changes. This implies that gastrointestinal hamartomas of PJS patients are not precursor lesions, although malignant transformation does occur in sporadic cases.

Seven (64%) hamartomas contained foci of low grade dysplasia (LGD) and four showed foci of high grade dysplastic (HGD) changes (Table 2). Median size of the dysplastic hamartomas

Table 1. *Clinical characteristics of Peutz-Jeghers syndrome patients with gastrointestinal carcinomas.*

No.	*LKB1* germline mutation	Carcinoma location	Age at diagnosis (year of diagnosis)	Age at death	Distant metastasis at diagnosis	Therapy	FFPE tissue available?	Remarks
1.	c.464+1dupG	Sigmoid	61 (1988)	73	No	Curative	Yes	
2.	Tested but no mutation found[1]	Colon	56 (2007)	-	Yes	Curative	Yes	
3.	codon 66 ins t, stop in codon 162[2]	Colorectum not otherwise specified	40 (1937)	40	Unknown	Unknown	No	Confirmed by previous reports [383, 384]
4.	codon 66 ins t, stop in codon 162[2]	Colon	43 (1975)	43	Yes	Unknown	No	
5.	codon 66 ins t, stop in codon 162[2]	Sigmoid	76 (1937)	76	Unknown	Unknown	No	Confirmed by previous reports [383, 384]
6.	Deletion exon 1	Colon	37 (1996)	-	Yes	Curative	Yes	Detected during surveillance
7.[a]	c.468C>G[2]	Colon	64 (1996)	68	Unknown	Curative	Yes	
8.	c.370A>T[2]	Pancreas	57 (1994)	58	Yes	Palliative	Yes[3]	
9.[a]	c.468C>G[2]	Pancreas	66 (1998)	68	Yes	Palliative	No	
10.	Deletion exon 6, 7 and 8	Pancreas	54 (2004)	54	Yes	Palliative	No	
11.	Tested but no mutation found[1]	Pancreas	62 (2005)	62	Yes	Palliative	Yes	
12.[4]	c.582C>A	Pancreas	45 (2008)	46	Yes	Palliative	Yes	
13.	c.991dupC	Pancreas	36 (1997)	36	Yes	Palliative	Yes[3]	
14.[b]	Unknown	Pancreas	35 (1998)	35	Yes	Palliative	Yes[3]	

F: female; FFPE: formalin-fixed paraffin-embedded; GI: gastrointestinal; M: male; n.o.s.: not otherwise specified.

[a, b, c, d] Same patient (see also Table 2, 3 and 4)

[1] Exon sequencing and Multiplex Ligation-dependent Probe analysis

[2] Germline mutation found in family member; patient not tested

[3] Tissue of tumour metastasis

[4] Previously described in Klumpen et al. [385]

Table 1. *Clinical characteristics of Peutz-Jeghers syndrome patients with gastrointestinal carcinomas.*

No.	*LKB1* germline mutation	Carcinoma location	Age at diagnosis (year of diagnosis)	Age at death	Distant metastasis at diagnosis	Therapy	FFPE tissue available?	Remarks
15.	c.370A>T	Small bowel	43 (2005)	45	Yes	Curative	No	
16.[c]	c.291-2A>G	Small bowel	16 (1962)	58	No	Curative	No	Pseudo-invasion mimicking an invasive carcinoma could not be ruled out
17.	c.735-1G>A[2]	Small bowel	54 (1980)	55	Yes	Palliative	No	
18.	codon 66 ins t, stop in codon 162[2]	Stomach	61 (1933)	61	Unknown	Unknown	No	Confirmed by previous reports [383, 384]
19.	c.580G>A[2]	Stomach	30 (1979)	30	Yes	Palliative	Yes	
20.	c.910delC	Stomach	26 (2008)	-	No	Curative	Yes	Detected during surveillance
21.[d]	c.156_157dupGG[2]	Distal bile duct	57 (2006)	57	Yes	Palliative	Yes[3]	
22.	Not tested	Distal bile duct	73 (2008)	74	Yes	Palliative	Yes[3]	
23.	c.291-2A>G[2]	Ampulla Vateri	53 (2006)	58	No	Curative	Yes	
24.	codon 66 ins t, stop in codon 162[2]	GI tract	31 (1947)	31	Unknown	Unknown	No	Confirmed by previous reports [383, 384]
25.	c.464+1dupG[2]	Upper GI tract n.o.s.	35 (1987)	35	Yes	Palliative	Yes	
26[c]	c.291-2A>G	GI tract n.o.s.	58 (2004)	58	Yes	Palliative	No	

F: female; FFPE: formalin-fixed paraffin-embedded; GI: gastrointestinal; M: male; n.o.s.: not otherwise specified.

[a, b, c, d] Same patient (see also Table 2, 3 and 4)

[1] Exon sequencing and Multiplex Ligation-dependent Probe analysis

[2] Germline mutation found in family member; patient not tested

[3] Tissue of tumour metastasis

[4] Previously described in Klumpen et al. [385]

Table 2. *Clinical characteristics of Peutz-Jeghers syndrome patients with dysplastic hamartomas.*

No.	*LKB1* germline mutation	No. of hamartomas histologically assessed	No. of dysplastic hamartomas	Age at diagnosis (year of diagnosis)	Location	Maximal diameter (mm)	Amount of dysplastic epithelium (mm)	Grade of dysplasia	Indication	FFPE tissue available?
1.	c.580G>A	1	1	61 (2010)	Duodenum	18	2 (11%)	LGD	Surveillance	Yes
2.	c.298C>T	6	2	30 (2004)	Colon	30	7 (23%)	HGD	Anemia	Yes
				30 (2004)§	Rectum	15	3 (20%)	HGD	Anemia	Yes
3.	c.735-1G>A	21	2	41 (2000)	Duodenum (ampulla)	60	4.5 (7.5%)	LGD	Weight loss	Yes
				50 (2009)	Stomach	23	3 (13%)	LGD	Surveillance	No
4.	c.468C>G	10	1	35 (2007)	Colon	11	3.5 (33%)	LGD	Surveillance	Yes
5.[d]	c.156_157dupGG	1	1	56 (2006)	Small bowel	55	14 (25%)	HGD/ adenoca	Intussusception	Yes
6.	c.291-2A>G	15	1	21 (1998)	Small bowel	40	15 (38%)	LGD	Intussusception	Yes
7.[b]	Unknown	6	1	35 (1998)	Small bowel	40	1.5 (4%)	LGD	Liver metastasis	Yes
8.	c.290+1G>A	252	1	49 (2008)	Stomach	10	8 (80%)	HGD	Surveillance	Yes
9.	Unknown	9	1	41 (2005)	Small bowel	40	6 (15%)	LGD	Surveillance	No

F: female; FFPE: formalin-fixed paraffin-embedded; HGD: high-grade dysplasia; LGD: low-grade dysplasia; M: male.

[a, b, c, d] *Same patient (see also Table 1, 3 and 4)*

Table 3. *Genetic and molecular characteristics of gastrointestinal carcinomas of Peutz-Jeghers syndrome patients.*

No.	Tumour type	Mib1[1]	*LKB1* germline mutation	LKB1	LOH*LKB1*	Epithelial phospho-S6 (IHC)[2]	P53 IHC[3]	*P53* mutation (exon)	LOH *P53*	*BRAF* mutation (codon 600)	*KRAS* mutation (codon 12-13)	Nuclear ß-catenin (IHC)	*ß-catenin* mutation (exon 3)[4]	Loss of nuclear SMAD4 (IHC)
1.	Sigmoid	~25%	c.464+1dupG	Frameshift	NA	- (focal +)	+++	His179Tyr (5)	no	no	no	yes	Gly69Glutam	no
2.	Colon	~5%	Tested but no mutation found[5]	Unknown	NI/NA	-	+++	Arg273Cys (8)	yes	no	no	no	NA	no
3.	Colon	70-80%	Deletion exon 1	Unknown	NA	+/++	+/++	no	NI	no	no	no	no	no
4.	Colon	~60%	c.468C>G	Tyr156Stop	no	+/++	++	no	NI	no	Gly12Val	yes	no	no
5.	Pancreas	~5%	c.370A>T	Lys124Stop	NA	+/++	+++	His296Tyr (ex 8)	yes	no	no	no	NA	yes
6.	Pancreas	60-70%	Tested but no mutation found[5]	Unknown	NI/NA	++	++	no	NI	no	no	no	NA	no
7.	Pancreas	~10%	c.582C>A	Asp194Glu	yes	+	+	no	NI	no	no	no	NA	no
8.	Pancreas	40-50%	c.991dupC	Frameshift	yes	+	++	no	NI	no	no	no	NA	no
9.[b]	Pancreas	~25%	Unknown	Unknown	NI/NA	+	-	no	NI	no	no	yes	no	yes
10.	Stomach	5-10%	c.580G>A	Asp194Asn	yes	+/++	-	no	NI	no	no	no	NA	no
11.	Stomach	~80%	c.910delC	Frameshift	no	++	+++	Pro190Leu (6)	No	no	no	no	NA	no
12.[d]	Distal bile duct	60-70%	c.156_157dupGG	Frameshift	no	++	+++	Ser127Phe (5)	yes	no	no	no	NA	NA
13.	Distal bile duct	5-10%	Not tested	Unknown	NI/NA	++	+	no	NI	no	no	no	NA	no
14.	Ampulla Vateri	~40%	c.291-2A>G	Splice variant	NA	+/++	+/++	no	NI	no	no	no	NA	no
15.	Upper GI tract	~50%	c.464+1dupG	frameshift	NA	++	+++	Ile195Ser (6)	yes	no	NI	no	NA	no

IHC: immunohistochemistry; LOH: loss of heterozygosity; NA: not analysed; NI: not informative.

[b, d] Same patient (see also Table 1, 2 and 4)

[1] Mean of three 20x microscopic fields

[2] -; negative, +; positive, ++; overexpression

[3] -, negative; +, positive/normal; ++, moderate overexpression; +++, strong overexpression

[4]Mutation analysis of ß-catenin (exon 3) was only performed if nuclear expression of ß-catenin was observed with immunohistochemistry.

[5]Exon sequencing and Multiplex Ligation-dependent Probe analysis

was 30 mm (IQR 15-40). Dysplastic hamartomas were detected throughout the gastrointestinal tract and five (45%) hamartomas were found during surveillance endoscopies.

Molecular alterations in gastrointestinal carcinomas

To investigate whether molecular alterations in genes known to be involved in sporadic and hereditary gastrointestinal cancer can also be identified in PJS-associated cancers, FFPE tissue of 15 available gastrointestinal carcinomas was selected (Table 3). Proliferation of tumour cells differed widely between the tissue samples. Loss of the wild-type allele of *LKB1*, indicative for LOH, was detected in 3/6 (50%) informative gastrointestinal carcinomas (Figure 1A), which is in line with previously reported frequencies. Inactivation of LKB1 impairs the inhibition of mammalian target of rapamycin (mTOR), resulting in phosphorylation of the ribosomal protein S6. All 15 carcinomas showed epithelial phospho-S6 expression, although in a heterogeneous manner. Phospho-S6 expression did not correlate with loss of wild-type *LKB1*. This suggests that complete loss of LKB1 occurs in PJS-associated gastrointestinal carcinomas, but is not required. Also, complete loss of LKB1 does not correlate with hyperactivation of mTOR in these tumours.

In 8/15 (53%) gastrointestinal carcinomas, aberrant P53 expression was observed. Six samples showed strong overexpression of P53 and two others showed total absence of P53 expression, suggesting that in these carcinomas P53 was genetically altered (Figure 1B). In 6/15 (40%) carcinomas, a mutation in *P53* could be detected, which was accompanied by loss of the remaining wild-type allele in 4/6 (67%) cases (Figure 1C). These data suggest that genetically altered P53 is involved in gastrointestinal carcinogenesis in PJS.

In none of the gastrointestinal carcinoma samples a mutation in the *BRAF* gene was observed, and only one somatic *KRAS* mutation was found in a colon carcinoma. Nuclear ß-catenin, indicative for active Wnt-signalling, was detected in some tumour cells scattered throughout glandular structures of two colorectal carcinomas and one pancreatic carcinoma. A mutation in exon 3 of ß-catenin was found in one colorectal carcinoma. Loss of nuclear SMAD4, indicative for the inactivation of TGF-ß/BMP signalling, was observed in two pancreatic carcinomas. This might implicate that *BRAF, KRAS*, Wnt-signalling and the TGF-ß/BMP pathway, involved in sporadic and other hereditary gastrointestinal cancers, are not frequently altered in PJS-associated cancer of the digestive tract.

Molecular alterations in dysplastic hamartomas

To compare molecular alterations of PJS-associated carcinomas and dysplastic hamartomas of the gastrointestinal tract, we also collected FFPE tissue of nine dysplastic hamartomas (Table 4). In all hamartomas, the characteristic branching structure of smooth muscle cells could be observed by H&E and αSMA stainings (Figure 2A,B). As expected, Mib1 staining showed increased proliferation rates in all dysplastic foci compared to the non-dysplastic epithelial compartments of the same hamartomas (Figure 2C).

Loss of the wild-type allele could not be detected in the non-dysplastic areas of any of the hamartomas available for analysis. In contrast, loss of the wild-type *LKB1* allele was detected in the dysplastic segments (2 LGD, 1 HGD) of 3/5 (60%) of these hamartomas (Figure 3A). Phospho-S6 expression was more abundant in the dysplastic epithelium of four hamartomas (three with HGD) (data not shown), and in two cases this correlated with loss of the wild-type

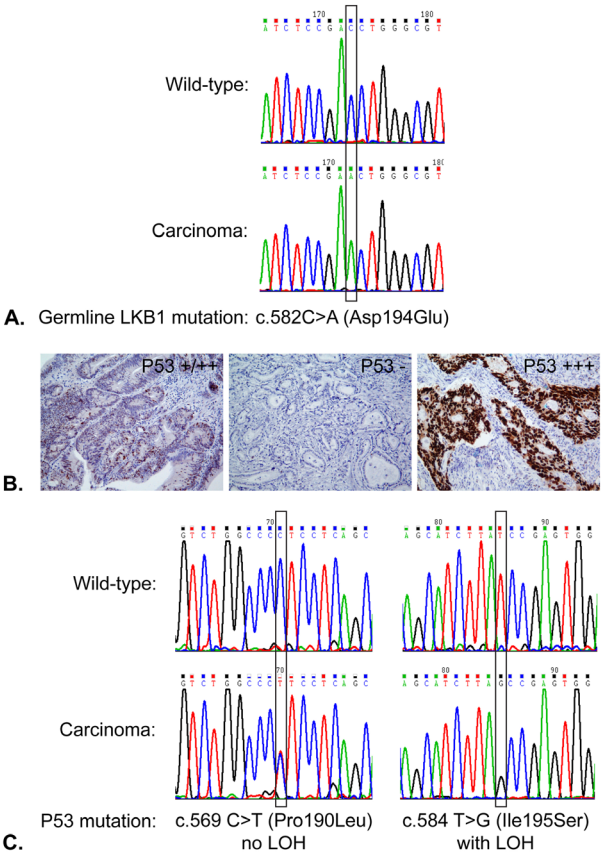

Figure 1. *Molecular alterations in gastrointestinal carcinomas of Peutz-Jeghers syndrome patients.* ***A.*** *LKB1 nucleotide sequence analysis of a carcinoma, showing the known germline mutation in LKB1 (Asp194Glu) with loss of the wild-type LKB1 allele (lower panel).* ***B.*** *Left (100x): carcinoma tissue showing P53 expression scored as +/++. Middle (100x): carcinoma tissue showing complete loss of P53 expression scored as –. Right (100x): carcinoma tissue showing overexpression of P53 scored as +++.* ***C.*** *Left: P53 nucleotide sequence analysis showing a mutation in P53 (Pro190Leu) without loss of the wild-type P53 allele (lower panel). In upper panel the wild-type sequence is shown. Right: P53 nucleotide sequence analysis showing a mutation in P53 (Ile195Ser) with loss of the wild-type P53 allele (lower panel). In upper panel the wild-type sequence is shown.*

allele of LKB1 (Table 4). Stromal cells were also positive for phospho-S6 staining, but there was no difference in stromal expression between the non-dysplastic and the dysplastic parts. These results suggest that complete loss of LKB1 and activation of mTOR are involved in dysplastic degeneration of gastrointestinal hamartomas in PJS. However, loss of LKB1 did not correlate with mTOR hyperactivation.

Since we observed that P53 was frequently genetically altered in PJS-associated gastrointestinal carcinomas, we investigated P53 expression in the dysplastic gastrointestinal hamartomas. Aberrant expression of P53 was specifically observed in the HGD parts of 3/4 (75%) hamartomas, of which two showed strong overexpression and one showed complete loss of P53 expression (Figure 3B). Aberrant expression of P53 could not be observed in

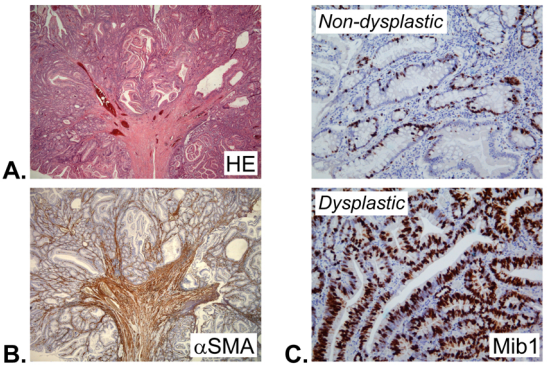

Figure 2. *Immunohistochemical staining of dysplastic Peutz-Jeghers syndrome hamartomas.* ***A.*** *H&E staining of a PJS hamartoma (50x).* ***B.*** *α-SMA staining of a PJS hamartoma (50x), showing the characteristic branching smooth muscle bundles.* ***C.*** *400x magnification of Mib1 staining, showing increased proliferation in the LGD area (~80%) (lower panel) compared to the non-dysplastic area (~40%) of the same hamartoma (upper panel), indicating increased cell proliferation in the dysplastic lesion of the hamartoma.*

the LGD or non-dysplastic parts of the hamartomas. In the two HGD hamartomas showing strong overexpression of P53, a *P53* mutation with loss of the wild-type allele was specifically detected in DNA isolated from the dysplastic cells (Figure 3C). This might indicate that genetically altered P53 is involved in HGD transformation of PJS-hamartomas. In contrast, no *BRAF* or *KRAS* mutations could be detected, suggesting that these oncogenes do not play a role in dysplastic transformation of hamartomas. In the low-grade dysplastic epithelium of 3/5 (60%) hamartomas with LGD, focal nuclear expression of β-catenin was detected, in contrast to the four hamartomas with HGD, where no nuclear expression of β-catenin was found (Figure 3B). However, the expression pattern was not abundant and no mutation in exon 3 of β-catenin was detected in DNA of these hamartomas. Notably, 2/4 (50%) HGD hamartomas showed absence of nuclear SMAD4 in the dysplastic foci, in contrast to the non-dysplastic epithelium of these hamartomas (Figure 3B). This was not observed in any of the LGD hamartomas. Therefore, loss of TGF-ß/BMP signalling might be involved in HGD transformation of gastrointestinal hamartomas in PJS.

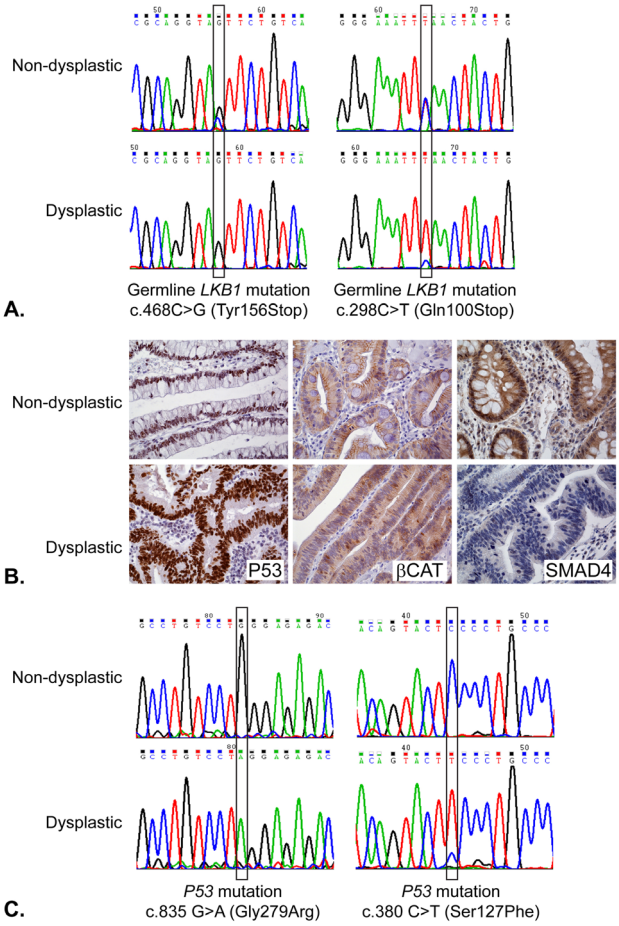

Figure 3. *Molecular alterations in dysplastic Peutz-Jeghers syndrome hamartomas.*
A. *Left: LKB1 nucleotide sequence analysis of a hamartoma with dysplasia, showing the known germline mutation in LKB1 (Tyr156Stop) as found in the non-dysplastic cells (upper panel) and loss of the wild-type LKB1 allele in the dysplastic LGD lesion of the hamartoma.*
Right: LKB1 nucleotide sequence analysis of a hamartoma with HGD, showing the known germline mutation in LKB1 (Gln100Stop) without loss of the wild-type LKB1 allele in both the non-dysplastic (upper panel) and dysplastic (lower panel) cells of the hamartoma. ***B.*** *Left (400x): P53 staining of hamartoma showing overexpression of P53 (scored as +++) in the HGD area (lower panel) in contrast to the non-dysplatic part (upper panel). Middle (400x): ß-catenin staining of a hamartoma with LGD, showing focal nuclear expression of ß-catenin in the LGD area (lower panel) but absence of nuclear ß-catenin in the non-dysplastic part (upper panel). Right (400x): SMAD4 staining of a hamartoma with HGD, showing nuclear expression of SMAD4 in the non-dysplastic hamartomatous cells (upper panel) but absence of nuclear SMAD4 in the HGD lesion of the same hamartoma.* ***C.*** *Left: P53 nucleotide sequence analysis of a hamartoma with HGD, showing a mutation in P53 (Gly279Arg) without loss of the wild-type P53 allele in the HGD lesion (lower panel), while the mutation could not be detected in DNA isolated from the non-dysplastic area of the same hamartoma (upper panel). Right: P53 nucleotide sequence analysis of a hamartoma with HGD, showing a mutation in P53 (Ser127Phe) with loss of the wild-type P53 allele in the HGD lesion (lower panel), while the mutation could not be detected in the non-dysplastic area of the same hamartoma (upper panel). HGD; high-grade dysplasia, LGD; low-grade dysplasia.*

Table 4. *Genetic and molecular characteristics of dysplastic hamartomas in Peutz-Jeghers syndrome patients.*

No.	Location	Grade of dysplasia	Mib1[1]	*LKB1* germline mutation	LKB1	LOH *LKB1*	Epithelial phospho S6 (IHC)[2]	P53 IHC[3]	*P53* mutation (exon)[4]	LOH *P53*	*BRAF* (codon 600)	*KRAS* (codon 12-13)	Nuclear ß-catenin (IHC)	*ß-catenin* mutation (exon 3)	Loss of nuclear SMAD4 (IHC)
1.	Duodenum	Ham	~40%	c.580G>A	Asp194Asn	no	+	+	NA	NA	no	no	no	no	NI
		LGD	~80%			yes	+	+	NA	NA	no	no	yes	no	NI
2.	Colon	Ham	50-60%	c.468C>G	Tyr156Stop	no	-	+ (focal ++)	NA	NA	no	no	no	NA	no
		LGD	90-100%			yes	+	+	NA	NA	no	no	no	NA	no
3.	Jejunum	Ham	~10%	c.291-2A>G	Splice variant	no	+	+	NA	NA	no	no	no	NI	no
		LGD	20-25%			no	+	++	NA	NA	no	no	yes	no	no
4.[b]	Small bowel	Ham	25-30%	Unknown	Unknown	NI	+ (focal ++)	+	NA	NA	NI	NI	no	NI	no
		LGD	60-70%			NI	+ (focal ++)	++	NA	NA	NI	NI	yes	NI	no
5.	Duodenum/ papil	Ham	20-25%	c.735-1G>A	Splice variant	no	+	+	NA	NA	no	no	no	NA	no
		LGD	40-60%			no	+ (focal ++)	+	NA	NA	no	no	no	NA	no
6.	Stomach	Ham	NA	c.290+1G>A	Splice variant	no	NA	+	NA	NA	no	no	no	NA	no
		HGD	NA			no	NA	++	NA	NA	no	no	no	NA	no
7.	Colon	Ham	40-50%	c.298C>T	Gln100Stop	NI	-	+	-	-	no	no	no	NA	no
		HGD	80-90%			NI	++	+++	Gly279Arg (8)	Yes	no	no	no	NA	yes
8.	Rectum	Ham	25-30%	c.298C>T	Gln100Stop	no	- (focal +)	+	no	NI	no	no	no	NA	no
		HGD	60-70%			yes	++	-	no	NI	no	no	no	NA	no
9.[d]	Small bowel	Ham	~10%	c.156_157dupGG	Frameshift	NA	+	+	no	NI	no	no	no	NA	no
		HGD	~70%			NA	++	+++	Ser127Phe (5)	yes	no	no	no	NA	yes

-: no mutation; ham: hamartomatous epithelium; HGD: high-grade dysplasia; IHC: immunohistochemistry; LGD: low-grade dysplasia; LOH: loss of heterozygosity; NA: not analysed; NI: not informative.

[b, d] Same patient (see also Table 1, 2 and 3)

[1] Mean of three 20x microscopic fields

[2] -; negative, +; positive, ++; overexpression

[3] -, negative; +, positive/normal; ++, moderate overexpression; +++, strong overexpression

[4]P53 mutation analysis was only performed if aberrant P53 expression (i.e. strong overexpression or absence) was observed with immunohistochemistry.

[5]Mutation analysis of ß-catenin (exon 3) was only performed if nuclear expression of ß-catenin was observed with immunohistochemistry.

DISCUSSION

Patients with PJS are prone to develop cancer at a young age, especially in the gastrointestinal tract. In a large cohort of Dutch PJS patients, 17% of patients developed a primary malignancy in the gastrointestinal tract at a median age of 49 years. Although tumours developed throughout the digestive tract, they were mainly found in the colorectum and pancreas. Survival after gastrointestinal cancer diagnosis was strikingly short in our cohort, suggesting a more aggressive tumour phenotype in PJS. However, the pathogenesis of PJS-associated cancers remains unknown.

Inactivating germline mutations in the *LKB1* tumour suppressor gene, causing PJS, were detected in the majority of patients or their affected family members of our cohort. Loss of the remaining wild-type allele of *LKB1* was found in only three out of six gastrointestinal carcinomas, which is in line with previously reported frequencies [119,120,382]. In contrast, in three out of five hamartomas, loss of the wildtype *LKB1* allele was detected in the dysplastic epithelium but not in the non-dysplastic epithelium of the same hamartomas. Therefore, complete loss of LKB1 might not be a prerequisite for gastrointestinal carcinogenesis in PJS, but is involved in dysplastic transformation of hamartomas. However, we cannot exclude loss of LKB1 protein expression since suitable antibodies for immunohistochemistry are not available. Furthermore, somatic inactivation by epigenetic silencing due to promoter methylation has been described for the *LKB1* gene. LKB1-deficiency impairs the inhibition of mTOR, and it has been suggested that specific mTOR inhibitors (e.g. rapamycin) might be an effective therapy for treatment of both hamartomas and carcinomas in PJS patients [256,385]. However, activation of mTOR - by means of higher phospho-S6 protein levels - did not correlate with genetic loss of LKB1 in the majority of carcinomas and dysplastic hamartomas, indicating that alternative pathways (e.g. PI3K/AKT signalling) might be involved in mTOR activation in PJS tumours as well.

We detected mutations in the *P53* tumour suppressor gene - in most cases with loss of the wildtype *P53* allele and concomitant P53 overexpression - in both gastrointestinal cancers and hamartomas with high-grade dysplastic lesions. Because some of the *P53* mutations were identified exclusively in the dysplastic parts of the hamartomas, and since our patients, although intensively monitored, do not show any signs of suffering from Li-Fraumeni syndrome, we strongly believe that these mutations are somatic rather than germline. These data suggest that mutant *P53* is involved in gastrointestinal carcinogenesis and late stages of dysplastic transformation of hamartomas in PJS. In *LKB1* mutant mice, additional loss of *p53* increased and accelerated gastrointestinal hamartoma growth without changing the histological grade of these hamartomas [100,101]. Notably, additional loss of *p53* could not induce intestinal carcinoma development in *LKB1*-deficient mice. Inactivation of *LKB1* might collaborate with mutant *P53* rather than with loss of *P53* for PJS-associated carcinogenesis.

In sporadic colorectal cancer (CRC) cases, both activation of the Wnt/ß-catenin pathway and the oncogene *KRAS* are essential early events in development [386]. At a later stage in the adenoma-carcinoma sequence of sporadic CRC, mutations in the *P53* tumour suppressor gene play an important role. In the present study, 2/4 (50%) of colorectal carcinomas showed nuclear ß-catenin, indicative for activation of the oncogenic Wnt/ß-catenin signalling pathway. However, nuclear ß-catenin was not as abundant as observed in sporadic CRC or in premalignant adenomas. Previously, ß-catenin mutations rather than mutations in *APC* have been detected in PJS tumours [120,387]. We observed no mutations in the ß-catenin gene in the carcinomas and dysplastic hamartomas showing nuclear ß-catenin. In one of four CRCs, a *KRAS* mutation was detected, which coincided with nuclear ß-catenin. In another CRC specimen, nuclear

ß-catenin coincided with a *P53* mutation. These results suggest a synergism between active Wnt/ß-catenin signalling, KRAS and P53 in PJS-associated CRC formation, like in sporadic CRC. However, no *KRAS* mutations were observed in the other GI carcinomas and nuclear ß-catenin was observed in only one pancreatic cancer, while *P53* mutations were abundant in the other carcinomas. Thus, although similar molecular mechanisms may be involved in PJS-associated CRC as well as in sporadic cases of CRC, our results indicate a strong link between LKB1 and P53 in other GI cancers, which may be specific for PJS-associated cancer.

Strikingly, in none of the investigated pancreatic carcinomas a *KRAS* mutation was detected, while mutations in this gene are found in more than 90% of sporadic pancreatic carcinomas [388]. This suggests that oncogenic activation of *KRAS* in addition to inactive *LKB1* is not required for pancreatic carcinogenesis in PJS. This is also shown in mice, where specific loss of *LKB1* in pancreatic epithelium is sufficient for pancreatic carcinoma formation [124]. In sporadic pancreatic cancers, the *SMAD4* gene is inactivated in approximately 60% [389]. Here, we observed loss of nuclear SMAD4 in two out of five pancreatic cancers. Notably, nuclear SMAD4 was absent in the nuclei of dysplastic cells of two out of four HGD intestinal hamartomas, while it was present in all intestinal carcinomas. This might indicate involvement of inactive TGF-ß/BMP signalling in dysplastic transformation of PJS hamartomas, but not in intestinal carcinoma development, and suggest that these are two different entities of PJS.

Although dysplastic and carcinomatous changes do occur in hamartomas, it is rare. In the present study only about 1% of hamartomas showed some degree of dysplasia. Because not all hamartomas found in our patients were revised, this could be an underestimation. However, also in previous studies malignant transformation was found in a minority of PJS hamartomas (<1-9%) [111,112]. Furthermore, signs of hamartomatous tissue that could indicate a hamartomatous origin were not observed in the PJS-associated carcinomas. Although the incidence of GI carcinomas is high in PJS patients, a much higher incidence would be expected if hamartomas were indeed precursor lesions, given the high polyp load in most PJS patients. In addition, the location of the gastrointestinal malignancies in PJS patients does not always correlate with the location of the hamartomatous polyps [381]. Also in the present study, most gastrointestinal cancers were found in the colorectum and pancreas, while dysplastic hamartomas were mainly found in the small bowel, in line with the preferred location of PJS hamartomas. Finally, although some molecular alterations were identified in both PJS-associated gastrointestinal carcinomas and dysplastic lesions of hamartomas, we mainly detected substantial differences. Based on these observations, we believe that an absolute hamartoma-carcinoma sequence in PJS patients is unlikely.

In conclusion, our findings suggest a role for mutant *P53* in PJS-associated gastrointestinal carcinogenesis, in addition to a haploinsufficient function of the *LKB1* tumour suppressor gene. Loss of nuclear SMAD4 and complete loss of *LKB1* may be involved in dysplastic transformation of gastrointestinal hamartomas specifically.

Our conclusions are based on a small number of heterogeneous tumour samples, which is explained by the rarity of PJS. Though, to our knowledge, we describe the largest sample set of PJS-associated carcinomas of the gastrointestinal tract specifically. In addition, the set of gastrointestinal hamartomas with different grades of dysplasia used in this study is of amount not incorporated in such a study elsewhere. With these unique sample sets, we were able to identify molecular alterations associated with gastrointestinal tumour development in PJS. Whether these alterations contribute to the development of these PJS tumours is suggestive, but needs to be investigated further in functional studies.

ACKNOWLEDGEMENTS

We thank intern Anja ten Hoeve-van Bergeijk for technical assistance.

SUPPLEMENTARY MATERIAL

***Supplementary Table** 1. Baseline characteristics of the Dutch Peutz-Jeghers syndrome cohort.*

Total	**144**
Gender	
Male	70 (49%)
Female	74 (51%)
Families	62
Family history	
Familial PJS	109 (76%)
Sporadic	24 (17%)
Family history unknown	11 (7%)
DNA mutation analysis	90 (63%)
LKB1 mutation carrier	83/89 (93%)
Deceased	48 (33%)
Median age at death	46 years (IQR 32-58 years)
Lost to follow up	6 (4%)
Cancer	48 (33%)
Median age at diagnosis of first cancer	46 years (IQR 35-55 years)
2 primary cancers	8

IQR: interquartile range; PJS: Peutz-Jeghers syndrome.

Chapter 5

GNAS is not involved in gastrointestinal tumour formation in Peutz-Jeghers syndrome

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ABSTRACT

Peutz-Jeghers syndrome (PJS), caused by germline mutations in *LKB1*, is characterized by the development of hamartomatous polyps in the gastrointestinal (GI) tract. McCune Albright syndrome (MAS) is caused by somatic activating mutations in *GNAS* and presents with cutaneous, skeletal, and endocrine manifestations. Recently, hamartomatous GI polyps with histological features similar to those in PJS were observed in MAS patients, suggesting a role for GNAS in the pathogenesis of PJS. This study reports the first somatic *GNAS* mutation analysis in GI tumours of PJS patients. No mutations were observed, suggesting that *GNAS* is not involved in the pathogenesis of GI tumours in PJS.

Peutz-Jeghers syndrome (PJS) is a rare autosomal dominant inherited disorder caused by germline mutations in *LKB1* (also known as *STK11*). PJS is clinically characterized by mucocutaneous pigmentations and the development of hamartomatous polyps, i.e. hamartomas, in the gastrointestinal (GI) tract [28]. Furthermore, PJS patients have a highly increased risk for developing cancer, mainly in the GI tract [28]. Although the genetic background of PJS is known, the pathogenesis of hamartomas and carcinomas in this syndrome is yet unclear.

McCune Albright syndrome (MAS), another rare genetic disorder, is characterized by café-au-lait spots, polyostotic fibrous dysplasia, and endocrine hyperfunction. MAS is caused by postzygotic mutations in the *GNAS* gene [390]. *GNAS* encodes the α subunit of the stimulatory G protein (Gsα) involved in G-protein signalling. The most frequently observed mutations in MAS occur in exon 8, resulting in amino acid substitutions in codon 201. These mutations, often mosaicisms, change the Gsα activity preventing downregulation of cAMP/PKA signalling [390]. It was recently reported that hamartomatous polyps were observed in the stomach and duodenum of four MAS patients [391]. The polyps were histologically similar to PJS polyps, showing prominent bundles of smooth muscle fibres in the stromal cores covered by well differentiated gastric and duodenal epithelium. Activating *GNAS* mutations were found in the polyps or adjacent mucosa in 3 of 4 subjects. The authors suggested a putative interaction between the *LKB1* and *GNAS* genes in the pathogenesis of MAS and PJS.

To investigate this hypothesis, we performed a somatic *GNAS* mutation analysis on GI tumours of PJS patients. We obtained archive material of 6 GI hamartomas and 12 GI carcinomas of our Dutch PJS patient cohort. DNA was extracted from this macro-dissected (carcinomas) or laser capture micro-dissected (hamartomas) formalin-fixed paraffin-embedded tissue. We successfully amplified and sequenced exon 8 of *GNAS*. No mutations at codon 201 were detected in the PJS-associated hamartoma and carcinoma samples (Table 1). These results suggest that *GNAS* is not involved in the pathogenesis of PJS-associated tumours.

Activating mutations in *GNAS* have been identified in various endocrine tumours [390], but its function in GI tumours is unclear. Recently, activating *GNAS* mutations were found in 83% of villous adenomas, but in only 3% of the more common tubulovillous adenomas [392]. Only 3% of colorectal adenocarcinomas contained a mutation in *GNAS*, consistent with previous findings (0.5-2%) [393,394]. In 8% of pancreatic intraepithelial neoplasms, the most common precursor of pancreatic ductal adenocarcinoma, the *GNAS* gene was mutated [395]. However, no *GNAS* mutations were detected in 32 pancreatic ductal adenocarcinomas [396]. Notably, in 41% of 118 intraepithelial papillary mucinous neoplasms, another common form of pancreatic carcinomas, *GNAS* mutations were observed [396].

These data suggest that GNAS could be involved in the pathogenesis of specific subtypes of GI carcinomas or their precursor lesions. In our study, we did not detect any *GNAS* mutation in GI hamartomas and carcinomas of PJS patients. Although hamartomas are not considered premalignant lesions of GI carcinomas in PJS, and should therefore as yet be regarded as two distinct entities of this syndrome, our results suggest that *GNAS* is not involved in GI tumourigenesis in PJS.

Table 1. *GNAS (codon 201) mutation analysis in gastrointestinal hamartomas and carcinomas of PJS patients.*

Tissue	Origin	*LKB1* germline mutation	LKB1	*GNAS* mutation (codon 201)
Hamartomas	Stomach	c.290+1G>A	Splice variant	no
	Small bowel	c.156_157dupGG	Frameshift	no
	Duodenum	c.580G>A	Asp194Asn	no
	Colon	c.468C>G	Tyr156Stop	no
	Colon	c.298C>T	Gln100Stop	no
	Rectum	c.298C>T	Gln100Stop	no
Adenocarcinomas	Stomach	c.580G>A	Asp194Asn	no
	Stomach[1]	c.910delC	Frameshift	no
	Colon	Unknown	Unknown	no
	Colon	c.464+1dupG	Frameshift	no
	Colon	Deletion exon 1	Unknown	no
	Colon[2]	c.468C>G	Tyr156Stop	no
	Pancreas[3]	c.582C>A	Asp194Glu	no
	Pancreas	Unknown	Unknown	no
	Pancreas	c.991dupC	Frameshift	no
	Distal bile duct	Unknown	Unknown	no
	Distal bile duct	c.156_157dupGG	Frameshift	no
	Ampulla Vateri	c.291-2A>G	Splice variant	no

[1]*Carcinoma in situ*

[2]*Adenocarcinoma derived from a villous adenoma*

[3]*Acinar cell type*

Chapter 6

Small bowel endoscopy and Peutz-Jeghers syndrome

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ABSTRACT

Peutz-Jeghers syndrome (PJS) is a rare, autosomal dominant inherited disease. It is clinically characterized by the development of gastrointestinal hamartomas, mainly located in the small bowel. These hamartomas are prone to complications such as intussusceptions, abdominal complaints and anaemia. Furthermore, patients are at increased risk for developing small bowel cancer. Therefore, regular surveillance of the small bowel is indicated. However, the optimal strategy for surveillance has not been determined yet. This review gives an overview of the different techniques that have been described to examine the small bowel of PJS patients. First, a number of radiologic and endoscopic imaging modalities with diagnostic value are discussed. Secondly, recently developed advanced endoscopy techniques are described that can serve both as a diagnostic and therapeutic tool in the surveillance of the small bowel. Finally, a recommendation is given how to apply these individual techniques for small bowel surveillance in a step-up approach.

INTRODUCTION

Peutz-Jeghers syndrome (PJS) is an autosomal dominant inherited disorder, characterized by gastrointestinal (GI) polyposis and mucocutaneous pigmentations. The syndrome is rare and incidence has been estimated between 1:8,500 and 1:200,000 births [397]. PJS is caused by germline mutations in the serine threonine kinase 11 tumour suppressor gene (*STK11* or *LKB1* gene), located on chromosome 19p13.3 [10,11].

The predominant clinical feature of PJS is GI polyposis. PJS polyps are often pedunculated and have typical histological features, consisting of a branched, tree-like smooth muscle core covered with normal epithelium (see chapter 1, Figure 4, page) [21]. They are referred to as hamartomas. Hamartomas can develop throughout the GI tract, but are mainly located in the small intestine, usually in the jejunum [19,20]. They may cause complaints of abdominal pain, blood loss, or acute intestinal obstruction, in particular resulting from intussusception of a small bowel segment carrying a large hamartoma (≥15mm) [20]. The risk of intussusception before reaching the age of 20 years up to 50% [20].

Apart from GI polyposis, PJS is also recognized as a cancer predisposition syndrome. Patients carry a high risk for the development of both gastrointestinal and extra-intestinal malignancies already at a young age [24,380]. For GI cancer, lifetime cumulative cancer risks of 38-66% have been reported [23]. For small bowel cancer in particular, a lifetime cumulative risk of 13%, and a highly increased relative risk compared to the general population (RR 520) has been described [380]. It should be pointed out however that these estimates are derived from one meta-analysis and no prospective studies are available to confirm this.

At present, the mechanism of carcinogenesis in PJS patients remains unknown. With regard to cancer development in the GI tract, there is controversy whether the malignancies originate from pre-existing hamartomas or from coexisting adenomas or normal mucosa. Although malignant transformation is found occasionally in PJS polyps and a hamartoma-adenoma-carcinoma sequence has been described [112,120], hamartomas are currently not considered precancerous lesions.

The initial main purpose of surveillance is prevention of small bowel obstruction and intussusception by removal of hamartomas of significant size (≥15 mm). With advancing age, this focus may shift to the early detection of small bowel cancer or precancerous lesions; however, the preventive effect of surveillance on development of small bowel cancer remains to be proven. Up till now, only one recently published paper described the yield of long-term GI surveillance in PJS patients [111]. In this retrospective study concerning 51 patients no luminal cancers were diagnosed during a median follow-up period of 10 years.

Currently, small bowel surveillance is recommended in patients with PJS every 2-3 years from the age of 8-10 years. However, because of its length and tortuous course, visualization of the small bowel is technically challenging and the optimal strategy for surveillance has not been determined yet. This review gives an overview of the different techniques that have been described to examine the small bowel of PJS patients. First, a number of radiologic and endoscopic imaging modalities with diagnostic value are discussed. In the second part, recently developed advanced endoscopy techniques are described that can serve both as a diagnostic and therapeutic tool in the surveillance of the small bowel.

IMAGING OF THE SMALL BOWEL

Long before any enteroscopy technique was available, the small bowel was visualized by radiological techniques. *Small bowel follow-through (SBFT)* has been the classic diagnostic tool for the assessment of small bowel polyps. However, studies have revealed that the sensitivity of SBFT for the detection of small bowel tumours is low (33%-61%) [398,399]. *Enteroclysis* is far more sensitive than SBFT for the detection of small bowel lesions [399]. Enteroclysis is a double-contrast radiographic study that is performed by passing a small tube into the proximal small intestine, followed by fast infusion of both barium and methylcellulose. The fluid infusion causes distension of the bowel lumen and improves the detection of mucosal pathologies. In a retrospective study, results of SBFT were abnormal in 11 of 18 patients while enteroclysis showed abnormalities in 19 of 20 patients [399]. However, the placement of the naso-enteric tube for contrast application can be burdensome. *Enterography* is a similar technique as enteroclysis, but the contrast fluid is ingested by the patient, thereby obviating naso-enteric intubation. However, distension of especially the proximal small bowel with enterography is more cumbersome in comparison with enteroclysis, thereby reducing the detection rate of proximally located lesions. Apart from the fact that both conventional enteroclysis and enterography are associated with significant exposure to ionizing radiation, even to a greater extent than with SBFT, overlapping bowel loops on a two-dimensional X-ray image may interfere with the distinctive capacity of these techniques to detect mucosal lesions. To overcome this problem, enterography and enteroclysis have been combined with cross-sectional imaging by means of computed tomography (CT) or magnetic resonance imaging (MRI). These techniques offer multiplanar imaging and transmural visualization and provide additional information of extraluminal structures. Although CT enteroclysis has high sensitivity and specificity rates for the detection of diverse small bowel pathology, including polyps [400], this technique is not advisable as surveillance tool due to high exposure to ionizing agents.

MR enterography and MR enteroclysis

The major advantage of using MRI is the absence of radiation exposure, except for very limited exposure for positioning the naso-enteric tube at Treitz in case of enteroclysis. High sensitivity (86%) and specificity (98%) rates are reported with MR enteroclysis for the identification of small bowel neoplasms, even for lesions as small as 8 mm [401,402]. Although no firm data are available concerning the diagnostic yield of MR enteroclysis in PJS patients, the use of MR enterography as surveillance method for PJS has been studied [403,404]. In a British prospective study of 19 patients, large PJS polyps (>15 mm) – even those within incompletely distended segments – were visible on MR enterography [403]. The authors postulate that poor luminal distension could reduce the sensitivity of MR enterography for smaller polyps (≤15 mm). However, as only large sized polyps determine further therapeutic strategies in PJS, this may be of less clinical relevance. In another small retrospective study, a 73% concordance between MR enterography and enteroscopy (i.e. double balloon enteroscopy, laparoscopic endoscopy or surgery) for polyps <15 mm was found, and a 93% concordance for polyps >15 mm [404]. The authors suggest the use of MR enterography as a yearly surveillance tool in PJS patients.

Video capsule endoscopy

With video capsule endoscopy (VCE) a pill-size capsule is swallowed and passes passively through the gastrointestinal tract via peristalsis. In an ambulatory setting, the device makes images of the mucosa without the use of ionizing radiation. Visualization of the entire small bowel is obtained in approximately 80% of patients [405] The major potential risk of this technique is capsule retention within a postsurgical stricture, adhesion, or by a large polyp occluding the small bowel lumen. However, this is a rare complication with a reported frequency of 1%-2% [406,407].

The diagnostic value of VCE has been studied in PJS patients (Table 1). In general it is safe, well tolerated and valuable for the detection of polyps in the small bowel. Although VCE is able to detect polyps smaller than 5 mm [408,409], it cannot accurately determine the location and the exact size of the polyps. Furthermore, VCE is not able to accurately visualize the proximal region of the small intestine, most probably due to rapid capsule transit and relatively poor luminal distension at this level. Moreover, false-negative results of VCE are frequently reported and there is increasing evidence that even large lesions may be missed by VCE [410,411]. A Portuguese study reported 20% (5/26) of polyps larger than 11 mm missed by VCE in 14 PJS patients [410]. Another case report described a PJS patient with a 37-mm polyp in the proximal ileum, not detected by VCE [411].

The use of VCE has also been assessed in children with PJS [412,413]. It is considered a safe and sensitive method for small bowel polyp surveillance. Younger children may have difficulty swallowing the capsule which can be overcome by placing the capsule in the stomach.

Small bowel endoscopy techniques

All the above mentioned imaging techniques share the disadvantage of only having diagnostic value. When large polyps (≥15 mm) are detected, intervention is required to remove these polyps. Various techniques have been described for small intestinal polypectomy in PJS patients. For a long time, push enteroscopy (PE) was the most frequently used non-surgical technique. It uses a long endoscope to reach the small bowel beyond the ligament of Treitz by fairly standard endoscopic techniques. Although several studies have described successful small bowel investigation and polypectomy of hamartomas with PE in PJS [421-423], the use of the push method is limited. Deep insertion of the enteroscope is hampered by the tortuous small intestine, in which the applied force is inefficiently transferred to the distal part of the enteroscope. The depth of insertion is at most 160 cm beyond the ligament of Treitz while small bowel length can range from 2 to 6.5 metres [424,425]. For surveillance in PJS patients, examination of the entire small intestine is desirable. To achieve this, in 1985 a combined endoscopic-surgical approach in 5 PJS patients was described [426]. During open laparotomy, passage of a scope was assisted by the surgeon. This approach allows direct visualization of the whole length of the small bowel and removal of hamartomas at the same time. Though, the investigation is invasive. It requires general anaesthesia and laparotomy with its associated risks, and the possibility of adhesion formation. Although some improvement has been made with laparoscopic assistance for insertion of the enteroscope, this still requires general anaesthesia and both a surgeon and an endoscopist.

In the last decade, several advanced endoscopic techniques have been developed that allow visualization of the entire small bowel and therapeutic interventions without the need for surgery. In the following section, two of these techniques are described; balloon assisted enteroscopy and spiral enteroscopy.

Table 1. *Overview of studies reporting the use of video capsule endoscopy in Peutz-Jeghers syndrome patients.*

Ref.	Patient characteristics	PJS patients	Transit time (minutes)	Complete entero scopy	Outcome	Remarks
408	N=20 16-54 yrs 10 patients history of abdominal surgery	N=4 A: 38-yrs old male; B: 40-yrs old male; C: 16-yrs old male; D: 22-yrs old male. 2/4 patients history of abdominal surgery.	NA	NA	VCE: 448 polyps ranging 1-30 mm in 8 patients. MRI: 24 polyps >5 mm in 4 PJS patients. Polyps >15 mm were detected with both techniques, whereas smaller polyps were seen much more often with VCE. Location and exact sizes more accurate by MRI.	Comparison of VCE and MRI.
410	N=20	N=14, n=6 first-degree relatives. Median age 33.8 yrs (range 18-61) 8 patients previous small bowel surgery.	33 (median 18, SD 27.3) to pylorus, 244.8 (median 260, SD 85) to ileocecal valve.	18 (90%)	VCE correctly identified all patients with large polyps, but had missed 20% of total number of large polyps. Counting of polyps cumbersome and imprecise. Capsule expelled within 24h, no complications, well tolerated.	
414	N=40 FAP and PJS patients	N=11 Median age 34 yrs (range 23-58). 10/11 history of abdominal surgery.	7.14 hours	9/11	In 10/11 patients polyps detected, superior to all other procedures.	VCE results were compared with OGD, PE, MRI enteroclysis and surgical specimen.
415	N=24 Mean age 35 ± 10 yrs 13 patients previous abdominal surgery	N=4 1 patient previous abdominal surgery.	Mean gastric transit 33 (range 1-287). Mean small bowel transit 233 (range 138-330). SBFT series: mean time 70 (range 60-90).	23/24	VCE detected small bowel polyps in 7/24 patients (29%). SBFT detected small bowel polyps in 3/24 patients (12%). All of these were also identified by VCE. In PJS patients SBFT and VCE identified the same proportion of polyps but VCE detected a larger number of lesions. No complications.	Comparison with SBFT.

FAP: familial adenomatous polyposis; h: hours; MRI: magnetic resonance imaging; NA: not available; OGD: oesophagogastroduodenoscopy; PE: push enteroscopy; PJS: Peutz-Jeghers syndrome; SBFT: small bowel follow-through; SD: standard deviation; VCE: video capsule endoscopy; yrs: years.

Table 1. *Overview of studies reporting the use of video capsule endoscopy in Peutz-Jeghers syndrome patients.*

Ref.	Patient characteristics	PJS patients	Transit time (minutes)	Complete entero scopy	Outcome	Remarks
409	N=19 FAP and PJS patients. Mean age 43 yrs. All previous abdominal surgery.	N=4 A: 30-yrs old; B: 55-yrs old; C: 29-yrs old; D: 32- yrs old.	NA	3/4 (75%)	3/4 (75%) PJS patients had small bowel polyps, 0.2->30 mm. Small bowel radiography underestimated the polyp burden. No complications.	
416	N=22 Mean age 41 yrs (range 19-55)	N=1 19-yrs old male.	NA	100%	129 polyps in 13 patients. 1-26 polyps/patient. Diagnostic yield 59%. Well tolerated by all patients.	
417	N=19	N=19 Mean age 37 yrs (range 20-60).	Mean small bowel transit to caecum 273.	7/19	10 patients had SBFT. VCE: median 4 (0-18) polyps ≥10 mm; SBFT: median 1 (0-4) polyp ≥10 mm. P-0.008 VCE less reliable for accurate sizing of polyps at the limit of 10 mm.	Comparison of VCE with SBFT
412	N=28 Median age 12.59 yrs (9.4-15.9)	N=3	NA	2/3 VCEs positive.	1 PJS patient unable to swallow the capsule. 2 PJS patients had small bowel polyps, not detected at SBFT. SBFT: 26% sensitivity compared with VCE. Endoscopic investigations: 43.4% sensitivity compared with VCE.	Children
411	N=5	N=1 32-yrs old female.	NA	NA	Carpeting of insignificant polyps throughout the small bowel; 2 largest polyps in ileum were 5 and 10 mm in maximal diameter, respectively. MRI enteroclysis showed a 37 mm polyp in proximal ileum, missed by VCE.	

FAP: familial adenomatous polyposis; h: hours; MRI: magnetic resonance imaging; NA: not available; OGD: oesophagogastroduodenoscopy; PE: push enteroscopy; PJS: Peutz-Jeghers syndrome; SBFT: small bowel follow-through; SD: standard deviation; VCE: video capsule endoscopy; yrs: years.

Table 1. *Overview of studies reporting the use of video capsule endoscopy in Peutz-Jeghers syndrome patients.*

Ref.	Patient characteristics	PJS patients	Transit time (minutes)	Complete entero scopy	Outcome	Remarks
413	N=11	N=11 Mean age 11.2 yrs (6.0-16.1). 3 previous abdominal surgery.	226	9/11 (82%)	No difference for >10mm polyp detection, more <10 mm polyps identified by VCE than enterography. VCE more comfortable and preferred. No complications.	Children, comparison with enterography.
418	N=19 Age 16-25 yrs	N=4 A: 16-yrs old female; B: 25-yrs old female; C: 22-yrs old female; D: 23-yrs old female.	NA	23/28 (82%) complete. 27/28 excretion.	In all PJS patients spontaneous excretion of capsule and detection of polyps. VCE missed two polyps (20mm) in proximal jejunum of 1 PJS patient.	
403	N=20	N=20 Median age 39.6 yrs (range 20.7-66.8) 16 patients previous abdominal surgery.	NA	17/19 1 withdrawal after MR enterography.	Large polyps (>15 mm) were missed in 3 patients. No significant difference between two techniques for the detection of clinically relevant (> 10 mm) polyps. No complications.	Comparison of MR enterography versus VCE
419	N=18	N=18 14 patients with history of laparotomy	467 ± 64	34/38 (89%)	Failed ingestion (n=3, 7.9%)	Comparison of FE, VCE and DBE.
420	N=165 Mean age 45.9 ± 16.7 yrs (range 14-81)	N=7	NA	NA	Diagnostic yield 44.2%. Capsule retention in 1 patient.	

FAP: familial adenomatous polyposis; h: hours; MRI: magnetic resonance imaging; NA: not available; OGD: oesophagogastroduodenoscopy; PE: push enteroscopy; PJS: Peutz-Jeghers syndrome; SBFT: small bowel follow-through; SD: standard deviation; VCE: video capsule endoscopy; yrs: years.

Balloon-assisted enteroscopy

As a modification of the push method, Yamamoto described total enteroscopy with a double-balloon method [427]. This technique uses two balloons, one attached to the tip of the endoscope and another at the distal end of an overtube. The balloons are used to grip the intestinal wall so that the endoscope can be inserted without forming redundant loops in the small bowel. In a series of push and pull steps, the small bowel is pleated over the enteroscope. In comparison with PE, the double-balloon endoscope (DBE) can be advanced much further into the small intestine. A combined oral and anal approach enables visualization of the entire small bowel with a success rate of 40%-80% [428]. However, the procedure takes more time to perform than a conventional push enteroscopy. Dependent on the indication, DBE has a diagnostic yield of at least 60% for small bowel disease, including neoplastic lesions [429].

Ohmiya *et al.* were the first to describe the use of DBE for the diagnosis and treatment of PJS polyps in the small intestine [430]. In two patients, multiple polyps (10-60 mm) were detected in the jejunum and ileum and resected without subsequent bleeding or perforation. Since then, various studies have described a high diagnostic yield of DBE in PJS patients with successful polypectomy (Table 2).

In general, the complication rate after diagnostic DBE is estimated at 0.8% [445]; the most severe being acute pancreatitis with a risk of approximately 0.2% to 0.3% after oral approach [446,447]. As in conventional endoscopy, the risk of severe complications is higher in therapeutic enteroscopy (4.3%), mainly attributable to bleeding (3%). The reported complication risk of DBE in PJS patients differ widely (Table 2). Dutch authors described 29 diagnostic and therapeutic DBE procedures in 13 patients with PJS, with removal of multiple polyps of ≥ 10 mm [440]. No complications occurred during the procedures and during follow-up. However, two other studies, concerning 15 and 18 PJS patients respectively, report a complication rate of up to 6.8%, including acute pancreatitis (2.7%) [444] and post-polypectomy syndrome (5%) [419].

It should be noted that DBE might be challenging in patients with a history of abdominal surgery, due to altered anatomy and the presence of adhesions. Furthermore, it is speculated that, apart from the usual risks associated with DBE, PJS patients might be more prone to perforation after polypectomy. As the serosa invaginates the stalk of the polyp, removing a polyp near its base might cause a bowel perforation. However, this is only based on expert opinion and no studies are available to confirm this theory.

One study assessed the feasibility and utility of DBE in the management of small bowel diseases in children (median age 12.9 years), including 5 patients known with PJS and small bowel polyps [438]. In two of the 5 PJS patients complete enteroscopy was achieved and polypectomy was successful in all. No enteroscopy-related complications occurred; however, one patient who underwent a laparoscopic-assisted DBE developed a pelvic abscess without intestinal perforation, most likely as complication of the laparoscopy.

Apart from the double-balloon method, a similar system exists with a single balloon. In contrast to the DBE system, no balloon is attached to the tip of the overtube but stable positioning in the small bowel is achieved by angling the tip of the endoscope or by so-called power suction. A recent study showed that DBE and single-balloon enteroscopy (SBE) have a comparable performance and diagnostic yield for the evaluation of the small bowel [448].

One study described the experience with SBE in PJS patients [449]. In 7 patients, multiple polyps (4-20 polyps; 0.5-40 mm in diameter) were detected and removed or biopsied. No major complications occurred. According to the authors, the handling of SBE seems to be much easier compared to that of DBE.

Table 2. *Overview of studies reporting the use of double-balloon enteroscopy in Peutz-Jeghers syndrome patients.*

Ref.	Patient characteristics	PJS patients	No. of procedures	Duration of procedure (minutes)	Insertion depth (cm)	Polypectomie	Complications	Remarks
430	N=2	N=2 A: 29-yrs old male; B: 50-yrs old female.	A: oral and anal approach; B: oral and 2x anal approach.	Mean 148		19 polyps resected in jejunum and ileum, 10-60 mm in size.	Post-polypectomy syndrome after anal approach (patient B).	
431	N=22	N=2 A: 42-yrs old female; B: 21-yrs old male.	22 17 oral approach 5 anal approach A: anal approach; B: oral approach.	71 (25-112) A: 54 B: 15	NA	NA A: 23 polyps detected; B: no significant findings	None.	Comparison of VCE and DBE.
432	N=3	N=3 A: 33-yrs old male; B: 53-yrs old male; C: 29-yrs old male.	3	A: 4 hours B: 3 hours C: 1 hour	Complete enteroscopy in all procedures.	A: 3 polyps, 0.5-50 mm; B: 6 polyps, max. 40 mm; C: 4 polyps, 10-30 mm.	Post-operative ileus (patient A and B).	Laparoscopy-assisted DBE.
433	N=44 Mean age 53.5 yrs (range 21-89).	N=2	44	90 ± 40	240 ± 50 (range 40-500) during a single approach. The procedure was finished after finding the lesion.	Diagnostic yield 75% (33/44). Therapeutic yield 63,6% (28/44).	No major. Minor (sore throat) 9.1% (4/44).	No interventions in PJS.

DBE: double-balloon enterosocpy; FE: fluoroscopic enteroclysis; NA: not available; IOE: intraoperative enteroscopy; PJS: Peutz-Jeghers syndrome; SBE: single-balloon enteroscopy; SD: standard deviation; VCE: video capsule endoscopy; yrs: years.

Table 2. *Overview of studies reporting the use of double-balloon enteroscopy in Peutz-Jeghers syndrome patients.*

Ref.	Patient characteristics	PJS patients	No. of procedures	Duration of procedure (minutes)	Insertion depth (cm)	Polypectomie	Complications	Remarks
434	N=16	N=16 17-65 yrs. 9 history of abdominal surgery.	47 39 oral approach 8 anal approach 15 diagnostic 32 therapeutic	80 (44-130) diagnostic 75 (30-127) therapeutic	153 (50-390) oral approach; 135 (70-170) anal approach.	47 polypectomies; polyps 15-50 mm in size.	Bleeding (n=2), perforation (n=1), all associated with polypectomy of polyps > 30 mm.	
435	N=178 Mean age 59 yrs (12-93).	N=23 PJS or familial polyposis patients.	225	50 (20-150)	180 (5-650). 240 (20-650) oral approach; 65 (10-150) anal approach.	Therapeutic intervention in 64% (115/178).	NA	Only abstract available.
436	N=47 51.6 ± 19.5 yrs (11-85).	N=4 polyposis patients, 3/4 PJS patients. Mean age 42.3 yrs (SD 19.1).	52 35 oral approach 7 anal approach 5 bidirectional Polyposis patients: 7 6 oral 1 both	90 (70-180) from oral route 100 (30-140) from anal route	Average 213 (50-480, SD 114). Polyposis patients: 188 (SD 107).	Polypectomie in 2/3 PJS patients.	No severe complications.	
437	N=29	N=3 A: 28-yrs old female; B: 54-yrs old male; C: 65-yrs old male.	40 A: 3 oral approaches; B: oral and anal approach; C: 4 oral approaches.	75 (25-115)	Mean 300 (30-540).	10 per session (range 3-32) in PJS patients.	3%; bleeding after polypectomy (patient B).	One failed DBE in PJS because of multiple adhesions.

DBE: double-balloon enterosocpy; FE: fluoroscopic enteroclysis; NA: not available; IOE: intraoperative enteroscopy; PJS: Peutz-Jeghers syndrome; SBE: single-balloon enteroscopy; SD: standard deviation; VCE: video capsule endoscopy; yrs: years.

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Ref.	Patient characteristics	PJS patients	No. of procedures	Duration of procedure (minutes)	Insertion depth (cm)	Polypectomie	Complications	Remarks
438	N=14 Median age 12.9 yrs (8.1-16.7).	N=5 A: 13-yrs old male; B: 12-yrs old male; C: 16-yrs old male; D: 11-yrs old male; E: 9-yrs old male.	A: oral and anal approach; B: oral approach; C: intra-operative; D: oral approach; E: oral approach.	118 (95-195)	A: complete; B: 320 cm post-pylorus distance; C: complete; D: 250 cm post-pylorus distance; E: incomplete.	Successful in all 5 PJS patients.	None.	Children
439	N=10	N=10 Mean age 25 yrs (12-48)	14, oral approach	113 (20-270)	NA	205 (1-37 per session), maximum diameter 60 mm.	No serious complications, one case of acute substantial arterial bleeding after polypectomy.	Comparison of DBE and IOE.
440	N=13	N=13 Median age 31 yrs (10-51) 11 history of abdominal surgery	29 (1-6/patient) 26 oral approach, 3 anal approach	70 ± 15	Mean 230 (140-320) oral approach; mean 145 (140-150) distal approach.	79 polyps resected, 10-50 mm in size.	None.	

DBE: double-balloon enterosocpy; FE: fluoroscopic enteroclysis; NA: not available; IOE: intraoperative enteroscopy; PJS: Peutz-Jeghers syndrome; SBE: single-balloon enteroscopy; SD: standard deviation; VCE: video capsule endoscopy; yrs: years.

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Ref.	Patient characteristics	PJS patients	No. of procedures	Duration of procedure (minutes)	Insertion depth (cm)	Polypectomie	Complications	Remarks
441	N=139 51.1 ± 18.6 yrs.	N=8	150	NA	213 (70-480)	Yes	NA	Only abstract available. Comparison with previous VCE in 27 patients. Concordance of findings 51.8% (14/27), in 2 cases DBE provided significantly new information including 1 malignancy.
419	N=18	N=18 14 patients with history of laparotomy	80 42 oral approach 38 anal approach	100 ± 46	Complete enteroscopy. In 13/25 (52%) patients.	387 at 71 DBE examinations in 16 patients (265 >10 mm).	Perforation (n=1, 1.3%); pancreatitis (n=1, 1.3%); post-polypectomy syndrome (n=4, 5.0%).	Comparison of FE, VCE and DBE.
442	N=6	N=6 19.5 ± 6.25 yrs.	17 12 DBE, 5 SBE 13 oral approach, 4 anal approach	96.2 ± 19.6	Complete small bowel examination in all patients in 1 or 2 steps.	10-20 polyps removed per patient, 5-60 mm in diameter.	None during follow-up of 32 ± 17.5 months.	

DBE: double-balloon enterosocpy; FE: fluoroscopic enteroclysis; NA: not available; IOE: intraoperative enteroscopy; PJS: Peutz-Jeghers syndrome; SBE: single-balloon enteroscopy; SD: standard deviation; VCE: video capsule endoscopy; yrs: years.

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443	N=1765 Mean age 64 ± 17 yrs (6-94).	N=44	2245	75 ± 30 (range 5-360)	Complete enteroscopy in 93/436 (21%) patients with oral and anal approach; in 31/1052 (2%) patients with oral approach.	137 polypectomies in 529 patients.	Overall 1.2%. 2 perforations after polypectomy in PJS patients.	
444	N=15	N=15 Mean age 34 ± 15.8 years (range 11-63)	88, mean no. of sessions per patient 3.0 ± 1.0	108.5 ± 39.4	Complete enteroscopy in 7/10 (70%) patients in whom total enteroscopy was attempted; in 5/8 patients with history of laparotomy; in 2/2 patients without history of laparotomy.	341 polyps resected in 71 procedures.	Total 6.8%; pancreatitis 2.7%; delayed bleeding 2.7%; perforation 1.4%.	

DBE: double-balloon enterosocpy; FE: fluoroscopic enteroclysis; NA: not available; IOE: intraoperative enteroscopy; PJS: Peutz-Jeghers syndrome; SBE: single-balloon enteroscopy; SD: standard deviation; VCE: video capsule endoscopy; yrs: years.

Spiral enteroscopy

Recently, spiral enteroscopy (SE) was introduced [450]. This method uses a special overtube with raised helices at the distal end. Clockwise rotations pleat the small bowel over the overtube. To perform interventions or take biopsies, the rotation of the overtube is discontinued and instruments are inserted through the accessory channel of the enteroscope. Because of the novelty of the technique, there is little experience with spiral enteroscopy in PJS patients yet. In 2010, the efficacy of the technique was described in a study of 11 patients, including 1 PJS patient [451]. This 35-year-old woman had previously undergone a small bowel resection because of an intussusception. During the procedure many small polyps and several large pedunculated polyps (20-30 mm) in the jejunum and ileum were detected and removed. The duration of the procedure was 30 minutes and the insertion depth was 240 cm. No major complications occurred. More recently, a case of a small bowel perforation after therapeutic SE in a PJS patient was reported [452]. Given this scarce experience, further prospective studies with SE are needed for a reliable assessment of the use of this technique in PJS patients.

SUMMARY

Small bowel surveillance is indicated every 2-3 years for patients with PJS from the age of 8-10 years. Removal of large small bowel hamartomas reduces the need for elective and emergency laparotomy. There are no firm data yet that surveillance of the small bowel reduces the risk of small bowel cancer.

Various radiologic and endoscopic techniques are available to examine the small intestine (Table 3). However, a golden standard is lacking. Currently, it is recommended to use a step-up approach for surveillance, making complementary use of the different techniques. As a first step, MRI enteroclysis is a sensitive method for visualisation of the small bowel lumen and the detection of polyps. In children, VCE might be a feasible procedure for the first step of surveillance, although large polyps may be missed.

When significant polyps are detected (>10-15 mm), balloon-assisted enteroscopy (BAE) should be the preferred method for polypectomy as a second step. BAE could be considered the first surveillance step in patients known to have a high polyp load. In case a polyp is too large for safe removal with BAE or when a polyp cannot be reached with BAE, (laparoscopic) intraoperative enteroscopy could be considered for polypectomy or enterotomy. However, elective surgical interventions should be avoided in PJS patients because of the risk of adhesions.

For patient convenience, one investigation for both detection and removal of significant lesions is preferable. This may be feasible with double balloon enteroscopy, although it is not known yet whether the diagnostic yield of DBE for the detection of PJS hamartomas is sufficient to serve as a diagnostic tool alone. Future research should clarify the diagnostic value of DBE in PJS patients.

Table 3. *Pros and cons of different radiologic and endoscopic techniques for small bowel surveillance in Peutz-Jeghers syndrome.*

	Small bowel follow-through	**MRI enteroclysis / enterography**	**Video Capsule Endoscopy**	**Intraoperative Endoscopy**	**Push Enteroscopy**	**Balloon Assisted Enteroscopy**
Pros	- Fast procedure - Not invasive - No sedation required	- Not invasive - No radiation exposure - No sedation required	- Minimally invasive - No radiation exposure - No sedation required - On outpatient basis	- Single-procedure - Inspection and total clearance of the entire small bowel	- Moderately invasive - Biopsies and therapeutic interventions possible	- Moderately invasive - Biopsies and therapeutic interventions possible - Total enteroscopy possible
Cons	- Radiation exposure - No interventions possible - Low sensitivity	- No interventions possible - Size estimation and exact localization of polyps inaccurate - Placement of naso-enteric tube	- No interventions possible - Significant lesions may be missed, especially in the proximal small bowel - Size estimation and exact localization of polyps inaccurate - Caecum not always reached	-Laparotomy/laparoscopy required - General anesthesia required - Surgeon and gastroenterologist required	- Sedation required - Time consuming - Limited to duodenum and proximal jejunum - Burdensome	- Sedation required - Time consuming - Often ≥ 2 sessions required for total enteroscopy - Burdensome

Chapter 7

Pancreatic cancer risk in Peutz-Jeghers syndrome patients; a large cohort study and implications for surveillance

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ABSTRACT

Background: Although Peutz-Jeghers syndrome (PJS) is known to be associated with pancreatic cancer (PC), estimates of this risk differ widely. This hampers counselling of patients and implementation of surveillance strategies. We therefore aimed to determine the PC risk in a large cohort of Dutch PJS patients.

Methods: PJS was defined by diagnostic criteria recommended by the WHO, a proven *LKB1* mutation, or both. All patients with a presumptive diagnosis of pancreatic, ampullary or distal bile duct cancer were identified. Cases were reviewed clinically, radiologically and immunohistochemically. Cumulative PC risks were calculated by Kaplan-Meier analysis and relative risks by Poisson regression analysis.

Results: We included 144 PJS patients (49% male) from 61 families (5640 person years follow-up). Seven (5%) patients developed PC at a median age of 54 years. Four patients (3%) were diagnosed with distal bile duct (n=2) or ampullary cancer (n=2) at a median age of 55 years. The cumulative risk for PC was 26% (95% CI 4% to 47%) at age 70 years and relative risk was 76 (95% CI 36 to 160; $p<0.001$). The cumulative risk for pancreatico-biliary cancer was 32% (95% CI 11% to 52%) at age 70, with a relative risk of 96 (95% CI 53 to 174; $p<0.001$).

Conclusions: PJS patients have a highly increased risk for pancreatico-biliary cancer. Therefore, patients are eligible for surveillance within well-defined research programmes to establish the benefit of such surveillance.

INTRODUCTION

Despite the relative low incidence of pancreatic cancer (PC) (8-10 per 100 000 per year, with an approximate 1% life time risk in western populations [453]), PC is among the top five causes of cancer related deaths in both the USA and Europe [454,455]. The mean survival after diagnosis is less than 6 months and the overall 5-year survival is less than 5% [456]. This poor prognosis is mainly due to the late onset of symptoms and anatomic location of the disease. Consequently, less than 20% of all patients presents with localised disease and are therefore eligible for curative treatment. Unfortunately, this intended curative treatment proves only to be effective for the minority of patients with an overall 5-year survival after surgical resection of less than 10% [457]. Despite recent advantages in the field of surgery and oncology, this dismal prognosis has not significantly changed over the past decades [458].

Detection of precursor lesions or malignancies at an early asymptomatic stage by surveillance with endoscopic ultrasound (EUS) and/or MRI could offer a way to improve the prognosis [459]. In particular, when surveillance is directed towards populations of individuals that carry a high risk for developing PC, the potential health gain could be substantial.

One such high-risk population consists of patients with Peutz-Jeghers syndrome (PJS). PJS is an autosomal dominant inherited disorder, caused by germline mutations in the *LKB1* tumour suppressor gene (also known as *STK11*) [30]. It is characterised by gastrointestinal hamartomas and mucocutaneous pigmentations. Furthermore, patients with PJS are at risk for developing various types of cancer, including PC [24,115,380]. The actual risk of developing PC for PJS patients is currently unclear. Previous studies reported relative risks ranging from 0 to 132-fold increase and an average age of PC onset ranging from 41 to 60 years of age [115,380,460-462]. Consequently, this hampers counselling of PJS patients and implementation of surveillance strategies.

These disparate risk estimates were mainly derived from heterogeneous multicentre populations, small single centre cohort studies, and meta-analyses of these same studies. It is therefore key to perform such a study in a large homogenous population. In 2011, our research group reported on the high overall cancer risk in a unique, large pedigree based homogenous cohort of Dutch PJS patients with a substantial prospective period of follow-up [24]. For the present study, we performed a thorough re-evaluation of all reported cancers in the pancreatico-biliary region in this patient cohort, including 2 years of extended follow-up. Thus, we aimed to conclude the ongoing debate regarding the true PC risk in PJS, and to provide a more scientific rationale for the implementation of surveillance strategies.

MATERIALS AND METHODS

Peutz-Jeghers syndrome database

This nationwide cohort study was initiated by two Dutch academic hospitals. Between 1995 and July 2011, PJS patients throughout the Netherlands were included without selection for medical history. All patients had a definite diagnosis of PJS, defined by diagnostic criteria recommended by the WHO (see chapter 1, Box 1, page 12), a proven *LKB1* mutation, or both. Informed consent was obtained from all patients and the study was approved by the Institutional Review Board of both participating hospitals. Patients were followed prospectively between January 1995 and July 2011. Patient information at baseline and during follow-up was obtained by interview and chart review. Clinical data from the period before 1995 as well as data of deceased family members fulfilling the diagnostic criteria for PJS were collected retrospectively.

Case selection and data collection

PC cases were identified from the PJS database. PC was defined according to the most recent WHO classification of tumours of the digestive system [456]. In addition, patients with a diagnosis of distal bile duct cancer or ampullary cancer were included. Surveillance of the pancreas might also detect these malignancies and accurate distinction between these three tumours often proves difficult. From all selected cases, medical records were reviewed by two MDs (SEK and FH). The following data were collected: gender, date of birth, cancer diagnosis and death, mutation status and type of mutation, family history of PJS, and family history of PC. The recorded cancer characteristics included tumour type and origin, tumour invasion, data on confirmation (medical record or histology), and presentation (surveillance, accidentally or symptomatic). Radiological images were reviewed by an expert abdominal radiologist (NK). Available formalin-fixed and paraffin-embedded tissue was reviewed by two expert pathologists independently (KB and GJAO). Immunohistochemical staining for SMAD4, CDX2 and cytokeratins was performed to ascertain the diagnosis [456]. Eventually, all available information was re-assessed by an expert panel (KB, GJAO, NK, ED, EMHMV, MEvL and MJB) to determine a definite diagnosis of PC, distal bile duct cancer or ampullary cancer.

Statistical analysis

Data were analysed using the SPSS V.17.0 statistical software for Windows (IBM, Somers, New York, USA). All risks were calculated for two groups: (1) PC cases; (2) cases of cancer in the pancreatico-biliary region, including cases with PC, distal bile duct cancer or ampullary cancer. Cumulative risks were estimated as a function of time using the Kaplan-Meier method and the Cox regression model. For these cumulative risk analyses, all subjects of the cohort were included. For relative cancer risk calculation, the tumour specific cancer incidence observed in our study population was compared to the age specific and gender specific incidence rates of the Dutch general population from 1960 to 2011 by Poisson regression analysis (log linear analysis) using the package R [463]. Subjects were studied with respect to their risk of developing cancer from birth until the date of death, date of last contact or the closing date of the study (1 July 2011). Sociodemographic data and incidence rates of the Dutch general population were derived from the Eindhoven Cancer Registry (1960-2009). These data are representative for the Netherlands. Incidence rates for 2009 were assumed to be representative for 2010 and 2011.

RESULTS

Study population

In total, 144 PJS patients from 61 families were included in the cohort with a total of 5640 person-years of follow-up (including 1757 person-years of prospective follow-up). Forty-nine per cent were male (3050 person-years). At the closing date of the study six patients had been lost to follow-up (4%), and 48 (33%) had died at a median age of 46 years (IQR 32-58 years); the median age of the 90 patients still alive (63%) was 37 years (IQR 21-52 years). The baseline characteristics of the cohort are shown in Table 1.

Table 1. *Baseline characteristics of the Dutch Peutz-Jeghers syndrome cohort.*

Total	**144**
Gender	
Male	70 (49%)
Female	74 (51%)
Families	61
Family history	
Familial PJS	109 (76%)
Sporadic	24 (17%)
Family history unknown	11 (7%)
Fulfilling WHO criteria[1]	142 (99%)
DNA mutation analysis	92 (64%)
LKB1 mutation carrier	82/92 (89%)
Deceased	48 (33%)
Median age at death	46 years (IQR 32-58 years)
Lost to follow up	6 (4%)
Cancer	48 (33%)
Median age at diagnosis of first cancer	46 years (IQR 35-55 years)
2 primary cancers	8

IQR: interquartile range; PJS: Peutz-Jeghers syndrome; WHO: World Health Organisation.
[1]Two patients not fulfilling the WHO-criteria carry a proven LKB1 germline mutation.

Pancreatic cancer cases

The case selection process is shown in Figure 1. During follow-up, seven (4.9%) PJS patients from seven families developed PC, six male and one female. None of the cases was detected within the framework of a PC screening/surveillance programme. Adenocarcinoma of the pancreas was found in 6 patients and acinar cell carcinoma in one. Six cases were confirmed by revision of histology, and no histological material was available for the seventh case. Median age at diagnosis was 54 years (IQR 37-62 years). Six patients presented with symptoms. In one patient a tumour mass was incidentally found during a laparotomy because of small bowel polyps. None of the patients could be treated curatively. Median survival of patients after diagnosis was 6 months (IQR 4-17 months). Mutation analysis for the *LKB1* gene was performed in five patients, detecting a pathogenic mutation in four of them. For the other two cases, a pathogenic mutation in the *LKB1* gene was detected in affected family members, but mutation analysis was not performed in the PC patients. Two of the patients had been treated curatively for another malignancy (colorectal cancer and liposarcoma) prior to the development of PC. Individual patient characteristics are shown in Table 2A.

Ampullary cancer and distal bile duct cancer cases

In addition to the patients with PC, distal bile duct cancer was diagnosed in a male patient at the age of 57 and in a female patient at the age of 73 years. Both patients only underwent palliative treatment; survival after diagnosis was 3 and 8 months, respectively. Furthermore,

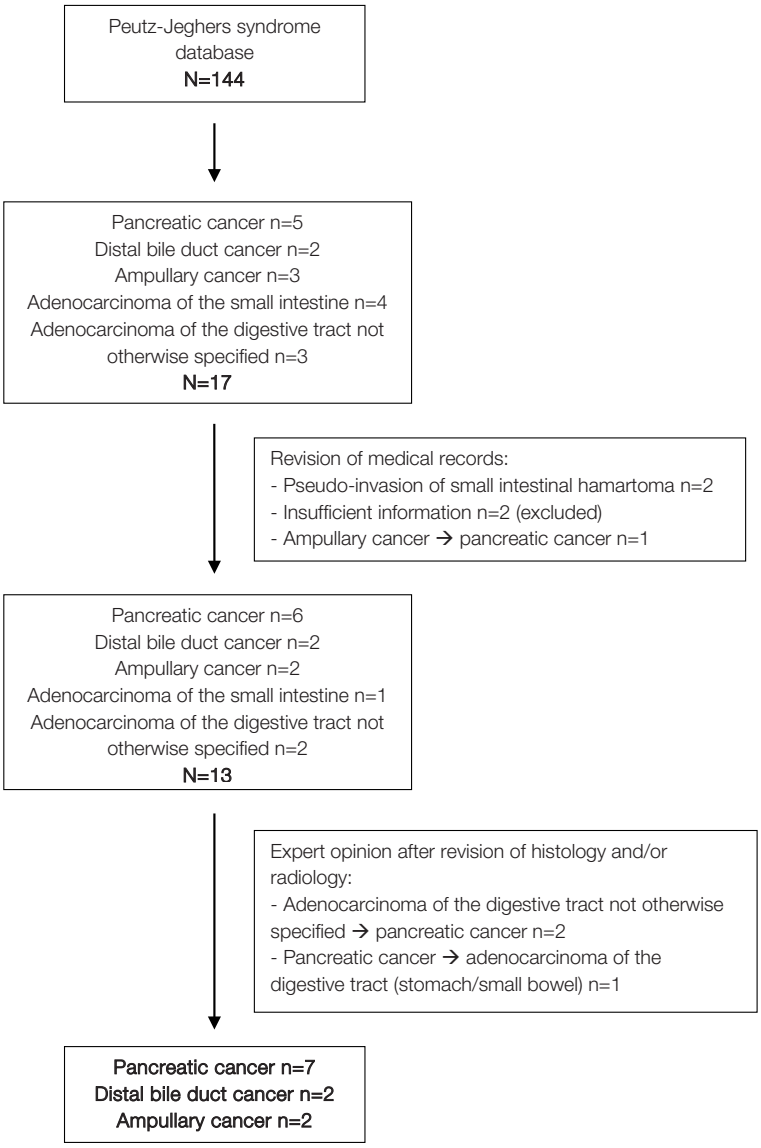

Figure 1. *Case selection*

ampullary cancer/cancer involving the ampulla was detected in two male patients at the age of 41 and 53 years. Both patients underwent a pylorus preserving pancreaticoduodenectomy as curative treatment. One patient died of metastasized disease 5 years after diagnosis; the other patient is still alive 11 years after diagnosis. Patient characteristics are shown in *Table 2B*. The median age at diagnosis for the group of patients with pancreatic, distal bile duct or ampullary cancer (n=11) was 54 years (IQR 42-62).

Table 2. *Characteristics of Peutz-Jeghers syndrome patients with pancreatic cancer (A) and with distal bile duct cancer and ampullary cancer (B).*

A	Germline *LKB1* mutation in patient (germline *LKB1* mutation in family)	Age at diagnosis (year of diagnosis)	Age at death	Type of cancer	Confirmation	Location tumour	Distant metastases	Other malignancy in medical history (year of diagnosis)
1.	+ (+)[1]	35 (1998)	35	Adenocarcinoma	HI	Head	Yes	No
2.	NT (c.370A>T, p.Lys124X)	57 (1994)[2]	58	Adenocarcinoma	HI	Head	Yes	Unknown
3.	NT (c.468C>G, p.Tyr156X)	66 (1998)	68	Adenocarcinoma	HI	Head	Yes	Colon cancer pT2NxMx (1996)
4.	c.991dupC, p.Arg331fs	36 (1997)	36	Adenocarcinoma	HI	Head	Yes	No
5.	c.582C>A, p.Asp194Glu	45 (2008)	46	Acinar cell carcinoma	HI	Tail	Yes	Liposarcoma (1992)
6.	Deletion exon 6, 7 and 8	54 (2004)	54	Adenocarcinoma	MR	Head	Yes	No
7.	- (-)	62 (2005)	62	Adenocarcinoma	HI	Head	Yes	No
B								
1.	c.156_157dupGG, p.Asp53fs	57 (2006)	57	Bile duct cancer	HI	Distal bile duct	Yes	No
2.	NT (unknown)	73 (2008)	74	Bile duct cancer	HI	Distal bile duct	Yes	No
3.	c.291-2A>G, p.98_155del	53 (2006)	58	Ampullary NET	HI	Ampulla	Yes	Non-Hodgkin Lymphoma grade 3 (2001)
4.	c.735-1G>A, p.?	41 (2000)	-	Dysplastic hamartoma near the ampulla with invasive growth in the ampulla	HI	Ampulla	No	No

F: female; HI: histology; M: male; MR: medical record; NET: neuro-endocrine tumour; NT: not tested; +: proven germline LKB1 mutation; -: tested but no germline mutation detected.

[1]Specific LKB1 germline mutation unknown

[2]Occurred in retrospective follow-up period

Cumulative cancer risk

Pancreatic cancer (n=7)

The Kaplan-Meier estimate for the cumulative PC risk was 2.4% (SE 1.7%; 95% CI -0.9% to 5.7%) at age 40; 3.9% (SE 2.2%; 95% CI -0.4% to 8.2%) at age 50; 11.1% (SE 5.3%; 95% CI 0.7% to 21.5%) at age 60; and 25.6% (SE 10.8%; 95% CI 4.4% to 46.8%) at age 70 (Figure 2, left). There was no significant difference in risk between males and females (p = 0.272).

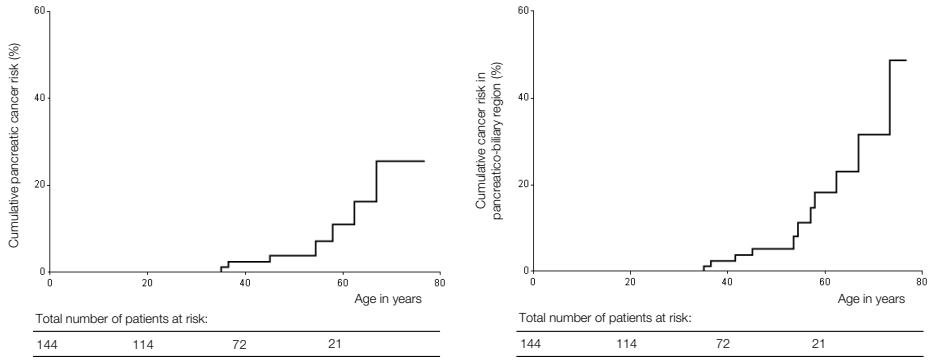

Figure 2. *Cumulative cancer risk in Peutz-Jeghers syndrome patients according to age.*

Pancreatic, ampullary or distal bile duct cancer (n=11)

For pancreatic, distal bile duct or ampullary cancer the cumulative risk was 2.4% (SE 1.7%; 95% CI -0.9% to 5.7%) at age 40; 5.2% (SE 2.6%; 95% CI 0.1% to 10.3%) at age 50; 18.2% (SE 6.5%; 95% CI 5.5% to 30.9%) at age 60; and 31.6% (SE 10.6%; 95% CI 10.8% to 52.4%) at age 70 (Figure 2, right). There was no significant difference in risk for these malignancies between males and females (p = 0.248)

Relative cancer risk

From 1960, 131 patients contributed to 4430 person-years at risk (males 2259 person-years, females 2171 person-years). Poisson regression analysis showed that the relative risk for PC (HR 76.2, 95% CI 36.3 to 160.0) as well as for pancreatic, ampullary or distal bile duct cancer (HR 95.8, 95% CI 52.8 to 173.7) was significantly higher in PJS patients than in the general population (p<0.001).

DISCUSSION

This nationwide, long term follow-up cohort study shows that patients with PJS have a highly increased PC risk. We found a cumulative cancer risk of more than 25% at the age of 70 years and a 76-fold increased risk compared to the general population. The cumulative risk for developing any type of malignancy in the pancreatico-biliary region, including pancreatic, distal bile duct, or ampullary cancer, is as high as 29% at the age of 70 years and the relative risk for these cancers is 96. These data emphasise the relevance and clinical potential of

surveillance of the pancreas for PJS patients, provided a suitable and effective surveillance program is available.

Estimates on the risk of PC in patients with PJS vary widely within the literature. Our data are most in line with those from Giardiello *et al.* [380]. In a meta-analysis in which 210 PJS patients from six American and European studies were included, six cases of PC were identified which amounted to a cumulative risk of 36% by the age of 64 years and a relative risk of 132. The mean age at PC onset was 40.8 years (SD 16.2). Because the source data were contributed by multiple centres worldwide, the authors were not able to give extensive information about the intricacies of case selection or confirmation of the cancer diagnosis, including revision of pathology specimens and potential confounding issues relating to the problematic distinction between pancreatic, distal bile duct, and ampullary cancer. Furthermore, incidence rates of the US population were used for relative risk calculation, while the study population consisted of both US and European (UK and Dutch) patients. This might have led to biased relative cancer risks, as differences in cancer risk between PJS and control populations could exist due to variations in geography, race, culture and diet.

The extremely elevated risk found by Giardiello *et al.* has not been reproduced by more recent studies. An international collaborative study concerning 419 PJS patients from eight centres worldwide found a cumulative risk for developing PC of 11% by the age of 70 [460]. Relative risk was not reported. Another collaborative study found no PC in a total of 149 PJS cases [461]. Interpretation of the results of these two series is difficult, since no information is provided on the average age of the cohort or follow-up period. Albeit speculative, the lower risk found in these series could be the result of the participants being too young or the lack of sufficient follow-up time. The same data on age and follow-up period is missing in most current nationwide or single centre studies, which are often limited by a small sample size.

To our knowledge this is the first study to investigate the PC risk within a large, nationwide PJS patient cohort with long term follow up. This cohort goes back to the original family described by Jan Peutz in 1921 [4], and encompasses a substantial period of prospective follow-up time amounting to 5640 person-years including 1757 person-years of prospective follow-up. Because it is well known that differentiation between pancreatic, distal bile duct and ampullary cancer is a diagnostic challenge, we attempted to address the issue of case selection by careful expert revision of clinical, radiological, and histological materials. This enabled us to provide reliable risk estimates for PC alone and for cancers of the pancreas and pancreatico-biliary region including distal bile duct and ampullary cancer. The latter are sometimes misclassified or impossible to differentiate from PC. As such, these numbers could be looked upon as absolute minimum and maximum risk estimates. Another important clinical consideration when making such a separate risk assessment is that distal bile duct and ampullary cancer, just like PC, have a potential for early detection in surveillance programs of the pancreas.

A few limitations of our study warrant consideration. Firstly, because this PJS patient cohort was initiated by two tertiary referral centres, selection bias could potentially have led to an overestimated incidence of PC. Secondly, we were unable to gather reliable information about the smoking behaviour of our patients. This is unfortunate because smoking is one of the most important risk factors for the development of PC [464,465] and therefore a probable confounding factor between different PJS populations.

The evidence is slowly accumulating that surveillance of high risk individuals leads to the detection of high grade precursor lesions and asymptomatic early stage PC [466-474].

However, we currently still lack definite evidence that surveillance has a net benefit in terms of mortality reduction of PC related mortality and gain in life years, and whether this benefit outweighs the potential negative side effects of overtreatment, including associated complications and costs. We and others therefore suggest that surveillance of PJS patients should only be performed within the framework of well-established research protocols [475,476]. Results of the international Cancer of the Pancreas screening (CAPS) summit meeting in 2011 indicate that surveillance in high risk individuals should be regarded as a promising development, though more evidence is needed to address its real value [477]. During this meeting, 49 experts in the field of PC voted on statements with respect to PC surveillance. This resulted in a number of outstanding questions that still need to be addressed, including questions with respect to who to screen, when to start screening, the optimal frequency of screening, and particularly the optimal management of the asymptomatic pancreatic lesions detected.

Based on the results of our current study, we recommend that PJS patients should be offered surveillance regardless of family history for PC, since all subjects with PC in our series had a negative family history of PC. Although the median age of PC onset in our cohort was 54 years, we propose that surveillance starts at the age of 30 years. This suggestion is based on the fact that two patients in our series developed cancer in the pancreatico-biliary region at a very young age. If screening had started 10 years earlier than the median age of PC onset, these cases would have been missed.

It has been noted that some patients with PJS develop intestinal-type intraductal papillary mucinous neoplasms (IPMNs) [153]. IPMNs are well defined premalignant lesions of PC. One pancreatic adenocarcinoma in our study showed histological indication for development out of an IPMN lesion. Future research should be directed towards unravelling the molecular pathway of PC development in PJS patients. Such knowledge may tailor surveillance recommendations even more. Furthermore, the efficacy and cost effectiveness of PC surveillance must be further studied.

In conclusion, absolute and relative risks of developing pancreatic, distal bile duct and ampullary cancer are very high in patients with PJS. This observation, and the prospect that detection of these malignancies, or preferably their precursor lesions, might be possible at an early and potentially curable point in time, render PJS patients eligible for surveillance by yearly EUS and/or MRI within well-defined research protocols.

ACKNOWLEDGEMENTS

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Chapter 8

Breast cancer in female Peutz-Jeghers syndrome patients: risk assessment and surveillance recommendations

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ABSTRACT

Background: Female Peutz-Jeghers syndrome (PJS) patients have a highly increased cancer risk, including breast cancer (BC). Risk estimates for BC in these patients vary, while information on clinical and histological characteristics is scarce. In the present study, we assessed the BC risk and clinicopathological features of BC in a large PJS cohort, and sought to formulate a present-day BC surveillance recommendation.

Methods: Cases were identified from the Dutch PJS cohort. PJS was diagnosed according to international criteria. Clinical data were collected and radiological and histological data were reviewed. Cumulative BC risks were calculated by Kaplan-Meier analysis and relative risk by Poisson regression analysis.

Results: Of 145 PJS patients, 75 (52%) were female. Nine women from 8 families were diagnosed with BC at a median age of 50 years (range 34-61). Mammography allowed good visibility of all but one BC. The majority was of good or intermediate differentiation grade. All BCs were oestrogen-receptor positive and Her2-negative. Cumulative BC risk was 62% (95% CI 31%-93%) at age 65, and relative risk was 6 (95% CI 3 to 13, $p<0.001$) compared to the general population.

Conclusions: BC risk for female PJS patients is highly increased, approaching that of *BRCA*-mutation carriers, while PJS associated BC seem to have a later onset and more favourable clinicopathological characteristics. We propose to start annual BC surveillance with mammography in female PJS patients as of 30 years, with additional MRI in patients with dense breast tissue. Prospective evaluation of this schedule is required to determine its effectiveness.

INTRODUCTION

Peutz-Jeghers syndrome (PJS) is a rare, autosomal dominant inherited disorder caused by a germline mutation in the *LKB1* (also known as *STK11*) tumour suppressor gene. Clinical features include mucocutaneous pigmentations and gastrointestinal hamartomas. In addition, PJS patients have an increased risk for developing cancer in adult life, including gastrointestinal cancers (colorectum, stomach, and pancreas) and extra-gastrointestinal malignancies (lung, breast and gynaecological organs). A cumulative lifetime risk (CLTR) for developing cancer of up to 76% has been described [24]. Consequently, mortality in PJS patients is significantly increased compared to the general population [24]. Assuming that detection of precursor lesions or malignancies at an early and asymptomatic stage might decrease cancer-related mortality, cancer surveillance of various organs is recommended for this patient group [30].

Due to the additional risk of breast cancer and gynaecological malignancies, female PJS patients carry a higher CLTR for developing cancer than male patients [24]. Several studies reported on the risk of breast cancer (BC) in PJS (Table 1), describing CLTRs for BC of 29-54% at age 65 years, and relative risks (RR) of 6-15 [17,380,460,478]. However, these risk estimates were mainly derived from heterogeneous multicentre populations, small cohort studies, or meta-analyses of these studies. Furthermore, important clinical and histological information of PJS-associated BC cases and data on the method of detection is currently lacking.

In the present study, we assessed the BC risk for female PJS patients and investigated the features of PJS-associated BC by revision of clinical, pathological and radiological data of the Dutch PJS cohort. Based hereon, we propose a present-day recommendation for BC surveillance for female PJS patients.

MATERIALS AND METHODS

Peutz-Jeghers syndrome database

Between 1995 and July 2012, Dutch PJS patients were included in a nationwide cohort study, initiated by two academic hospitals (Academic Medical Centre in Amsterdam and Erasmus MC University Medical Centre in Rotterdam). The diagnosis of PJS was defined by diagnostic criteria as recommended by the World Health Organisation (WHO) (see chapter 1, Box 1, page 12) and/or a proven *LKB1* mutation. Patients were included without selection for medical history. Informed consent was obtained from all patients or from their next of kin if patients had passed away. Patient information was obtained by interview and chart review. Clinical data from the period before 1995 as well as data of deceased family members fulfilling the diagnostic criteria for PJS were collected retrospectively from hospital files. Institutional Review Boards of both coordinating hospitals approved the study.

At the closing date of the current analysis, 145 PJS patients from 62 families were included in this cohort study. Baseline characteristics are shown in Table 2.

Case selection and data collection

BC cases (both invasive and ductal carcinoma in situ; DCIS) were identified from the PJS database. The following data regarding BC patients were collected: dates of birth, BC diagnosis and death; tumour type (DCIS or invasive cancer); family history of BC and PJS; and *LKB1* mutation status and type of mutation.

Table 1. *Overview of studies reporting about the breast cancer risk in Peutz-Jeghers syndrome.*

Reference	Study design	Pts/families	Breast cancers	Breast cancer age (years)	Breast cancer risk	Remarks
380	Meta-analysis	210/79 Females:104	11	Range 19-48	RR: 15.2 (95%CI 7.9-27) CR: 54% from age 15 to 64yrs	Three cases of bilateral breast cancer
478	Multicentre study (UK)	33/33 Females:19	2	52 and 35	SMR: 13.9 (95%CI 0.2-50.3) CR: 29% (95%CI 12-62) at 65yrs	One case with positive family history for breast cancer
17	Collaborative study of 8 centres form Europe, Australia and USA	240/101 Females:131	9	Range 38-62	RR: 7-fold increased CR: 8% (95%CI 3-28) at 40yrs 11% (95%CI 4-27) at 50yrs 32% (95% CI 15-59) at 60yrs	
460	Collaborative study of 8 centres from Europe, Australia and USA	419/225 Females:226	17	Range 35-61	RR: ~6-fold increased CR: 8% (95%CI 4-17) at 40yrs 13% (95%CI 7-24) at 50yrs 31% (95%CI 18-50) at 60yrs 45% (95%CI 27-68) at 70yrs	One case of male breast cancer
461	Collaborative study of 12 centres from Europe, Asia, South America, North Africa and USA	149/41 Females:73	1	NR	CR: 5% (95%CI 0-13) at 40yrs	

CR: cumulative risk; NR: not reported; pts: patients; RR: relative risk; SMR: standardized mortality ratio; yrs: years.

Table 2. *Baseline characteristics of the Dutch Peutz-Jeghers syndrome cohort.*

	Total	Males	Females
Number of patients	145	70 (48%)	75 (52%)
Families	62	36/62	46/62
Family history			
Familial PJS	110 (76%)	55	55
Sporadic	26 (18%)	9	17
Family history unknown	9 (6%)	6	3
DNA mutation analysis	91 (63%)	42	49
LKB1 mutation carrier	82/91(90%)	37	45
Deceased	50 (35%)	27	23
Median age in years at death (IQR)	46 (32-58)	54 (33-60)	45 (31-53)
Follow-up (person-years)	5796	3091	2705
Prospective follow-up (person-years)	1852	860	992
Lost to follow up	5 (3%)	3	2

IQR: interquartile range; PJS: Peutz-Jeghers syndrome.

Radiological analysis

Available radiological images were reviewed by two expert breast radiologists (HMZ and IMO), being informed on clinical symptoms of the cases. Images consisted of mammographies and in one case an additional ultrasound was available. No magnetic resonance imaging (MRI) scans were performed. The radiologists assessed the overall breast composition using the following patterns: almost entirely fat (American College of Radiology score 1; ACR 1), scattered fibroglandular densities (ACR 2), heterogeneously dense (ACR 3) and extremely dense (ACR 4). Radiological findings were described and the level of suspicion for malignancy was assessed according to the Breast Imaging Reporting and Data System (BI-RADS): no abnormality (BI-RADS 1), normal finding (BI-RADS 2), probably benign finding (BI-RADS 3), suspicious abnormality (BI-RADS 4) or finding highly suggestive for malignancy (BI-RADS 5) [479].

Histological analysis

Available formalin-fixed and paraffin-embedded breast tissue was reviewed by an expert breast pathologist (CvD). Invasive carcinomas were classified according to the criteria of the WHO Classification, and graded according to the modified Bloom and Richardson grading system [480]. DCIS cases were graded based on cytonuclear characteristics. Slides stained for steroid receptors (oestrogen receptor (ER) and progesterone receptor (PR)) were scored as positive (≥ 10% tumour nuclei positive) or negative (< 10%) according to Dutch guidelines (available at www.oncoline.nl) [481]. Her2 status was determined according to the current guidelines.[482] Steroid receptors and Her2 status were only determined on invasive BCs.

STATISTICAL ANALYSIS

Data were analysed using the SPSS 20.0 statistical software for Windows (IBM, Somers, New York, USA). Cumulative BC risks were estimated as a function of time using the Kaplan-Meier method, including all females of the cohort. For relative cancer risk calculation, the BC incidence observed in the female study population was compared to the age-specific and gender-specific incidence rates of the Dutch general population from 1960 to 2011 by Poisson regression analysis (loglinear analysis) using the package R [463]. Patients were studied with respect to their risk of developing cancer from birth till date of BC diagnosis, death, date of last contact or closing date of the study (July 1, 2012), whichever came first. Socio-demographic data and incidence rates of the Dutch general population were derived from the Eindhoven Cancer Registry (1960-2011). These data were considered representative for 2012 as well.

RESULTS

Study population

In total, 75 female PJS patients of 46 families contributed to 2705 person-years of follow-up, including 992 person-years of prospective follow-up (64 patients). At the closing date of the current analysis, two patients had been lost to follow-up (2.7%) and 23 patients (31%) had died at a median age of 45 years (IQR 31-53 years). The median age of the 50 women (67%) still alive was 31 years (IQR 20-49 years). At the closing date of the study, 24 out of 30 patients being ≥ 25 years of age underwent some kind of BC surveillance.

Table 3. *Clinical characteristics of Peutz-Jeghers syndrome patients with breast cancer.*

Case number[1]	Age (year) at breast cancer diagnosis	Age at death	Type of breast cancer	Family history of breast cancer	Familial/ sporadic PJS	*LKB1* germline mutation
I	34 (2000)	-	*In situ* carcinoma	No	Familial	c.370 A>T
II	50 (2009)	-	Multifocal invasive carcinoma	Yes	Familial	c.752 G>A
III	34 (1997)	-	Invasive carcinoma	No	Sporadic	Not tested
IV	55 (2009)	58[5]	Invasive carcinoma	Unknown	Familial	c.735-1 G>A[6]
V	49 (2009)	-	Invasive carcinoma	No	Sporadic	Deletion exon 1
VI[2]	53 (1964)	69	Unknown	Yes	Familial	c.921-12 G>A[6]
VII[2]	61 (2006)	-	Invasive carcinoma	Yes	Familial	c.921-12 G>A
VIII	61 (1996)	62[5]	*In situ* carcinoma	No	Familial	c.991dupC
IX[3,4]	47 (1944)	47[5]	Unknown	No	Familial	codon 66insT, stop in codon 162[6]

[1]Case numbers correspond with the case numbers in Table 4 and Figure 1
[2]First-degree family members
[3]Malignancy confirmed by previous case-reports [383, 384]
[4]Case excluded from the relative risk analysis because of diagnosis before 1960
[5]Deceased because of breast cancer
[6]Mutation detected in affected first-degree relative

Breast cancer cases

During follow-up, nine (12%) PJS patients from eight families developed BC at a median age of 50 years (range 34-61 years), including two cases of DCIS. No cases of bilateral BC were observed. Data on patient characteristics are depicted in Table 3. At the end of the study period, four BC patients had died, of which three died due to BC. One of these patients (case VIII) was diagnosed with DCIS (poorly differentiated) but presented with metastatic disease 5 months after diagnosis. Mutation analysis for the *LKB1* gene was performed in five patients,

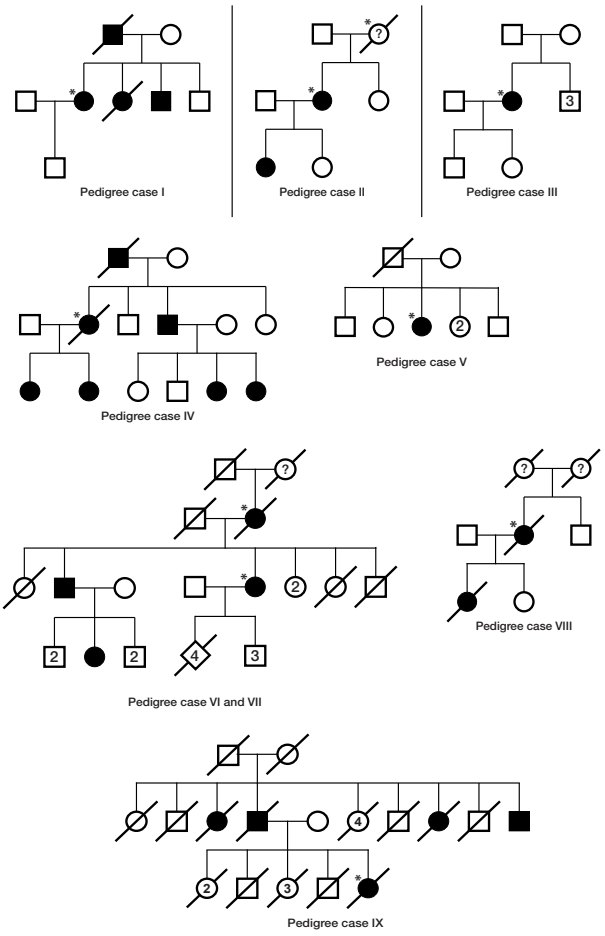

Figure 1. *Pedigrees of breast cancer patients.*
Breast cancer patients are marked with an asterisk. Case numbers are depicted in roman numerals according to Table 3 and 4.
Squares: males; circles: females; diamonds: unknown sex; solid symbols: PJS affected individual; open symbols: unaffected individual; symbol with question mark: unknown PJS status; symbol with slash: individual is deceased.

detecting a pathogenic mutation in four. In three other patients fulfilling the diagnostic WHO criteria for PJS, a pathogenic mutation in the *LKB1* gene was detected in affected first-degree relatives. Two BC patients belonged to the same family (case VI and VII). A first-degree family member of case II also suffered from BC, but the diagnosis of PJS in this affected family member was uncertain and this patient was therefore not included in the current analysis. Figure 1 shows the pedigrees of all patients.

Radiology of breast cancer cases

Radiological images of seven patients (78%) were available for revision. Results are shown in Table 4. Two patients underwent BC surveillance on a yearly basis, and three patients presented with a palpable mass. In five of the seven patients (71%), the mammographies showed a low breast density (ACR score 1 or 2). Of note, all cancers but one were identifiable on mammography; one BC was identified on additional ultrasound.

Histology of breast cancers

BC tissue of six patients (67%) was available for revision. Two tumours concerned DCIS, and four were invasive carcinomas, including a multifocal carcinoma in one patient. All five invasive carcinomas, with a median size of 12 mm, were classified as invasive ductal carcinoma not otherwise specified. Three of these invasive carcinomas showed adjacent DCIS. Results of grading, immunohistochemical staining for steroid receptors and Her2 status, and TNM stage at time of diagnosis are shown in Table 4.

Cumulative cancer risk

At age 35 years, the Kaplan-Meier estimate for the cumulative breast cancer risk was 5% (95% CI -1.7-11.7) (Figure 2). This BC risk increased from the age of 50 years on, with cumulative breast cancer risks being 13.7% (95% CI 0.8-26.6) at age 50 years; 25.9% (95% CI 6.3-45.5) at age 55 years; 36.5% (95% CI 11.0-62.0) at age 60 years; and 61.9% (95% CI 30.5-93.3) at age 65 years (Figure 2).

Relative cancer risk

From 1960 onwards, 69 patients contributed to 2239 person-years at risk. One BC case was excluded from the relative risk analysis because of diagnosis before 1960. Poisson regression analysis showed that the relative risk for BC in female PJS patients was 6.4 (95% CI 3.2 - 12.9) compared to the general Dutch female population ($p<0.001$).

DISCUSSION

The present study, performed in a homogenous, large cohort of Dutch PJS patients, shows a highly increased risk for developing BC in female patients. We found a cumulative risk of 62% at the age of 65 years, mainly rising from 50 years on, and a 6-fold increased risk compared to the general female population. These results confirm the very high breast cancer risk in women with PJS, in fact approaching the BC risk of patients with *BRCA1/2*-gene mutations

Table 4. *Radiological and histological characteristics of PJS-associated breast cancer cases.*

Case number[1]	Indication for breast imaging	Mammographic assessment		Breast cancer subtype and grade based on histology	Tumour size (mm)	Immunohistochemistry[2]			Conclusion and tumour stage at diagnosis[3]
		ACR	BI-RADS			ER	PR	Her2	
I	Suspicion of unknown primary	2	4 Microcalcifications	Ductal carcinoma *in situ* grade II	6	na	na	na	Incidental finding pTis
II	Palpable mass Previous mammography 7 months earlier (BI-RADS 2)	4	1[4]	Multifocal ductal carcinoma Lesion 1: grade II Lesion 2: grade I	Lesion 1: 12 Lesion 2: 8	Lesion 1 and 2: positive (95%)	Lesion 1: positive (30%) Lesion 2: positive (50%)	Lesion 1 and 2: negative	Interval carcinoma pT1N0Mx
III	Palpable mass No previous breast imaging	2	5 Mass lesion	Ductal carcinoma grade III	35	positive (90%)	positive (90%)	negative	Symptomatic carcinoma pT2N0M0
IV	Yearly surveillance	2	5 Mass lesion	Unknown	unknown	unknown	unknown	unknown	Screening carcinoma pT2N0M0[5]
V	Palpable mass Previous mammography 6 years earlier[6]	3	4 Mass lesion	Ductal carcinoma grade I	9	positive (70%)	positive (40%)	negative	Symptomatic carcinoma pT1N0M0
VII	Surveillance Previous mammography 3 years earlier	1	5 Mass lesion	Ductal carcinoma grade II	12	positive (90%)	negative	negative	Screening carcinoma pT1N0M0
VIII	First surveillance No previous breast Imaging available	2	4 Microcalcifications	Ductal carcinoma *in situ* grade III	na	na	na	na	Screening carcinoma pTis

ACR; American College of Radiology, BI-RADS; Breast Imaging Reporting and Data System, ER; oestrogen receptor, na: not applicable; PJS: Peutz-Jeghers syndrome; PR: progesterone receptor; Tis: in situ carcinoma.

[1]Case numbers correspond with the case numbers in Table 3 and Figure 1

[2]No immunohistochemistry was done on in situ carcinomas without an invasive component

[3]Pathological classification

[4]Palpable mass not visible on mammography, detected by additional ultrasound

[5]Pathological classification based on medical records. No tissue was available for histological revision.

[6]Surveillance because of fibroadenoma and adenomyo-epithelioma in breast in medical history

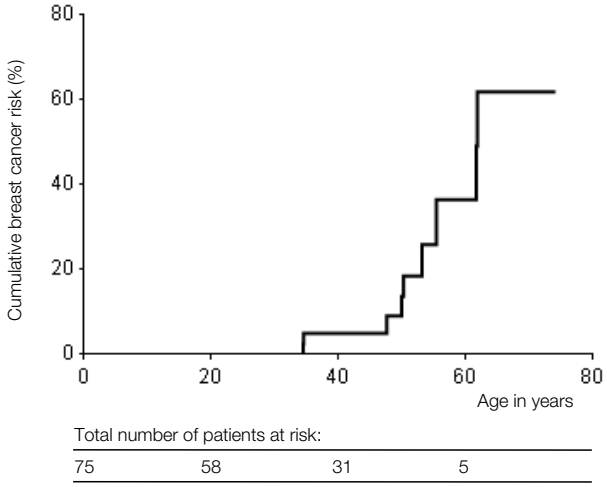

Figure 2. *Cumulative breast cancer risk in female Peutz-Jeghers syndrome patients according to age.*

(relative risk 6-8) [483,484]. It has previously been proposed that PJS patients should be offered BC surveillance as applied for *BRCA* mutation carriers [23,30]. In the Netherlands, this encompasses annual MRI from the age of 25 years, with annual mammography added from 30 till 60 years, after which enrolment in the population based BC screening programme is advised. However, histological and radiological features of breast tumours could provide valuable information in view of surveillance recommendations. To our knowledge, data heron of PJS-associated breast tumours has not been systematically described before in literature. Based on our observations, important clinicopathological differences between BC cases in PJS patients and *BRCA* mutation carriers seem to exist, which in our opinion require adjustment to provide a more tailor made BC surveillance regimen for female PJS patients.

First of all, age of BC onset in PJS patients is not as young as reported for *BRCA* mutation carriers. The median age at BC diagnosis in the present study was 50 years (range 34-61 years), which is in line with the age distribution of BC in other studies concerning PJS patients (Table 1). Younger onset of disease has only been described in single case reports [485,486]. For BRCA1 and BRCA2 carriers, mean age at BC diagnosis is 43.7 years and 46.8 years, respectively [487]. Furthermore, the cumulative incidence of BC in *BRCA* mutation carriers rapidly increases as of young age, being 20-25% at the age of 40 years [487,488]. We observed a cumulative BC incidence of 5% at 40 years in PJS patients, in line with the literature (Table 1), and this risk mainly increases from the age of 50 years on (Figure 2).

Secondly, our histological revision of PJS-associated breast cancers showed that the majority of carcinomas were of good or intermediate differentiation grade. Furthermore, all invasive carcinomas were steroid receptor positive, and Her2 negative. In contrast, most of the invasive *BRCA*-associated breast cancers are of poor differentiation grade and associated with a more rapid tumour growth, justifying intensive surveillance at young age [489-491]. Although we were not able to assess tumour growth rate in our sample of BC patients due to sample size and unavailability of serial imaging examinations, mammographies showed a low breast density (ACR score 1 or 2) in five of the seven PJS patients, allowing good visibility of

the suspicious lesions. Unfortunately, we were not able to compare our data with other data because of lack of reports.

Based on our observations, a panel of experts in the field of (hereditary) BC surveillance discussed the pros and cons of different surveillance recommendations for female PJS patients. In this panel, an oncologist, a pathologist, two radiologists, and a clinical geneticist were represented. Because of the highly increased RR of 6, we propose that BC surveillance should approach the annual surveillance recommendation of *BRCA* mutation carriers. Though, in view of the later age of BC onset, a later start of surveillance as of 30 years seems justifiable in female PJS patients. Furthermore, based on the finding of the radiological revision, it may be considered to only perform an MRI in patients with dense breast tissue (ACR 3 or 4), also to limit the amount of surveillance investigations in this patient group. After the age of 60 years, PJS patients can enrol in a population based BC screening programme. In the Netherlands, this encompasses mammography every two years till the age of 75 years.

It seems unlikely that the increased BC risk also affects male PJS patients. In our cohort no case of male BC occurred, and in previous literature only one male PJS patient with BC has been described [460]. Although this might be an underestimation, the BC risk will always be too low to justify surveillance in male PJS patients.

Several limitations of this study should be discussed. First of all, because two tertiary referral centres initiated this PJS patient cohort, selection bias could potentially have led to an overestimation of the incidence of BC. Second, three BC cases had a family history for both PJS and BC. Having a first-degree relative with BC is known to increase the risk for BC with a factor 1-4 [483,484,492]. This might have distorted the increased risk as described in the current study. Third, reliable information about important risk factors for BC, such as obesity, diet, the use of oral contraceptives, the age of menarche and first pregnancy, or null parity are missing [483,484,492,493]. These missing data, in addition to the small sample size, did not allow correcting for confounding factors possibly influencing the BC incidence.

Our results are in line with the results of other collaborative studies (Table 1) [17,460,461,478]. However, these studies showed extensive overlap in included patients, who were enrolled from specialised centres throughout the Western world. Furthermore, in these studies risk estimates for BC were not always complete, and lacked important information about clinical and histological characteristics. The extremely elevated risk found by Giardiello *et al.* [380] was based on a meta-analysis of available literature, including single case reports, and has not been reproduced by more recent and larger studies. The current study is the first to report on the occurrence of BC specifically in a large, pedigree-based nationwide cohort of PJS patients with a substantial period of prospective follow-up. None of the patients was included in this cohort because of cancer diagnosis, which refutes the possibility of ascertainment bias.

While the high relative risk estimate as described in this study justifies the proposed intensive BC surveillance for PJS patients, we are aware that our recommendation solely reflects expert opinion and is not yet evidence based. The relevant findings on clinical and histological features of PJS-associated BCs indicate that adaptations to the surveillance scheme for *BRCA* mutation carriers may be relevant. We therefore emphasize that prospective evaluation of the proposed surveillance regimen is mandatory, and central registration of this patient group might facilitate this aim.

In conclusion, the current study shows a highly increased absolute and relative risk for developing BC in female PJS patients, approaching the risk for *BRCA* mutation carriers. At the same time, clinical, histological and radiological characteristics of PJS-associated breast

tumours substantially differ from *BRCA* tumours, in favour of the PJS group. Therefore, we propose to start annual BC surveillance with mammography in female PJS patients at the age of 30 years, with additional MRI in patients with dense breast tissue (ACR 3 and 4). After the age of 60 years, patients can enrol in a population based BC screening programme. Central registration of female PJS patients and prospective evaluation of BC surveillance regimens is warranted.

Chapter 9

Peutz-Jeghers syndrome and family planning: the attitude towards prenatal diagnosis and pre-implantation genetic diagnosis

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ABSTRACT

Peutz-Jeghers syndrome (PJS) is a hereditary disorder caused by *LKB1* gene mutations, and is associated with considerable morbidity and decreased life expectancy. This study was conducted to assess the attitude of PJS patients towards family planning, prenatal diagnosis (PND) and pregnancy termination and pre-implantation genetic diagnosis (PGD). In a cross-sectional study, 61 adult PJS patients were asked to complete a questionnaire concerning genetic testing, family planning, PND and PGD. The questionnaire was completed by 52 patients (85% response rate, 44% males) with a median age of 45 (range 18-74) years. Thirty-seven (71%) respondents had undergone genetic testing. Twenty-four respondents (46%, 75% males) had children. Fifteen (29%) respondents reported that their diagnosis of PJS had influenced their decisions regarding family planning, including 10 patients (19%, 9/10 females) who did not want to have children because of their disease. Termination of pregnancy after PND in case of a foetus with PJS was considered 'acceptable' for 15% of the respondents, whereas 52% considered PGD acceptable. In conclusion, the diagnosis of PJS influences the decisions regarding family planning in one third of PJS patients, especially in women. Most patients have a negative attitude towards pregnancy termination after PND, while PGD in case of PJS is judged more acceptable. These results emphasize the importance of discussing aspects regarding family planning with PJS patients, including PND and PGD.

INTRODUCTION

Peutz-Jeghers syndrome (PJS) is a rare, autosomal dominant inherited disorder caused by germline mutations in the *LKB1* gene [10,11]. The syndrome is clinically characterized by gastrointestinal hamartomas and mucocutaneous pigmentation [4,494]. Hamartomatous polyps can develop already in the first decade of life and may cause various complications, including anaemia, bleeding and acute intestinal obstruction [19,495]. Furthermore, PJS is associated with an increased cancer risk in adult life. Lifetime cumulative cancer risks as high as 93% have been described [23,380]. These clinical aspects of the disease affect the psychological condition and quality of life of PJS patients. They suffer from mild depression and experience a poorer mental quality of life, more limitations in daily functioning due to emotional problems, and a poorer general health perception compared to the general population [496,497].

Performing genetic testing might influence family planning of patients. Diagnostic mutation analysis is available for patients clinically suspected of PJS. If a pathogenic mutation is confirmed, antenatal genetic testing of offspring is available through prenatal diagnosis (PND) (i.e. chorionic villus sampling and amniocentesis), which may result in the wish to terminate the pregnancy in case of an affected foetus. In addition, prei-mplantation genetic diagnosis (PGD) has become available. PGD involves in vitro fertilization (IVF). One or two cells of a 3-day old embryo created *in vitro* are analysed for the genetic defect, and only embryos with an unaffected genotype are selected for transfer to the uterus [498]. Although PND and PGD are available for hereditary cancer syndromes in most European countries, the application of these techniques remains controversial in the social, ethical and political domain [499].

Data concerning family planning of patients with PJS are lacking. Therefore, the aim of this study was to investigate the desire to have children in PJS patients, and their attitudes towards PND with the implication of pregnancy termination and towards PGD.

MATERIALS AND METHODS

Patients

A total of 61 PJS patients from 39 families from two Dutch academic hospitals were invited to complete a questionnaire on genetic testing, family planning, PND, and PGD. The study was approved by the Institutional Review Boards of both participating hospitals. Patients were eligible if they were aged 18 years or older and fulfilled the diagnostic criteria for PJS recommended by the World Health Organisation (see chapter 1, Box 1, page 12). The questionnaire, an information-folder, a consent form and a reply paid envelop were sent to all potential participants by mail. After 6 and 12 weeks a reminder was sent to non-respondents.

Measures

The questionnaire was earlier described in detail by van Lier *et al*. [497]. Briefly, it comprised a range of demographic variables including age, gender, and parenthood. As psychological determinants, concerns regarding cancer were assessed with the cancer worry scale (CWS) [500], and illness perceptions were evaluated by the Illness Perception Questionnaire-Revised (IPQ-R) [501]. Clinical variables including history of cancer and family history of PJS were derived from medical records.

In addition, respondents were asked whether or not they had undergone genetic testing and, if they had, what the result had been. Self-reported data regarding genetic testing were

confirmed by medical records where possible. Questions were posed about the current desire to have (more) children, and if the diagnosis of PJS had influenced the desire to have (more) children. Furthermore, after a short introductory text about PND and PGD, respondents were asked whether or not they considered termination of pregnancy after PND or the use of PGD acceptable; (1) in general, and (2) in case of PJS. Response categories were 'yes', 'no' or 'unsure' [502].

Statistical analysis

Data were analysed using the SPSS 17.0 statistical software for Windows. Descriptive statistics were used to characterize the study sample. Continuous variables were reported by means (and standard deviation) and medians (and range). Univariate analyses (χ^2, Fisher's exact test, independent t-test and Mann-Whitney U test) were used to evaluate which sociodemographic, clinical and psychological variables were related to attitudes towards genetic testing, PND and PGD. A two-sided p value < 0.05 was considered statistically significant. Multivariate logistic regression analyses using backward selection with a p value of 0.1 for removal of the variable was carried out to determine associations between possible confounders (sociodemographic, personal and family medical history, and psychosocial determinants) and three outcome measures: genetic testing ('yes' or 'no'), termination of pregnancy after PND acceptable in case of PJS ('yes' or 'no/unsure') and PGD acceptable in case of PJS ('yes' or 'no/unsure').

RESULTS

Baseline characteristics

The questionnaire was completed by 52 PJS patients (response rate 85%) from 34 families. Median age of respondents was 44.5 (18-74) years and 23 (44%) were male. Baseline characteristics of the respondents and non-respondents are shown in Table 1.

There were no significant differences in age (p = 0.056) or cancer incidence between male and female respondents (p = 0.144). However, women in our cohort scored significantly higher than men on the cancer worry scale (6.41 vs. 5.13, p = 0.038), and on the IPQ-R subscale *emotional representations* (16.21 vs. 12.87, p = 0.019). Scores on the other six IPQ-R subscales did not differ significantly between male and female respondents.

Genetic testing

Of the 52 patients who completed the questionnaire, 37 patients had undergone genetic testing, of which 33 (89%) were actually carrier of a pathogenic *LKB1* mutation. Multivariate logistic regression analysis showed female gender (p = 0.035) and parenthood (p = 0.016) as positive predictors for genetic test uptake (Table 2).

Parenthood and influence of PJS on family planning

Twenty-four respondents (46%; median age 50 years) had children. Female PJS patients less often had children than male patients (25% versus 75%, p<0.001).

Fifteen of the 52 respondents (29%, median age 44 years) reported that the diagnosis of PJS had influenced their desire to have children (i.e. less or no children). Ten of these 15

Table 1. *Baseline characteristics of respondents and non-respondents.*

	Respondents N (%)	**Non-respondents N (%)**
Total number	52	9
Median age (range)[1]	44 (18-74)	34 (18-67)
≤ 45 yrs (childbearing age)	29 (56)	5 (56)
> 45 yrs	23 (44)	4 (44)
Gender[1]		
Male	23 (44)	6 (67)
Female	29 (56)	3 (33)
Partner		
Yes	36 (69)	Unknown
No	16 (31)	Unknown
Children		
Yes	24 (46)	5 (56)
No	28 (54)	4 (44)
Educational level		
Low	29 (56)	Unknown
High	23 (44)	Unknown
Genetic testing performed		
Yes	37 (71)	9 (100)
No	15 (29)	0 (0)
Family history		
Familial PJS	33 (63)	5 (56)
Sporadic PJS/family unknown	19 (37)	4 (44)

PJS: Peutz-Jeghers syndrome; yrs: years.
[1]Age (p = 0.86) and gender distribution (p = 0.29) did not differ between respondents and non-respondents.

Table 2. *Determinants of genetic testing (N=52).*

	Univariate analysis		**Multivariate logistic regression analysis**	
	OR (95% CI)	*p* value	OR (95% CI)	*p* value
Gender; male/female	1.676 (0.501;5.611)	0.402	11.344 (1.183;108.805)	0.035
Age	1.042 (0.995;1.092)	0.080	-	
Children; yes/no	3.235 (0.869;12.043)	0.080	17.664 (1.726;180.818)	0.016
PJS familial; yes/no	1.333 (0.357;4.985)	0.669		
Malignancy; yes/no	1.517 (0.277;8.310)	0.631		
CWS score	0.962 (0.750;1.235)	0.763		

OR: odds ratio; 95% CI: 95% confidence interval; CWS: cancer worry scale.

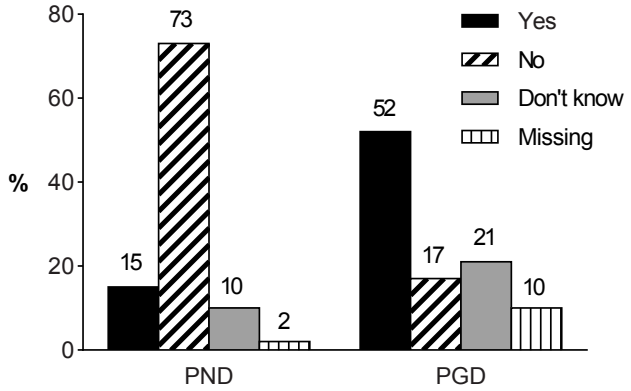

Figure 1. *Attitude of Peutz-Jeghers syndrome patients towards termination of pregnancy after PND, and towards pre-implantation genetic diagnosis in case of Peutz-Jeghers syndrome.*
PND: acceptance of termination of pregnancy after PND in case of PJS.
PGD: acceptance of the use of pre-implantation genetics diagnosis in case of PJS.

respondents (19%; median age 45 years) stated that they had decided to have no children because of PJS, including 9 females and one male, the latter whom had adopted a child. Cancer incidence was higher in these 10 patients (56% vs. 44%, $p = 0.011$), and they scored higher on the cancer worry scale (8.0 vs. 5.2, $p = 0.039$) compared to the other respondents. Twenty-three of the respondents (44%, median age 45 years) indicated that PJS had not influenced their desire to have children.

In general, the majority of respondents considered termination of pregnancy after PND and the use of PGD as 'acceptable' (62% and 61%, respectively). The attitude of respondents regarding these two techniques in relation to PJS is shown in Figure 1. Fifteen per cent of

Table 3. *Determinants of the attitude towards termination of pregnancy in case of a foetus with PJS (N=51).*

	Univariate analysis		Multivariate logistic regression analysis	
	OR (95% CI)	*p* value	OR (95% CI)	*p* value
Gender; male/female	2.609 (0.472;14.406)	0.271	-	
Age	0.936 (0.877;0.998)	0.042	-	
Aware of mutation status; yes/no[1]	0.680 (0.149;3.099)	0.618		
Children; yes/no	0.124 (0.014;1.098)	0.061	-	
PJS familial; yes/no	0.655 (0.133;3.218)	0.602		
Malignancy; yes/no	0.625 (0.067;5.822)	0.680		
CWS score	1.165 (0.881;1.540)	0.283		

OR: odds ratio; 95% CI: 95% confidence interval; CWS: cancer worry scale.
[1]Twenty-nine respondents were aware of their mutation status; 27 LKB1-mutation positive and 2 LKB1-mutation negative.

patients considered pregnancy termination after PND acceptable, while 52% accepted the use of PGD in case of PJS. Results of univariate and multivariate analyses are shown in Table 3 and 4. No significant associations were found for the attitude towards pregnancy termination after PND or towards PGD.

Table 4. *Determinants of the attitude towards preimplantation genetic diagnosis in case of PJS (N=47).*

	Univariate analysis		**Multivariate logistic regression analysis**	
	OR (95% CI)	*p* value	OR (95% CI)	*p* value
Gender; male/female	1.455 (0.454;4.664)	0.529	-	
Age	1.021 (0.978;1.067)	0.341	-	
Aware of mutation status; yes/no[1]	1.700 (0.525;5.500)	0.376		
Children; yes/no	1.135 (0.356;3.621)	0.831		
PJS familial; yes/no	0.343 (0.078;1.500)	0.155		
Malignancy; yes/no	0.375 (0.078;1.803)	0.221		
CWS score	1.187 (0.884;1.593)	0.254		

OR: odds ratio; 95% CI: 95% confidence interval; CWS: cancer worry scale.
[1]Twenty-nine respondents were aware of their mutation status; 27 LKB1-mutation positive and 2 LKB1-mutation negative.

DISCUSSION

This is the first survey among PJS patients that evaluated their decisions regarding family planning, and their attitude towards PND with possible pregnancy termination, and towards PGD. Twenty-four respondents (46%, 75% males) had children. Interestingly, there was a notable gender difference in our study population with respect to parenthood. Female patients less often had children than men with PJS. Furthermore, 90% of patients (9 / 10) who explicitly indicated that they did not want to have children because of PJS were female. The reason for this difference is not clear. As PJS is associated with an increased risk for the development of gynaecological tumours [23,460], disabilities (e.g. hysterectomy or oophorectomy) might have prevented female patients from having children. However, this was the case in only 2 females from our cohort (at the age of 36 and 39 years). In addition, there were no significant differences in age or cancer incidence between male and female respondents. One could postulate that psychosocial explanations for this difference exist. Women in our cohort did have more cancer worries than men, and had a higher emotional response to PJS. These findings could imply that women are more emotionally affected by their disease which can render to a higher sense of responsibility towards their offspring [503].

All respondents, irrespective of parenthood or not, were asked about their attitude towards termination of pregnancy after PND. More patients accepted the use of PGD in case of PJS than pregnancy termination after PND, suggesting a preference for PGD. This preference has been observed before in couples with different genetic disorders, including cancer susceptibility syndromes as hereditary breast and ovarian cancer, and familial adenomatous polyposis syndrome [504-508]. In a recent study among 210 couples with a broad spectrum of genetic disorders, the majority of couples preferred PGD over PND for diagnostic testing in a future pregnancy [509]. The preference for PGD can partly be explained by the fact that PGD

offers patients the possibility to have an unaffected genetically related child while termination of a pregnancy can be avoided. Furthermore, early reassurance is seen as an important advantage [504]. Though, many individuals with a hereditary condition for which PGD has been permitted, are unfamiliar with the technique or even unaware of its existence [509]. In practice, PGD is physically and psychologically burdensome [510]. Our questionnaire did not explore the knowledge of respondents about PND and PGD. Although both techniques were shortly described, the information might have been too limited. Furthermore, positive attitudes towards PND and PGD do not necessarily translate into actual use [511].

This study is hampered by some limitations. First of all, the cross-sectional study design makes evaluation of causal interactions impossible. Instead, we can only demonstrate statistical associations between determinants and the attitude towards genetic testing and reproductive decision making. Second, only affected individuals were asked to fill in the questionnaire, not their partners, yet it is likely that partners of PJS patients play an important role in the reproductive decision making and family planning. Third, the actual use of PND and subsequent pregnancy termination and PGD amongst PJS patients is not known and questions regarding religion were not included in our questionnaire, while religion can be of influence on the attitude towards both PND as well as PGD. Finally, in spite of the response rate of over 85%, our conclusions are drawn from a small sample size. Since PJS is a rare disorder it is difficult to assess a larger group. However, we managed to approach nearly all known Dutch PJS patients, thereby creating a heterogeneous cohort of patients enrolled in similar surveillance programs and with similar access to medical care. To our knowledge this is the first report concerning reproductive decision making and the attitude towards antenatal diagnostics amongst PJS patients.

In conclusion, this study demonstrates that the diagnosis of PJS influences decisions regarding family planning in approximately one third of PJS patients, especially in women. The majority of patients undergo genetic testing, and many PJS patients have a positive attitude towards PGD as an option to prevent transmission of PJS to their offspring. In contrast, the attitude of respondents was predominantly negative towards pregnancy termination after PND in case of a foetus affected with the syndrome. Our results emphasize not only the importance of accurate genetic counselling for these patients; it also indicates that medical specialists dealing with patients suffering from hereditary cancer syndromes, including PJS, should discuss aspects regarding family planning such as PND and PGD.

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We would like to thank all participating PJS patients.

Chapter 10

General discussion and future perspectives

A clear link exists between Peutz-Jeghers syndrome (PJS), the *LKB1* tumour suppressor gene, and the development of cancer. Within this context, the focus of this thesis was to investigate 1) the role of LKB1 signalling in cancer development and treatment, and 2) the prevalence and prevention of cancer in PJS. This final chapter discusses the novel insights obtained from our research projects and directions for future research.

THE ROLE OF LKB1 IN GASTROINTESTINAL CANCER

After the discovery of *LKB1* as the gene carrying the causative mutation in PJS in 1998 [10,11], its function was gradually unravelled in the following years. LKB1 appears to play a crucial role during early embryonic development [121]. Additionally, LKB1 is involved in various processes in the cell, including cell polarity, energy metabolism, and cell growth (**chapter 2)**. *LKB1* is classified as a tumour suppressor gene, although its tumour suppressing functions are still largely unclear.

Despite the strong association between *LKB1* germline mutations and the increased cancer risk in PJS patients, *LKB1* is not commonly mutated in sporadic cancers, except for non-small cell lung cancer (NSCLC) [141,160]. Also in sporadic colorectal cancer (CRC) somatic mutations in *LKB1* are rarely detected, although CRC is one of the most commonly found cancer types in PJS patients. Loss of heterozygosity (LOH) of the LKB1 locus 19p13.3 has been detected in up to 50% of sporadic CRC cases [143,145], suggesting that reduced levels of LKB1 might contribute to the development of these cancers. However, the exact tumour suppressor functions of LKB1 in sporadic CRC development remain elusive. Therefore, in **chapter 3**, we investigated the role of LKB1 in colon cancer by knocking down LKB1 expression in human colon cancer cells. To this aim, we used HT29 cells, which are well differentiated epithelial colon cancer cells with good adherent capacities. These cancer cells harbour a homozygous mutation in the tumour suppressor genes *P53* and *APC*, and an oncogenic *BRAF* mutation. We observed that additional loss of LKB1 in these cells did not significantly affect Wnt/β-catenin signalling and cell growth, but rather affected cellular motility by rearranging the actin cytoskeleton. This observation is in line with previous studies showing that loss of LKB1 increases migration and invasion in epithelial breast, lung and oesophageal cancer cells [136,369,373]. Together, these data suggest that LKB1 suppresses progression of sporadic CRC as well as of other cancers to a more invasive and thus more malignant phenotype. Overall, LKB1 might play a role in epithelial cancer cell metastasis rather than initiating sporadic cancer development. To further address this intriguing suggestion, the level of LKB1 expression in human CRC tissue should be evaluated and correlated to clinicopathological parameters such as tumour stage and patient survival. However, such analyses require a suitable antibody detecting endogenous LKB1 in immunohistochemical stainings, which is currently lacking. Therefore, research should focus on the development of such an antibody.

LKB1 is considered a haploinsufficient tumour suppressor gene. Haploinsufficiency means that one functional allele of the gene is not sufficient to bring about the wild-type condition, in contrast to a classical tumour suppressor gene. The haploinsufficiency of *LKB1* has been confirmed in mice. *Lkb1*$^{+/-}$ mice develop intestinal polyps identical to those seen in individuals affected with PJS (hamartomas), but neither loss of the wild-type *Lkb1* allele nor loss of expression of Lkb1 could be detected in most gastrointestinal (GI) polyps of these mice, indicating that partial loss of Lkb1 is sufficient for hamartoma development [34,35].

Haploinsufficiency of a tumour suppressor gene could be more pronounced in combination with additional oncogenic triggers. For Lkb1, additional loss of tumour suppressor genes *Pten*

or *p53*, or additional oncogenic activation of *Kras* synergizes with Lkb1 loss for tumour formation [95,100,101,106]. In addition, because Lkb1-deficient mouse models did not develop GI carcinomas, and because of the lack of a genotype-phenotype correlation in PJS patients, additional oncogenic events seem to be required for LKB1-associated CRC development. It remains yet unknown which additional triggers, together with loss of LKB1, are needed for CRC development.

To answer this question, we investigated the involvement of candidate genes and molecular pathways in tissue of PJS-associated GI cancers and dysplastic hamartomas in **chapter 4 and 5**. Firstly, we detected loss of the remaining wild-type allele of *LKB1* more frequently in carcinomas than in hamartomas of PJS patients, which is in line with previous reports [119,120]. However, loss of the wild-type *LKB1* allele was observed in dysplastic epithelium of a subset of the investigated hamartomas, but not in the non-dysplastic epithelium of the same hamartomas. This suggests that partial loss of LKB1 is sufficient for hamartoma development, and that complete loss of LKB1 may induce dysplastic transformation in the epithelium of PJS hamartomas.

Next, our findings suggest that mutant *P53*, rather than complete loss of this tumour suppressor gene, plays a major role in LKB1-associated GI carcinogenesis. It is remarkable that we did not find evidence for involvement of mutant *KRAS* in PJS-associated GI tumours, since previous research indicate that *LKB1* cooperates with *KRAS* in the development of both pancreatic cancer in mice [95] and human NSCLC [140,141]. In fact, NSCLC tumours with *LKB1:KRAS* mutations have a more aggressive phenotype compared to LKB1 wild-type tumours [142]. This discrepancy might be explained by different oncogenic mechanisms that cause tumour growth in different organs. In addition, LKB1-associated carcinogenesis in PJS patients and patients without a hereditary cancer predisposition may differ substantially. Furthermore, in the gastrointestinal tract, LKB1 has been suggested to act as a landscaper gene rather than being a tumour suppressor gene directly affecting the epithelium. Hamartomas as seen in PJS contain a prominent stromal component, raising the possibility of non-epithelial tissue origins in tumour growth. A previous study showed that heterozygous loss of *Lkb1* restricted to the smooth muscle lineage in mice was sufficient to induce gastrointestinal hamartoma formation [127]. Whether this so-called 'landscaper mechanism' is also associated with LKB1-associated GI carcinogenesis, and how this would cooperate with additional oncogenic events in the epithelial compartment of the GI tract, is yet unknown.

Because hamartomatous GI polyps with histological features similar to those in PJS were observed in patients with McCune Albright syndrome - MAS; caused by somatic activating mutations in *GNAS* - [391], we also investigated the effect of loss of LKB1 in the GNAS signalling pathway (**chapter 5**). We did not find any evidence for an interaction between *GNAS* and *LKB1*. It should be noted that smooth muscle proliferation, upon which the hypothesis of overlap between MAS and PJS was based, is not a histopathological feature specific for the PJS polyp. It can develop secondary to mechanical insults due to intestinal peristalsis during polyp growth.

To investigate whether the observed molecular alterations described in **chapter 4** truly cooperate with loss of LKB1 to induce GI cancer, mouse models are a useful research tool. By specific crossings, it is possible to generate *Lkb1*$^{+/-}$ mice that harbour intestinal epithelium-specific *p53* or *Kras* mutations, or Apc-deficiency; all mutations that also commonly occur in sporadic CRC. With such models, the additional role of Lkb1 loss in GI carcinogenesis can be investigated *in vivo*. In addition, these models will serve a valuable tool to investigate novel therapeutic strategies in the preclinical setting. Furthermore, it may clarify the controversy regarding the hamartoma-carcinoma sequence, similar to the well-known adenoma-carcinoma

sequence of sporadic CRC, as proposed for cancer growth in PJS [512]. Whether the carcinomas in the GI tract of PJS patients develop in sequence from hamartomas, or if they develop independently, remains one of the major questions within the PJS research field. Based on our own clinical experience and on available literature, we proclaim that GI hamartomas and carcinomas are two distinct entities of PJS. This opinion was strengthened by our study of molecular alterations in these two types of PJS lesions (**chapter 4**). Although there is some overlap in molecular alterations, particularly with regard to mutations in *P53*, substantial differences remain. Most strikingly, nuclear SMAD was absent in high-grade dysplastic foci of intestinal hamartomas, while it was present in all intestinal carcinomas. This might indicate involvement of inactive TGF-β/BMP signalling in dysplastic transformation of PJS hamartomas, but not in intestinal carcinoma development. Interestingly, Lkb1-deficient mesenchymal cells stop producing TGF-β, which is a crucial factor in suppressing tumour initiation and progression [127]. This resulted in the development of GI hamartomas, but not adenomas or carcinomas in the GI tract. Therefore, we suggest a role for aberrant TGF-β signalling in hamartoma development and transformation, which is not involved in PJS-associated CRC development.

TREATMENT OF LKB1-ASSOCIATED DISEASE

Up to now, PJS patients with cancer are treated according to standard treatment protocols for sporadic cancer. However, personalized treatment may avert the increased mortality in this patient group caused by the increased cancer risk. With the current molecular knowledge of LKB1-associated cancer, various treatment options have been suggested and investigated with variable success (reviewed in **chapter 2)**. The usual suspect as a target for therapy is inhibiting mammalian target of rapamycin (mTOR). Inactivation of LKB1 impairs the inhibition of mTOR, resulting in phosphorylation of the ribosomal protein S6. However, despite phospho-S6 expression in tissue of GI carcinomas and hamartomas of PJS patients, we could not identify a correlation between genetic loss of LKB1 and increased levels of phospho-S6 (**chapter 4**). This suggests that alternative pathways may be involved in mTOR activation, which could be targeted with other compounds (**chapter 2**). In 2010 however, a case report described the successful use of the mTOR inhibitor everolimus in a PJS patient with advanced pancreatic cancer [385]. This patient harboured a germline *LKB1* mutation and loss of the wild-type allele was detected in tumour tissue. The mTOR pathway was activated in the primary tumour, as evident from strong cytoplasmic staining for phospho-S6. Targeting the activated mTOR pathway with everolimus resulted in a good initial response of the tumour. In addition, no new large colorectal polyps developed in this patient during the treatment period of 9 months. Interestingly however, phospho-S6 protein levels did not change during everolimus treatment and after 9 months of treatment, progressive disease was found.

Nevertheless, this promising result led to the initiation of a clinical pilot study to treat PJS patients with everolimus (EVAMP study, ClinicalTrials.gov identifier: NCT01178151). The EVAMP study consists of two treatment arms. In the one arm, PJS patients with advanced malignancies are included for everolimus treatment. In the other arm, patients with high-risk GI hamartomas (defined as fast growing, recurrent polyps >15 mm, not accessible for safe endoscopic resection) are included for chemopreventive treatment with everolimus. In both arms, treatment consists of 10 mg everolimus orally daily. At the time of this writing, one

PJS patient was included in the chemopreventive arm of the EVAMP study. The effect on hamartoma growth was not evaluated yet.

Metformin, an activator of AMP-activated kinase (AMPK), is suggested as another promising drug for targeted treatment of cancer associated with loss of LKB1, since LKB1 is a direct activator of AMPK (**chapter 2**). However, metformin failed to inhibit cell growth in cells completely lacking LKB1, indicating that LKB1 is required for metformin to sort a growth inhibitory effect [106,217]. Therefore, instead of being a curative therapy, metformin might be a suitable drug to prevent hamartoma and carcinoma development and outgrowth in PJS patients. Studies to assess this in Lkb1-mouse models and in PJS patients have not been performed yet, but there are plans in the near future to start a chemoprevention trial with metformin in PJS patients.

A new approach for the treatment of LKB1-associated cancer could be inhibition of p21-activated kinases (PAKs, isoforms 1-6). PAKs are downstream effectors of the small G-proteins of the Rac and cdc42 family. They are involved in the regulation of cell survival, proliferation and migration. PAK overexpression and activation is reported in various types of cancer, including CRC [513]. Our *in vitro* data, described in **chapter 3**, also suggest that loss of LKB1 in CRC results in cell migration through cytoskeletal rearrangement mediated by PAK signalling. Specific PAK inhibitors could thus serve as potential therapeutics for cancer, including LKB1-associated cancer. To date, a handful of highly selective and potent small-molecule PAK inhibitors have been developed, including IPA-3 (PAK1 inhibitor) and PF-3758309 (PAK4 inhibitor) [514,515]. The efficacy and applicability of these agents is still investigated. Research showed that PF-3758309 blocked the growth of multiple human tumour xenografts in mice [516]. However, a phase I study investigating oral administration of PF-3758309 in patients with advanced solid tumours (ClinicalTrials.gov identifier: NCT009321126) was prematurely terminated due to the undesirable pharmacokinetic characteristics and the lack of an observed dose-response relationship.

In conclusion, the molecular background of LKB1-associated tumour growth is slowly being unravelled, leading to targeted treatment options for LKB1-associated cancer. PJS is a perfect model to study the relationship between genetics and disease phenotype, and to design rational therapy strategies. However, given the high levels of complexity in biological systems, the road to the development of adequate personalized treatment of LKB1-associated disease is still long and winding.

SURVEILLANCE IN PJS

Early detection is still the best option to treat cancer. Therefore, care for PJS patients should focus on surveillance. Surveillance of the GI tract (e.g. stomach, small bowel, and colon) should start at a young age, to prevent complications of (large) hamartomas. At later age, the detection of precursor lesions or malignancies at an early stage becomes important and surveillance should also involve other organs, such as the breast, the pancreas and the gynaecological tract.

Small bowel surveillance

Surveillance of the stomach and the colon of PJS patients is possible with common endoscopic techniques, and is therefore quite feasible. PJS hamartomas however, are located in the small

intestine in >90% of patients, predominantly in the jejunum [8]. It is this part of the GI tract that is difficult to examine, because of its extensive length and tortuous course. The application of several non-surgical techniques to examine the small bowel of PJS patients was reviewed in **chapter 6.** The golden standard for small bowel surveillance in PJS patients does not exist. MRI combined with enterography or enteroclysis is a safe and sensitive method for the detection of especially large (>15 mm) hamartomas in the small bowel. Balloon-assisted enteroscopy (BAE) offers both diagnostic and therapeutic options for small bowel surveillance in PJS patients. Both techniques are available in various Dutch hospitals and thus are valuable tools for small bowel surveillance in PJS patients. But how do MRI enteroclysis and BAE relate to each other? Does one technique exclude the other, or are they complementary? A clinical trial is currently been carried out in two Dutch academic hospitals to answer these questions. This prospective trial compares the diagnostic accuracy of MRI enteroclysis (MRE) and proximal double balloon enteroscopy (DBE) for the detection of small bowel hamartomas in PJS patients. Included patients first undergo an MRE, followed by a DBE within two months. Diagnostic yield – number, location, and size of polyps in the small bowel – of both examinations are analysed and compared. Furthermore, patient burden is investigated using questionnaires. Bowel preparation, duration of the examination, side effects of sedation, and pain during and after the examination are variables that should also be taken into account when establishing a surveillance examination that patients need to undergo regularly. Results of this trial are still awaited.

Although repeated abdominal surgery should be avoided as much as possible in PJS patients, the value of intraoperative enteroscopy for detection and removal of small bowel hamartomas should be noted. This combined endoscopic-surgical approach was first described in 1985 [426], and enables endoscopic inspection and clearance of the entire small bowel with surgical assistance if needed. Nowadays, advanced laparoscopic techniques minimize surgical invasiveness and duration of the procedure, and facilitate the intra-abdominal assistance of the endoscopist by the surgeon. Especially in PJS patients with a high polyp load, laparoscopic intraoperative enteroscopy seems to be the most elegant method for both patient and doctor.

Pancreatic cancer surveillance

Although the need for GI surveillance in PJS patients is established, this does not hold true for pancreatic cancer (PC) surveillance. Given the very poor prognosis of PC (5-year survival <5% [517], surveillance might be feasible in a selected group of individuals at high risk for developing PC, based on their family history or identifiable genetic predisposition. Whether PJS patients also belong to this high-risk group, and should be eligible for surveillance, was subject to debate since accurate risk estimates for PC in PJS were not available. We were now able to provide a reliable risk for PC in PJS, as described in **chapter 7**. This risk was calculated from data of the Dutch PJS cohort; a large, nationwide patient cohort with long-term follow-up. We found a cumulative risk for PC of 26% (95% CI 4-47) at the age of 70 years, and a relative risk of 76 (95% CI 36-160). The risk for pancreatico-biliary cancer (including pancreatic, distal bile duct and ampullary cancer) was even higher, with a cumulative risk of 32% (95% CI 11-52) at age 70, and a relative risk of 96 (95% CI 53-174). Given this high risk, we recommend PJS patients to undergo PC surveillance from the age of 30 years, regardless of family history of PC, by yearly EUS and/or MRI. However, this should only be done in well-defined research protocols, since correct implementation of such a surveillance program is far from clear yet. Available evidence supporting screening and surveillance is

limited to observational studies, and many unanswered questions remain. Who should be screened? At what age should screening begin and end? Which screening test should be used? And, most challenging, what should be the management of asymptomatic pancreatic lesions detected with screening or surveillance? All these questions should be answered with careful consideration of the principles of screening and practice for disease as proposed by Wilson and Jungner and published by the World Health Organisation (Box 1) [368], as nicely explicated by Harinck and colleagues [459]. Although the International Cancer of the Pancreas Screening (CAPS) Consortium recently developed statements on these important matters [477], additional research and evidence is needed to optimize screening and subsequent management. Until then, PC surveillance for high-risk individuals including PJS patients, is performed only within a research setting. Such a trial has been conducted by four large hospitals in the Netherlands. High-risk individuals, defined as first-degree relatives of patients with familial PC or carriers of a PC-prone gene mutation (i.e. *CDKN2A*, *LKB1*, *BRCA1*/2 and *P53*), undergo yearly surveillance of the pancreas from the age of 45 years or from the age of at least 10 years younger than the age of the youngest relative with PC. Surveillance involves both endoscopic ultrasound and MRI. PJS patients were also included. The main objectives of this study are to determine whether surveillance results in the detection of early carcinomas and benign precursor lesions, and whether this early detection improves the prognosis of patients. In addition, advantages of such a surveillance program are outweighed against the disadvantages such as overtreatment, costs and psychological burden. Results of this trial are expected within the near future.

Box 1. *Principles of screening by Wilson and Jungner [368].*

1.	The condition sought should be an important health problem.
2.	There should be an accepted treatment for patients with recognized disease.
3.	Facilities for diagnosis and treatment should be available.
4.	There should be a recognized latent or early symptomatic stage.
5.	There should be a suitable test or examination.
6.	The test should be acceptable to the population.
7.	The natural history of the condition, including development from latent to declared disease, should be adequately understood.
8.	There should be an agreed upon policy on whom to treat as patients.
9.	The cost of case-finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relationship to possible expenditure on medical care as a whole.
10.	Case-finding should be a continuing process and not a ‘once and for all’ project.

Breast cancer and surveillance

In contrast to surveillance of the pancreas, breast cancer screening and surveillance is well established and seems beneficial, both in the general population as in high-risk individuals such as *BRCA1/2* mutation carriers. Additionally, there is no doubt that the breast cancer risk is increased in female patients with PJS. The first PJS patient officially described in literature, one sister of the so-called ‘Hutchinson twins’, died of breast cancer [3]. But it remained unclear where PJS patients should be placed in the spectrum of high-risk individuals. Accurate risk estimates for breast cancer in PJS were lacking and nothing was known about

tumour characteristics, which may influence the design of surveillance protocols. Therefore, we evaluated breast cancer cases detected in the Dutch PJS cohort and provide an accurate breast cancer risk estimate for female PJS patients in **chapter 8**. Although our study confirms that this risk is highly increased (relative risk 6, 95% CI 3-13), and even approaches that of *BRCA1/2* mutation carriers, there are important considerations that refrain from following the surveillance recommendations as formulated for this latter patient group. However, given the very small number of PJS patients, it is desirable to implement a surveillance program for PJS patients in the current, well organized breast cancer surveillance care as exists in our country. Extensive multidisciplinary deliberation led us to the formulation of a breast cancer surveillance recommendation for PJS patients, as depicted in Box 2. This recommendation is derived from the surveillance guidelines for *BRCA1/2* mutation carriers, as formulated in the Dutch breast cancer guidelines (available at www.oncoline.nl) Although extrapolating data can be misleading, sometimes one has to do with the means available. Though, future evaluation of the proposed recommendation for breast cancer surveillance in PJS patients is required.

Box 2. *Adjusted Dutch surveillance recommendations for Peutz-Jeghers syndrome patients.*

Examination[1]	Starting age	Interval
History, physical examination (including testicular palpation), and hemoglobin analysis	10 years	1 year (paediatrician)
MRI enteroclysis[2]	10 years	2-3 years
(chapter 6)		
Gastroduodenoscopy	20 years	2-5 years (depending on findings)
Colonoscopy	25-30 years	2-5 years (depending on findings)
MRI and EUS	30 years	1 year, only in a prospective ongoing trial
(chapter 7)		
Mammography and breast MRI[3]	30 years	1 year
(chapter 8)		
Pelvic exam, cervical smear, transvaginal ultrasonography, and CA-125	25-30 years	1 year

EUS: endoscopic ultrasound; MRI: magnetic resonance imaging.
[1]Earlier and/or more frequently in symptomatic patients or if clinically indicated.
[2]Polyps >10-15 mm in diameter are an indication for double-balloon enteroscopy with polypectomy. In addition, we recommend intra-operative enteroscopy with polyp removal in patients with a high-polyp load.
[3]If mammography provides a reliable assessment of the breast tissue, MRI can be omitted.

Family planning and prenatal testing

Testing an unborn child for PJS (prenatal diagnosis, PND), providing the causing germline mutation is known in the affected parent, and possibly terminating a pregnancy in case of an affected foetus could be seen as an extreme form of prevention. PND by means of chorionic villus sampling is widely available in the Netherlands. However, the effects of such a test, and especially the impact of the decision-making in case of an affected foetus, should not be underestimated. In case of pre-implantation genetic diagnosis (PGD), the decision to terminate a pregnancy needs not to be made because only unaffected embryos are transferred into the

uterus. However, the PGD procedure is physically and psychologically burdensome [510], mainly because of the need for pregnancy by *in vitro fertilization*. In **chapter 9** we describe that PJS seriously influences the desire to have children of patients because of the disease burden. Additionally, our results indicate that PJS patients consider PGD more acceptable than the use of PND with the probability of pregnancy termination.

The actual use of PND amongst PJS patients in the Netherlands is unknown, but data are available for the use of PGD. PGD has been performed in the Netherlands since 1995. Only one academic hospital (University Hospital Maastricht) is authorized to perform this treatment. In the period from 1995 until 2011, 3 couples were referred for PGD for the indication PJS, including 2 in 2011 [518]. This might indicate that the awareness of the possibility of antenatal genetic testing for PJS patients with a desire to have children increases in patients and doctors, which is a step forward in the care for PJS patients.

GENERAL CONSIDERATIONS

As mentioned repeatedly in this discussion, care for PJS patients should focus on surveillance. Box 2 shows the slightly modified Dutch surveillance recommendation, as a result of the new insights provided by this thesis (see also chapter 1, Box 2, page 15). But this recommendation only reflects expert opinion; the lack of data from randomized controlled trials as well as comparative observational studies remains a major drawback. A systematic review assessed the current literature on the management of PJS [30]. This revealed 15 studies concerning PJS and surveillance, all with low or very low category of evidence (III; non-experimental descriptive study or IV; expert opinion) and grading of recommendation (B or C). This highlights the paucity of good-quality data on which management recommendations are based.

It was attempted by colleagues in the UK to provide such data. In a retrospective study they aimed to assess the outcomes from GI surveillance in PJS patients [111]. Fifty-one young patients (median age at first contact 20 years) were followed for 683 patient years (median 10 years). During follow-up, patients underwent enteroscopies or surgical procedures during which 2461 polypectomies were performed (of which only 6 hamartomas contained atypia or dysplasia). No luminal GI cancers were diagnosed in any of these patients. The authors conclude that, although evidence of benefit from GI tract surveillance in terms of cancer detection or prevention is not robust, surveillance is still required to prevent polyp-related complications. These are valuable observations, but only well-designed, prospective studies can provide robust evidence for surveillance guidelines.

Another important question is whether the possible clinical benefit of surveillance outweighs the psychological burden of surveillance, as required according to the principles of Wilson & Jungner (Box 1). All currently proposed guidelines for PJS patients, including the Dutch surveillance guidelines, are highly intensive and burdensome. Surveillance should already start at a young age and needs to be performed regularly. It concerns multiple organs, and several of the surveillance modalities are invasive. A recent review evaluated all studies that investigated psychological distress and quality of life in individuals under surveillance for hereditary cancer, including a report of our research group concerning PJS patients [497,519]. Although participating in surveillance programs in most common hereditary cancers was generally associated with normal levels of distress, surveillance of hereditary cancer syndromes with a high risk of multiple tumours, such as PJS, appeared to be associated with a worse quality of life, suggesting that these patients may be in need of psychological support. Therefore, if patients

need multiple surveillance examinations, ideally one physician who knows all the aspects of the disease should coordinate these examinations, and additionally guide the patients through surveillance. Furthermore, given the very low prevalence of PJS, care for these patients should be centred in a limited number of (academic) hospitals to ensure uniformity of care.

Despite the above considerations and proposals, the rarity of PJS is an obstacle for further research. PJS is considered an orphan disease, defined in the United States as a condition that affects fewer than 200.000 people [520]. In Europe most countries use the European Orphan Drug regulation definition of a prevalence of less than 1 in 2000 [521]. PJS is, due to its low prevalence, not a hot topic in the field of biomedical research. This translates for example into difficulties in obtaining research funding, recruiting sufficient numbers of patients for meaningful research studies, finding valid statistical approaches when small numbers are involved, and finding a platform for the publication of results. One way to overcome a number of these problems is by joining forces. Currently, several groups of experts throughout the world are engaged in research of PJS individually. If these groups would form a network and cooperate, studies with high patient numbers can lead to convincing results concerning the nature of the disease, surveillance benefits, and treatment options. The establishment of such a multidisciplinary, international consortium is not easy and takes a lot of time and effort. But only in this way we will be able to contribute to a better future perspective for PJS patients.

CONCLUDING REMARKS

The tumour suppressor gene *LKB1* links the rare Peutz-Jeghers syndrome and the development of cancer. Disease characteristics and the natural course of PJS, as well as the function of LKB1, have been gradually unravelled in recent years. Novel insights as provided by this thesis, concerning the role of LKB1 in sporadic and PJS-associated cancer, have contributed to more understanding of this link. However, many uncertainties remain. Due to the elucidation of molecular mechanisms, some promising medical therapies for LKB1-associated cancer are on the horizon but are still being evaluated. In addition, evidence is accumulating that not only the luminal GI tract, but also other organs such as the pancreas and breasts require surveillance because of the considerably increased cancer risk. Though, the design of and adherence to surveillance guidelines is problematic due to the lack of robust evidence for the benefit of such surveillance programs. Therefore, research for the missing links between PJS and cancer should be continued.

Chapter 11

Appendix

References
Summary
Samenvatting
Dankwoord
List of publications
List of co-authors
Curriculum Vitae
PhD portfolio

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SUMMARY

Peutz-Jeghers syndrome (PJS) is a rare, autosomal dominant inherited disorder, first described by the Dutch physician Jan Peutz in 1921. It is clinically characterized by mucocutaneous pigmentations, gastrointestinal (GI) polyposis and an increased cancer risk in adult life. Hamartomatous polyps can develop already in the first decade of life and may cause various complications, including abdominal pain, bleeding, anaemia, and acute intestinal obstruction. Cancer can develop at a later age, both in the GI tract as in other organs. The medical management of PJS mainly consists of surveillance, firstly to detect and remove hamartomas, and secondly to detect cancer at an early stage.

PJS is caused by germ line mutations in the *LKB1* tumour suppressor gene. LKB1 is considered a "master kinase", regulating a range of cellular processes via the AMPK/mTOR signalling pathway, a possible target for treatment. In **chapter 2** the function and regulation of the LKB1/AMPK/mTOR signalling pathway is explained. In addition, we describe how aberrant signalling of this cascade can cause disease and cancer, and we reflect on the design and use of rational treatment targeting LKB1/AMPK/mTOR-associated cancer.

Despite the strong association between *LKB1* germline mutations and the increased cancer risk in PJS patients, *LKB1* is not commonly mutated in sporadic cancers, except for lung cancer. Its exact tumour suppressor functions in sporadic and PJS-associated colorectal cancer development remain elusive. In **chapter 3** of this thesis, we investigated the role of LKB1 in colon cancer *in vitro.* To this aim, LKB1 expression was knocked down in HT29 human colon cancer cells. Although reduced LKB1 expression did not affect growth-related properties, it did induce migration of these cells by affecting cytoskeletal structures. This might suggest that the tumour suppressor LKB1 acts on the cancerous process through cytoskeletal rearrangements.

In addition, we studied which molecular pathways could be involved in LKB1-associated GI carcinogenesis. A unique sample set of tissue of 15 GI carcinomas and 9 dysplastic hamartomas of PJS patients was analysed at DNA and protein level. Our findings as described in **chapter 4** suggest a role for mutant *P53* in PJS-associated GI carcinogenesis, in addition to a haploinsufficient function of the *LKB1* tumour suppressor gene. Loss of nuclear SMAD4 and complete loss of *LKB1* may be involved in dysplastic transformation of GI hamartomas. The *BRAF* and *GNAS* genes do not seem to be involved in the pathogenesis of GI tumours in PJS (**chapter 4 and 5**).

Chapter 6 provides a review of the literature concerning small bowel endoscopy in PJS patients. Radiologic and endoscopic imaging modalities with diagnostic value are discussed, as well as advanced endoscopy techniques that can serve as a diagnostic and therapeutic tool in the surveillance of the small bowel.

In **chapter 7 and 8** we provide reliable estimates for respectively pancreatic and breast cancer risks in PJS patients. In addition, clinical and histological characteristics of PJS-associated tumours are described in these chapters. For these studies, we used the Dutch PJS database, comprising of nearly all PJS patients in the Netherlands. Given the highly increased risk for pancreatico-biliary cancer (RR 96), PJS patients are eligible for surveillance within well-defined research programs (**chapter 7**). Based on the highly increased risk for breast cancer risk (RR 6), we advised to start annual breast cancer surveillance in female PJS patients at the age of 30 years, with mammography and MRI (**chapter 8**).

In our final study, described in **chapter 9,** we assessed the attitude of PJS patients towards family planning, prenatal diagnosis (PND) and pregnancy termination, and pre-implantation genetic diagnosis (PGD) with a questionnaire study. In conclusion, the diagnosis of PJS influences the decisions regarding family planning in one third of PJS patients, especially in women. In addition, most patients have a negative attitude towards pregnancy termination after PND, while PGD in case of PJS is judged more acceptable.

Finally, this thesis concludes with a general discussion and future perspectives for PJS in **chapter 10**.

SAMENVATTING

Het Peutz-Jeghers syndroom (PJS) is een zeldzame, autosomaal dominant overervende aandoening. De Nederlandse arts Jan Peutz beschreef in 1921 als eerste een familie waarin een aantal gezinsleden zowel gastro-intestinale poliepen als opvallende pigmentaties in het gezicht en op de slijmvliezen van de mondholte vertoonden. Tegenwoordig wordt PJS gekarakteriseerd door mucocutane pigmentaties, gastro-intestinale polyposis en een verhoogd kankerrisico op volwassen leeftijd.

De hamartomateuze PJS poliepen kunnen zich al op kinderleeftijd (< 10 jaar) manifesteren en kunnen klachten van buikpijn, bloedverlies, anemie of acute darmobstructie veroorzaken. Kanker kan zich op latere leeftijd ontwikkelen, zowel in het maag-darmkanaal als in andere organen. De zorg voor PJS patiënten bestaat voornamelijk uit surveillance; in eerste instantie om hamartomen te detecteren en te verwijderen en op latere leeftijd om kanker in een vroeg stadium te diagnosticeren.

PJS wordt veroorzaakt door een kiembaanmutatie in het LKB1 tumor suppressor gen. LKB1 wordt beschouwd als een “master-kinase” en reguleert cellulaire processen via de AMPK/mTOR signaleringscascade. Deze cascade is een mogelijk aangrijpingspunt voor therapie. In **hoofdstuk 2** wordt de functie en regulatie van de LKB1/AMPK/mTOR signaleringscascade uitgelegd. Tevens wordt beschreven hoe gestoorde signalering van deze cascade kan leiden tot kanker en wijden we uit over mogelijke rationele behandeling hiervan.

Hoewel kiembaanmutaties van *LKB1* sterk geassocieerd zijn met een verhoogd risico op het krijgen van kanker in PJS, is *LKB1* niet vaak gemuteerd in sporadische kanker, behalve in longkanker. Wat de exacte tumor suppressor functies van LKB1 zijn in colorectaal kanker is dan ook nog onbekend. In **hoofdstuk 3** van dit proefschrift hebben we *in vitro* de rol van LKB1 in darmkanker onderzocht. Hiervoor werd de expressie van LKB1 in humane HT29 darmkankercellen uitgeschakeld. Hoewel de verminderde expressie van LKB1 geen effect had op de groei-eigenschappen van deze cellen, leidde het wel tot meer migratie van deze cellen door aantasting van het cytoskelet. Dit zou kunnen suggereren dat de tumor suppressor LKB1 het kankerproces beïnvloed door modulatie van het cytoskelet.

In aanvulling op deze studie hebben we onderzocht welke moleculaire routes betrokken zijn bij LKB1-geassocieerde gastro-intestinale kankergroei. Weefsel van 15 gastro-intestinale carcinomen en 9 dysplastische hamartomen van PJS patiënten werd geanalyseerd op DNA- en eiwitniveau. Onze bevindingen (beschreven in **hoofdstuk 4 en 5**) suggereren een rol voor mutant *P53*, samen met de haplo-insufficiënte functie van LKB1, in PJS-geassocieerde gastro-intestinale kankergroei. Verlies van nucleair SMAD4 en volledig verlies van *LKB1* zou betrokken kunnen zijn bij de dysplastische transformatie van gastro-intestinale hamartomen. De *BRAF* en *GNAS* genen lijken echter niet betrokken te zijn bij de pathogenese van gastro-intestinale tumoren in PJS.

Hoofdstuk 6 geeft een overzicht van de beschikbare literatuur over dunne darmscopie bij PJS patiënten. Eerst bespreken we radiologische en endoscopische technieken met diagnostische waarde. Vervolgens komen geavanceerde endoscopie-technieken aan bod, die zowel diagnostisch als therapeutisch ingezet kunnen worden bij de dunne darmsurveillance.

Hoofdstuk 7 en 8 verstrekken betrouwbare risicoschattingen voor pancreas- en borstkanker in PJS patiënten. Tevens beschrijven we de klinische en histologische kenmerken van de tumoren. Voor beide studies hebben we gebruik gemaakt van de Nederlandse PJS database, waarin nagenoeg alle PJS patiënten in Nederland zijn opgenomen. Gezien het

sterk verhoogde risico op kanker in de pancreatico-biliaire regio (RR 96) lijkt het wenselijk om pancreassurveillance aan te bieden aan PJS patiënten, maar alleen in goed omschreven onderzoeksprogramma's (hoofdstuk 7). Daarnaast adviseren wij, gebaseerd op het sterk verhoogde risico op borstkanker (RR 6), jaarlijkse borstkanker surveillance voor vrouwelijke PJS patiënten vanaf de leeftijd van 30 jaar, met mammografie en eventueel MRI (**hoofdstuk 8**).

Voor de laatste studie (beschreven in **hoofdstuk 9**) hebben we door middel van vragenlijsten onderzocht hoe PJS patiënten denken over gezinsplanning, prenatale diagnostiek (PND) eventueel gevolgd door zwangerschapsbeëindiging en pre-implantatie genetische diagnostiek (PGD). Het blijkt dat de diagnose PJS voor een derde van de patiënten de gezinsplanning beïnvloedt, voornamelijk voor vrouwen. Daarnaast hebben de meeste patiënten een negatief beeld van zwangerschapsbeëindiging na PND, terwijl ze PGD in geval van PJS meer acceptabel achten.

Tot slot wordt dit proefschrift afgesloten met een algemene discussie en toekomstperspectieven (**hoofdstuk 10**).

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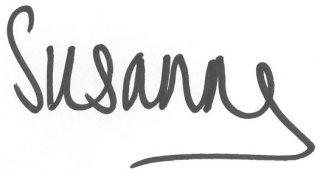

LIST OF PUBLICATIONS

Publications related to this thesis

Korsse SE, Biermann K, Offerhaus GJ, Wagner A, Dekker E, Mathus-Vliegen EM, Kuipers EJ, van Leerdam ME, van Veelen W. *Identification of molecular alterations in gastrointestinal carcinomas and dysplastic hamartomas in Peutz-Jeghers syndrome.*
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Tijdschrift voor Neurologie en Neurochirurgie 2007;8:383-388.

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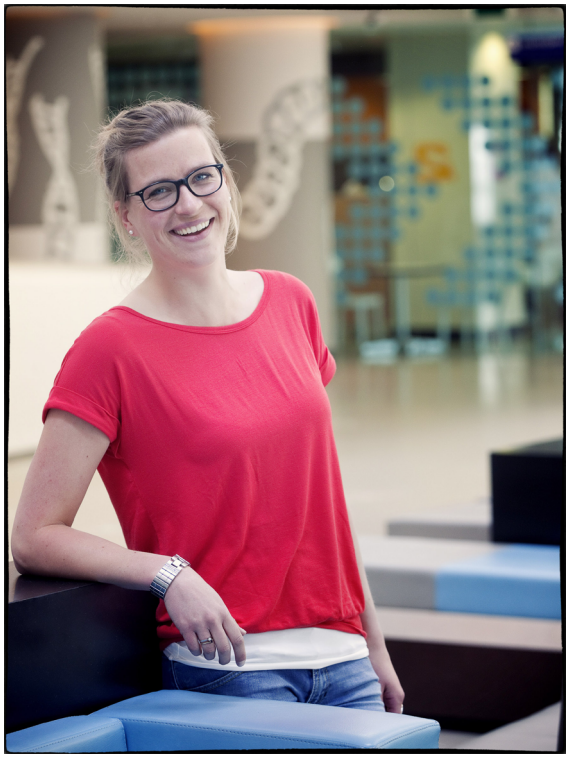
CURRICULUM VITAE

Susanne Elisabeth Korsse werd geboren op 14 januari 1985 te Apeldoorn. In 1996 startte zij met het VWO op het Veluws College Mheenpark. In de zomer van 1999 verhuisde zij naar Breda waar ze de laatste 3 jaar van het VWO volgde aan het Onze Lieve Vrouwe Lyceum. Na het behalen van haar diploma in 2002 woonde zij 5 maanden in Salamanca, Spanje, om de Spaanse taal te leren, waarna zij in 2003 startte met de studie Geneeskunde aan de Universiteit Utrecht. Tijdens het derde jaar van haar studie liep zij een klinische stage op de afdeling Chirurgie van het Hospital Civil de Guadalajara in Mexico en voor het co-schap Sociale Geneeskunde in het vijfde jaar van haar studie week zij uit naar het St. Luke's Hospital in Malawi. In 2009 verrichte zij haar wetenschappelijke stage op het laboratorium van de afdeling Maag-, Darm- en Leverziekten van het Erasmus MC te Rotterdam, onder begeleiding van dr. W. van Veelen. Na het behalen van haar artsexamen in december 2009 startte zij op 1 januari 2010 op dezelfde afdeling met promotieonderzoek naar het Peutz-Jeghers syndroom, in samenwerking met het AMC te Amsterdam. Tijdens dit onderzoek werd zij begeleid door haar promotoren professor E.J. Kuipers en professor E.M.H. Mathus-Vliegen en haar co-promotoren dr. W. van Veelen en dr. M.E. van Leerdam. De resultaten van dit onderzoek staan beschreven in dit proefschrift. Susanne werkt op dit moment als arts-assistent Interne Geneeskunde/MDL-ziekten in het Ikazia Ziekenhuis te Rotterdam.



PhD PORTFOLIO

Courses

2010 Moleculaire diagnostiek voor dokters, Molecular Medicine Erasmus Postgraduate School, Rotterdam, the Netherlands

Course on Molecular Diagnostics V, Molecular Medicine Erasmus Postgraduate School, Rotterdam, the Netherlands

Biostatistics for Clinicians, Netherlands Institute for Health Sciences, Rotterdam, the Netherlands

Basic and translational oncology, Molecular Medicine Erasmus Postgraduate School, Rotterdam, the Netherlands

2011 English Biomedical Writing and Communication, Erasmus MC University Medical Centre, Rotterdam, the Netherlands

Regression Analysis for Clinicians, Netherlands Institute for Health Sciences, Rotterdam, the Netherlands

BROK Cursus – Good Clinical Practice, Erasmus MC University Medical Centre, Rotterdam, the Netherlands

Seminars and workshops

2010 Talentendag, Nederlandse Organisatie voor Wetenschappelijk Onderzoek, the Netherlands

Young Investigator Workshop, ASNEMGE, Spain, Barcelona

Oral presentations at (inter)national conferences

2010 Peutz-Jeghers syndrome and family planning: the attitude towards prenatal genetic testing. Najaarsvergadering NVGE, Veldhoven, the Netherlands

2011 Peutz-Jeghers syndrome and family planning: the attitude towards prenatal genetic testing. 4th Biennial Meeting International Society for Gastrointestinal Hereditary Tumours (InSiGHT), San Antonio, Texas, USA.

2012 Gastrointestinal carcinomas of Peutz-Jeghers syndrome patients: a further look. Voorjaarsvergadering NVGE, Veldhoven, the Netherlands

Molecular alterations in dysplastic hamartomas of Peutz-Jeghers syndrome patients. Digestive Disease Week, San Diego, CA, USA

Poster presentations at (inter)national conferences

2010 Peutz-Jeghers syndrome and family planning: the attitude towards prenatal genetic testing. United European Gastroenterology Week, Barcelona, Spain.

2011 Reduced expression of LKB1 in colon cancer cells increases cell migration, and survival during metabolic stress. Voorjaarsvergadering NVGE, Veldhoven, the Netherlands

2012 Molecular alterations in gastrointestinal carcinomas of Peutz-Jeghers syndrome patients. Digestive Disease Week, San Diego, CA, USA

Molecular alterations in gastrointestinal carcinomas of Peutz-Jeghers syndrome patients. United European Gastroenterology Week, Amsterdam, the Netherlands

Molecular alterations in dysplastic hamartomas of Peutz-Jeghers syndrome patients. United European Gastroenterology Week, Amsterdam, the Netherlands

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Memberships

2010 Dutch Society of Gastroenterology and Experimental Gastroenterology and Hepatology

Tutoring

2012 Supervision internship Bsc student C. Voorsluijs, Opleiding Biologie en Medisch Laboratoriumonderzoek, Hogeschool Rotterdam

Tutoring bachelor students Faculty of Medicine, Erasmus University, Rotterdam, the Netherlands

Others

2010 Peutz-Jeghers syndrome information day for patients, Erasmus MC University Medical Centre, Rotterdam, the Netherlands

“ALWAYS LAUGH WHEN YOU CAN. IT IS CHEAP MEDICINE.”

LORD BYRON