Role of BRCA1 and BRCA2 mutations in pancreatic cancer

Julia B Greer, David C Whitcomb

Germline mutations in the tumour suppressor genes breast cancer antigen gene (BRCA1) and BRCA2 have been proven to portend a drastically increased lifetime risk of breast and ovarian cancers in the individuals who carry them. A number of studies have shown that the third most common cancer associated with these mutations is pancreatic cancer. BRCA1/2 mutations are characterised by “allelic” or “phenotypic” heterogeneity, in that they demonstrate differing cancer expressivity between and within pedigrees that segregate their mutations. If the same mutation is present in all our cells, why do some families with a given mutation display predominantly breast cancer? Why do other lineages show a preponderance of ovarian cancer? And why would some families have members who develop mostly or exclusively pancreatic cancer—a cancer that occurs more commonly in men and that lacks consistent evidence for a hormonal basis to its aetiology—which is clearly the case for breast and ovarian cancer? The answer is that other modifying genetic and environmental factors must interact to preferentially incite carcinogenesis in one organ over another. We are just beginning to elucidate what these factors are.

An unknown but significant proportion of cancers develop in people who carry a germline mutation in a cancer-predisposing gene. Identification of cancer-predisposing genetic mutations in susceptible individuals affords the opportunity to practise preventive medicine. In the realm of pancreatic cancer, whose case-fatality rate approaches 1, prevention may be our most potent weapon. Contrary to diseases that follow Mendelian genetics, pancreatic cancer is an aetiologically complex disease whose development is contingent on the independent and joint effects of genes and environment. Isolated case reports observed that pancreatic cancer aggregates in some families, and current data suggest that as many as 10% of cases of pancreatic cancer may be due to an underlying inherited component. A recent prospective study estimated the risk of developing pancreatic cancer among first-degree relatives of a patient with pancreatic cancer to be 18-fold in relatives with pancreatic cancer who do not fulfil the criteria for other familial cancer syndromes.

Familial pancreatic cancer (FPC) is a term applied to families with at least two first-degree relatives with pancreatic cancer who do not fulfil the criteria for other familial cancer syndromes. In the context of FPC, studies have shown that pedigrees with germline mutations in BRCA1 and BRCA2 have an increased lifetime risk of pancreatic cancer.

The quality of a cancer-predisposing genetic mutation is described in terms of penetrance (number of individuals with the gene who develop cancer) and expressivity (type of cancer that develops).

BRCA1 and 2 are tumour suppressor genes that are inherited in an autosomal dominant fashion with incomplete penetrance. Loss of the function of tumour suppressor genes is instrumental in the cascade of genetic changes that controls cell growth and differentiation and drives tumorigenesis. Both BRCA proteins engage in transcriptional regulation of gene expression as well as the recognition or repair of DNA damage, particularly double-strand breaks. In patients with sporadic pancreatic cancer, BRCA1/2 are mutated in the most advanced pancreatic intraepithelial neoplasia lesions, whereas a germline mutation in either gene represents the earliest risk factor in many FPC kindreds.

PROGRESS IN BRCA1/2 RESEARCH

Discoveries in BRCA1/2 mutation research have paralleled advancements in techniques of genetic analysis. Early studies focused on the founder BRCA1/2 mutations common to the Ashkenazi Jewish population—185delAG, 5382insC in BRCA1 and 6174delT in BRCA2—and their role in familial breast and ovarian cancer. In the US population, it is estimated that between 1 of 345 and 1 of 1000 individuals carry a BRCA mutation, compared with approximately 1 in 40 individuals of Ashkenazi Jewish descent. Approximately 10% of both breast and ovarian cancers are found in women with one or more mutations in the BRCA1 or BRCA2 genes. In a recent analysis of relatives of 1008 unselected cases with BRCA1/2 mutations, BRCA1 and BRCA2 germline mutations confer 54% and 23% cumulative risks of ovarian cancer, respectively, as well as over 80% cumulative risk of breast cancer by age 80.

The cloning of BRCA2 occurred after the identification of a homozygotic deletion in 13q12.3 in a pancreatic carcinoma, making BRCA2 a candidate tumour suppressor gene not only for early-onset breast carcinoma but also for pancreatic cancer. A family history of pancreatic cancer is a consideration in the evaluation of a patient who presents with a primary pancreatic tumour, as a familial component may explain the presentation or nature of the tumour.

Abbreviations: BRCA, breast cancer antigen gene; CHEK2, cell-cycle checkpoint kinase gene; FPC, familial pancreatic cancer; PRSS1, protease serine 1 (trypsin 1) gene; SPINK1, serine protease inhibitor Kazal type 1 gene

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cancer has been observed to predict the presence of a BRCA2 mutation, and pancreatic cancer is more frequent in breast cancer families with BRCA2 mutations than in families without mutations. In addition, research has estimated that BRCA2 mutation carriers have a 10-fold higher risk of developing pancreatic cancer than the general population.

A study of BRCA1/2 mutation positive patients with breast cancer and their families observed that the average age of onset of pancreatic cancer in their BRCA1 carriers was 65 years. The National Familial Pancreas Tumor Registry demonstrated that the average age of pancreatic cancer in BRCA2 mutation positive patients was 66 years, essentially the same as in their control study group of sporadic cases (age 66.7 years). Approximately 80% of tumours from BRCA1 and BRCA2 carriers undergo inactivation of the wild-type allele by loss of heterozygosity, a relatively late event in pancreatic cancer development, which reflects its older age of onset in comparison with other genetically related cancers.

BRCA1 AND BRCA2: A MULTI-CANCER PHENOTYPE

Attempts to quantify pancreatic cancer risk in germline BRCA1/2 mutation carriers vary, depending on study design and source population.

The overall relative risk of pancreatic cancer as determined by the Breast Cancer Linkage Consortium—which evaluated multiple breast cancer families—was 2.26 for BRCA1 mutation carriers and 3.55 for BRCA2 mutation carriers, although more recent estimates of risk for BRCA2 carriers are in the 2–9 region. In the 10 years since the discovery of these genes, hundreds of patients with hereditary breast and/or ovarian cancer have been observed to segregate these mutations, yet the incidence of pancreatic cancer in many of these families remains somewhat low. We are now aware of the existence of hundreds of BRCA1/2 mutations, and there is great phenotypic heterogeneity (ie, differing expressivity) between family lineages that share a common mutation. Interesting relationships have been observed between and within the BRCA genes. For example, a study of 179 pedigrees with identified germline mutations (104 BRCA1 and 75 BRCA2) from six Italian centres observed that the proportion of ovarian cancer was increased by mutations in the 5’ portion of BRCA1, and the presence of prostate or pancreatic cancer in a family was correlated with the presence of ovarian cancer in BRCA2 mutation carriers. Other research demonstrated that breast cancer cases who have relatives affected by only breast cancer (but not ovarian) appear to be preferentially mutated in BRCA2, whereas even a single case of ovarian cancer in the family is a predictor of a BRCA1 mutation.

Investigators reviewing first- and second-degree relatives of 440 ethnically diverse families who segregated a variety of BRCA2 mutations noted that (1) Ashkenazi Jewish families with the 6174delT founder mutation were more likely to have a family member with ovarian cancer and less likely to have family members with prostate cancer, (2) French-Canadian families had a reduced presence of ovarian cancer and (3) families of Polish ancestry had a reduced frequency of pancreatic cancer.

This group and others also recognised an association of mutations in exon 11 (nucleotides 3035–6629) in BRCA2—the so-called ovarian cancer cluster region—with a higher ratio of ovarian to breast cancer. In addition, a polymorphic stop codon in the coding region of BRCA2 (K3326X) was shown in a large case-control study to be significantly more prevalent in individuals with FPC than controls. Nonetheless, some families known to segregate the most common BRCA2 mutation—6174delT—recount no family history of any cancer and many patients with pancreatic cancer caused by a germline mutation in BRCA1/2 do not have a pedigree suggestive of FPC at all.

CATEGORISING BRCA1/2 PEDIGREES ON THE BASIS OF EXPRESSIVITY

In an attempt to classify BRCA1/2-linked cancer pedigrees, some researchers felt that families with breast and ovarian cancer but rarely pancreatic cancer would constitute one group, those with a preponderance of pancreatic cancer and a few other cancer types would be a second group, and the third group would be families with no documented cancer history who develop apparently “sporadic” pancreatic cancer but on evaluation are determined to have a BRCA1/2 mutation.

A theory on why, given identical mutations, some families tend to develop one type of tumour rather than another is that unknown genetic or environmental factors play a role in expressivity.

Because BRCA1/2 mutations were first identified just slightly over a decade ago, creating three mutually exclusive categories of BRCA1/2 families at this stage is likely to be premature. Broader-scope prospective and retrospective studies based on cancer registry data rather than questionnaires could show distinct trends in cancer expressivity within pedigrees. Additionally, because women in previous generations could have developed aggressively fatal breast or ovarian cancer before the age when pancreatic cancer in mutation carriers typically presents, the undertaking of preventive mastectomy and oophorectomy by female BRCA1/2 carriers may “unveil” more pancreatic cancer in certain families.

GENE–GENE INTERACTIONS: HIGH-RISK CANDIDATE MUTATIONS AND POLYMORPHISMS

Additionally, we do have some knowledge about selected genetic and environmental factors that can drive carcinogenesis in one organ over another in selected families (table 1).

The incidence of pancreatic cancer is increased in a number of other genetic and familial cancer syndromes with established

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Risk factors for cancers associated with BRCA1 and BRCA2 mutations</th>
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<tbody>
<tr>
<td>Cancer type</td>
<td>Increase risk</td>
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<tr>
<td>Pancreatic</td>
<td></td>
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<tr>
<td>Cigarette smoking</td>
<td>Low-fat, high fruit and</td>
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<tr>
<td>Chronic pancreatitis (hereditary,</td>
<td>vegetable diet</td>
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<tr>
<td>idiopathic, tropical, alcohol</td>
<td>Abstinence/quitting</td>
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<tr>
<td>related)</td>
<td>smoking</td>
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<tr>
<td>High-fat, meat-based diet</td>
<td>Pancreactectomy</td>
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<tr>
<td>Breast</td>
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<tr>
<td>Combined oestrogen–progestin</td>
<td>Prophylactic mastectomy</td>
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<tr>
<td>hormone replacement therapy</td>
<td>Prophylactic oophorectomy</td>
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<tr>
<td>Alcohol</td>
<td>SERMs and aromatase inhibitors</td>
</tr>
<tr>
<td>Obesity</td>
<td>Cigarette smoking?</td>
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<tr>
<td>Ionising radiation (especially in</td>
<td>Prophylactic oophorectomy</td>
</tr>
<tr>
<td>puberty)</td>
<td></td>
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<tr>
<td>Ovary</td>
<td></td>
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<tr>
<td>Hormone replacement therapy</td>
<td>Oral contraceptive use</td>
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<tr>
<td>Nulliparity (lack of childbearing)</td>
<td>Tubal ligation</td>
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<tr>
<td>Male, asbestos exposure</td>
<td>Breastfeeding</td>
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<tr>
<td>PCOS</td>
<td>Parity (childbearing)</td>
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<tr>
<td>Infertility</td>
<td>Prophylactic oophorectomy</td>
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</table>

HRT, hormone replacement therapy; PCOS, polycystic ovarian syndrome; SERMs, selective oestrogen receptor modifiers.

†Evident only in BRCA1/2 mutation carriers.
germline mutations such as Peutz–Jeghers syndrome and hereditary non-polyposis colorectal cancer. Hereditary pancreatic neoplasms, linked often to mutations in the cationic trypsinogen gene, protease serine 1 (trypsin 1) gene (PRSS1), is associated with a markedly increased lifetime risk of pancreatic cancer. If a BRCA1/2 mutation carrier and an individual with a PRSS1 mutation had children, some of their offspring might have an exponentially increased risk of pancreatic cancer. Similarly, serine protease inhibitor Kazal type 1 gene (SPINK1) mutations are associated with tropical pancreatic neoplasms, which carries a high lifetime risk of pancreatic cancer. If a BRCA1/2 mutation carrier also had a separate pancreatic cancer-predisposing genetic mutation, one might observe greater numbers of pancreatic cancer compared with other BRCA1/2-linked cancers. Along these same lines of reasoning, protein truncating mutations in the cell-cycle checkpoint kinase (CHEK2) are associated with an increased risk of breast as well as prostate cancers and might drive certain families to display a greater incidence of breast and/or prostate cancer than pancreatic cancer.

There are numerous other candidate genes as well as polymorphisms for cancers that are observed in BRCA1/2 positive carriers that might not play an important contributing role to pancreatic carcinogenesis.

GENE–ENVIRONMENT INTERACTIONS: THE SMOKING AND OESTROGEN CONNECTION

The single environmental factor shown to be of great risk for pancreatic cancer is cigarette smoking, which is estimated to account for approximately 25–30% of all pancreatic tumours. A recent interview-based study of 247 pancreatic cancer case probands (and their 1816 first-degree relatives) and 420 population-based controls (and their 3157 first-degree relatives) determined that a positive family history of pancreatic cancer or ever-smoking cigarettes more than doubled the pancreatic cancer risk (RR 2.49 and 2.04, respectively). Additionally, the risk of pancreatic cancer increased to 8.23 for relatives who ever smoked and who were related to a case diagnosed with pancreatic cancer before the age of 60 years.

To date, no studies have specifically examined the interaction of smoking and BRCA1/2 mutation with the risk of developing pancreatic adenocarcinoma. For female BRCA1/2 mutation carriers, however, smoking may actually decrease the risk of developing breast cancer. A 1998 case–control study of breast cancer among BRCA1/2 carriers found that subjects with BRCA1 or BRCA2 gene mutations and breast cancer were significantly more likely to have been non-smokers than subjects with mutations and without breast cancer (p = 0.007), and that women with BRCA1 or BRCA2 mutations who had smoked cigarettes for more than four pack-years had a lower breast cancer risk (OR 0.46, 95% CI 0.27 to 0.80; p = 0.006) than subjects with mutations who had never smoked. The reduction in breast cancer risk associated with smoking is specific to BRCA1/2 mutation positive smokers was hypothesised to be associated with the effect that cigarette smoking has on oestrogen levels. Cancer risk in BRCA1/2 mutation carriers peaks at about age 40–45 years, when oestrogen levels are still high before the majority of women experience menopause, and then declines, in contrast with the general population in which the risk steadily increases throughout the life course.

Owing to the fact that incidence rates for pancreatic cancer have consistently been shown to be lower in women than in men and the discovery of oestrogen receptors in exocrine pancreatic tissue—from which 95% of pancreatic tumours arise—a body of pancreatic cancer research has focused on the effects of exposure to oestrogen on pancreatic ductal tissue. Studies have suggested that reproductive factors, particularly parity, may reduce pancreatic cancer risk in women. Harvard researchers prospectively examined parity, breastfeeding history, age at first birth, menstrual factors and exogenous hormone use in relation to pancreatic cancer risk. During 22 years of follow-up (1976–1998), 115 474 women contributed 2.4 million years of person time, and 243 cases of pancreatic cancer were identified. After multivariate adjustment for known risk factors such as cigarette smoking, compared with nulliparous women, the relative risk of pancreatic cancer was 0.86 for women with 1–2 births, 0.75 for 3–4 births and 0.58 for those with ≥5 births. Linear trend analysis indicated a 10% reduction in risk for each birth (p trend = 0.008).

Additionally, in another study, data from 52 cases and 233 population-based controls in Ontario were used to assess the effects of parity, age at first birth and other hormonal factors on pancreatic cancer risk. Reduced risk was seen with three or more pregnancies (adjusted OR = 0.22, 95% CI 0.07 to 0.65) and use of oral contraceptives (adjusted OR = 0.36, 95% CI 0.13 to 0.96). Examining the interaction of exposure to oestrogen and smoking, a recent population-based case–control study assessed the role of menstrual factors, reproductive factors and hormone use in the aetiology of pancreatic cancer among 241 female cases of pancreatic cancer and 818 controls, and found that the adjusted OR for current smoking and pancreatic cancer was stronger for women who had never used oral contraceptives or oestrogen replacement therapy (OR = 11.5, 95% CI 3.5 to 38.1) than for those who reported using both (OR = 1.7, 95% CI 0.56 to 5.0). These results provide some evidence that the interaction of cigarette smoking and exposure to oestrogen influences pancreatic cancer risk, but in a direction opposite to that of breast cancer risk in BRCA1/2 mutation carriers.

ETHICAL ISSUES AND STUDY LIMITATIONS

Ethically, the decision to undergo BRCA1 or BRCA2 mutation testing must be made autonomously. Many individuals might want to know the extent of their chances of developing a potentially fatal disease; for others this knowledge would be burdensome.

The consequences of discovering that one harbours a BRCA1/2 mutation include increased health-related anxiety, difficulty in making certain life decisions and guilt over passing on the mutation to one’s children. Additionally, genetic health insurance discrimination is a not a threat in countries in which premiums are established on the basis of income level, but it is a threat in countries in which premiums are calculated on the basis of risk, as they are in the United States.

We should use caution in giving credence to risk estimates that are based on the results of a single study. The frequently cited statistic that pancreatic cancer risk is 57-fold for members of families with three or more pancreatic cancer cases was based on the observation of just three cases of cancer in 105 at-risk individuals, when 0.05 (essentially, none) were expected. Another noted statistic is that the risk of breast cancer by age 70 years for male BRCA2 carriers is 6–7%, or 80-fold greater than the risk for non-carriers—a value that was derived from a single study that observed four cases of cancer. Many studies of BRCA1/2 carriers have suffered from similar limitations, based on small sample sizes, differential criteria used to classify individuals as “high risk” and inconsistencies in methods of statistical analysis (table 2).

Problems arise in validating some studies’ findings because researchers may count the number of cancers rather than the
number of individuals with cancer, and families with multiple cases may skew results.

CONCLUSIONS
Pancreatic cancer-prone families are an ideal resource in which the aetiology, progression and treatment of a deadly disease can be studied. Obtaining a thorough family history is essential in predicting which families may be at increased risk, because it is virtually impossible to differentiate hereditary cases of pancreatic cancer from sporadic cases on the basis of clinical presentation or pathological features. Owing to the relatively low birth rate in western countries, small pedigrees may downstage the predictive value of family history. Genetic analysis for BRCA1/2 mutations may prove beneficial in establishing cancer risk profiles and instituting standards for surveillance measures.

Furthermore, it would be careless to generalise cancer penetration estimates from multiple-cancer-case families to the general population or to families lacking a significant history of cancer. Genetic counselling and preventive screening measures should probably be reserved for multiple-case families, families of Ashkenazi Jewish descent or families with proven germline BRCA1/2 mutations. At this time, the exact incidence of BRCA1/2 germline mutations in the general population has not yet been determined, effective screening measures for pancreatic cancer are mediocre at best and there is no current accepted protocol for screening. Although the identification of cancer-predisposing heritable mutations such as BRCA1/2 has led to prophylactic surgery in women to remove at-risk organs/tissues, total pancreatectomy leads to significant morbidity and mortality and is unlikely to be embraced as first-line prevention for a cancer with relatively low penetrance and late onset. The importance of referring BRCA1/2 mutation positive families to centres that have ongoing research, provide genetic counselling and are capable of implementing screening measures for relevant cancers cannot be stressed enough.

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Competing interests: None.

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REFERENCES
1 Harris M, Winship I, Spriggs M. Controversies and ethical issues in cancer- 
25 Schubert EL, Lee MK, Metford HC, et al. BRCA2 in American families with four or more cases of breast or ovarian cancer: recurrent and novel mutations, variable expression, penetrance, and the possibility of families whose cancer is not attributable to BRCA1 or BRCA2 [see comment]. Am J Hum Genet 1997; 60:1031–40.

Table 2 Study limitations

<table>
<thead>
<tr>
<th>Limitation</th>
<th>Example</th>
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<tbody>
<tr>
<td>Study design</td>
<td>High-risk hereditary breast/ovarian cancer families versus familial pancreatic cancer families versus population-based studies</td>
</tr>
<tr>
<td>Power</td>
<td>Inadequate number of subjects to detect statistical differences; wide CI</td>
</tr>
<tr>
<td>Method of BRCA detection</td>
<td>Differences in mutation analysis and linkage analysis, whole genome versus select mutation analysis</td>
</tr>
<tr>
<td>Statistical analyses</td>
<td>Calculating SIRs or RR on the basis of limited numbers; using χ² analysis inappropriately</td>
</tr>
<tr>
<td>Ascertainment bias</td>
<td>Not obtaining complete family history, cancers can be missed or overlooked</td>
</tr>
<tr>
<td>Recall bias</td>
<td>Using questionnaires rather than tumour registry information, family members of cancer cases tend to ‘recall’ more cancer; incorrect identification of cancers</td>
</tr>
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BRCA, breast cancer antigen gene; SIRs, standardised incident ratios.
EDITOR’S QUIZ: GI SNAPSHOT .................................................................

An unsuspected cause of chronic diarrhoea

Clinical presentation
A 45-year-old Italian man, living in Italy, was admitted to our clinic with a 6-month history of chronic diarrhoea (soft stool, 3–4 times/day), iron-deficiency anaemia, recurrent abdominal pain, weight loss (10 kg over 6 months) and weakness. Examination was normal apart from moderate pallor and a soft but diffusely tender abdomen. He was not febrile: there was no pathological sign apart from increased bowel sounds. Other investigations showed haemoglobin 10.8 g/dl, mean corpuscular volume 72 fl, full blood count normal with relative hyper eosinophilia (8.5%), platelets 230×10^9/l, erythrocyte sedimentation rate 60 mm/h, C reactive protein <1.0 mg/l, normal renal and hepatic biochemistry, albumin (30 g/l), serum iron 10 µg/dl, total iron-binding capacity 330 µg/dl (normal: 240–480 µg/dl) and ferritin 7 ng/ml (20–200 ng/ml).

Parasitological stool examination was non-diagnostic, with normal stool pH. Coproparasitological study was negative; stool specimens for faecal leucocytes, bacterial culture, ova and parasites were all negative and pH was normal. Occult blood was found repeatedly in the stool.

Routine biochemical examinations including thyroid function test, protein electrophoresis and serum immunoglobulin levels were all normal, and antigliadin antibodies (IgG, IgA) were negative. HIV-1 and HIV-2 were also negative.

Chest x ray and barium follow-through were all normal. Upper endoscopy with gastric and duodenal mucosal biopsies was unremarkable. Colonoscopy was difficult and performed only up to the mid-sigmoid colon because of poor luminal distension due to perivisceral phenomena resulting from previous diverticulitis. Double-contrast barium enema was therefore used to evaluate the colon, but did not yield significant findings. Abdominal ultrasound showed widespread abnormalities with ileal loops and moderately dilated, fluid-filled jejunal loops with oedematous wall thickening (fig 1). A few small (12mm) peri-intestinal lymph nodes were detected. Localised peritoneal fluid was present in the right iliac fossa. Despite extensive investigations the diagnosis remained uncertain.

Question
How would you explain the clinical context and sonographic findings and what further diagnostic procedures you would like to perform?

See page 668 for answer

This case is submitted by:

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Figure 1 The examination with a linear transducer shows bowel loops characterised by thickened ileal wall (transverse section, right; longitudinal section, left) and liquid endoluminal content.