British Journal of Cancer www.nature.com/bjc

REVIEW ARTICLE

Clinical Studies

Potential influence of the microbiome environment in patients with biliary tract cancer and implications for therapy

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Biliary tract cancers, including intra- and extra-hepatic cholangiocarcinoma as well as gallbladder cancer, are associated with poor prognosis and the majority of patients present with advanced-stage, non-resectable disease at diagnosis. Biliary tract cancer may develop through an accumulation of genetic and epigenetic alterations and can be influenced by microbial exposure. Furthermore, the liver and biliary tract are exposed to the gastrointestinal microbiome through the gut–liver axis. The availability of next-generation sequencing technology has led to an increase in studies investigating the relationship between microbiota and human disease. In particular, the interplay between the microbiome, the tumour micro-environment and response to systemic therapy is a prospering area of interest. Given the poor outcomes for patients with biliary tract cancer, this emerging field of research, through which new biomarkers may be identified, offers potential as a tool for early diagnosis, prognostication or even as a future therapeutic target. This review summarises the available evidence on the microbiome environment in patients with biliary tract cancer, including a discussion around confounding factors, implications for therapy and proposed future directions.

British Journal of Cancer (2022) 126:693–705; https://doi.org/10.1038/s41416-021-01583-8

BACKGROUND

Biliary tract cancers (BTC) are sub-classified into intra- and extra-hepatic cholangiocarcinoma (the latter being further subdivided into perihilar cholangiocarcinoma and distal cholangiocarcinoma [1]) and gallbladder cancer. Although cholangiocarcinoma is considered relatively rare in the Western world, 0.35–2 cases per 100,000 population [2], both incidence and mortality are rising, particularly of intrahepatic cholangiocarcinoma [3, 4]. Furthermore, estimated cancer-related deaths for the United States suggest that liver and intrahepatic bile duct cancer will surpass colorectal cancer to become the third most common cause of cancer-related death by 2040 [5].

Patients with BTC have a poor prognosis and the majority present with advanced-stage, non-resectable disease at diagnosis [6]. The Advanced Biliary tract Cancer (ABC)-02 study reported a median progression-free survival (PFS) and overall survival (OS) in the advanced setting of 8 and 11.7 months, respectively, with first-line standard-of-care cisplatin/gemcitabine chemotherapy [7]. Additionally, the ABC-06 study determined benefit from second-line FOLFOX (5-fluorouracil, folinic acid and oxaliplatin) chemotherapy compared with active symptom control alone, median OS of 6.2 months versus 5.3 months [8]. Given the modest survival gains observed thus far, new therapeutic options are required.

Risk factors for cholangiocarcinoma vary globally and collectively account for a small number of cases [9]. In East Asia, liver fluke infections with *Opisthorchis viverrini* or *Clonorchis sinensis*, due to the consumption of uncooked river fish, are the driving risk factor for cholangiocarcinoma [10]. A recent meta-analysis evaluated risk factors for cholangiocarcinoma in Eastern and Western world populations, excluding the established risk factors of primary sclerosing cholangitis (PSC) and liver fluke infection, and reported that the strongest risk factors for both intra- and extra-hepatic cholangiocarcinoma were biliary cysts and stones, cirrhosis, hepatitis B and hepatitis C [11]. Gallbladder carcinoma has a different range of established risk factors, including, but not limited to, cholelithiasis [12], PSC [13], structural biliary tree abnormalities [14] and obesity [15].

Biliary tract cancer may therefore develop through the accumulation of genetic and epigenetic alterations, and can be influenced by host immunity, diet, environmental and microbial exposure [16]. The 'microbiome' refers to the collective genomes of microorganisms within a particular environment, and the human intestinal microbiome comprises ~10^{14} microorganisms that have a crucial role in host functions, including modulating immunity, protecting the host against pathogenic microbes and regulating metabolic processes [17–20]. Microbial dysbiosis (an imbalance in the gut microbial community) has been associated

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Received: 14 April 2021 Revised: 16 September 2021 Accepted: 4 October 2021
Published online: 18 October 2021

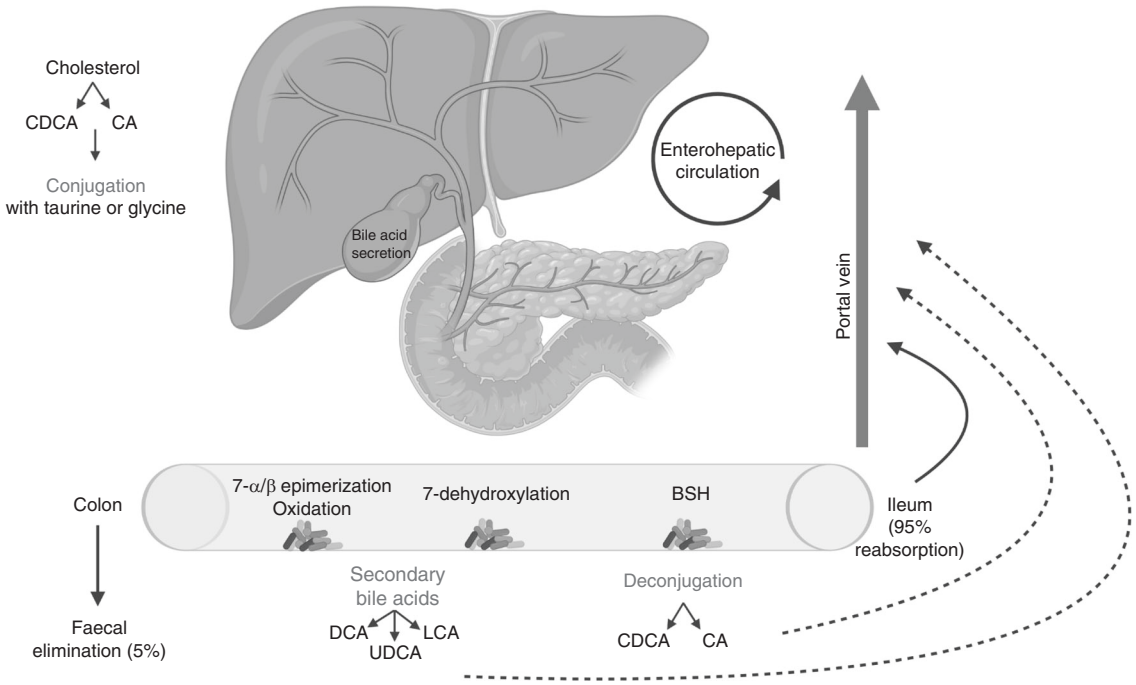

Fig. 1 Microbial metabolism of bile acids and the enterohepatic circulation [28–31]. (CDCA: chenodeoxycholic acid, CA: cholic acid, LCA: lithocholic acid, DCA: deoxycholic acid, UDCA: ursodeoxycholic acid, BSH: bile salt hydrolase). Created with BioRender.com.

with multiple diseases, including cancer [21, 22], obesity and insulin resistance [23, 24], inflammatory bowel disease [25] and neurodegenerative disease [26, 27].

The liver and biliary tract are exposed to the gastrointestinal microbiome through the gut–liver axis, which refers to the bidirectional communication between the gastrointestinal tract and the liver via the portal vein, biliary tract and systemic circulation [28]. Primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA) are conjugated with glycine or taurine in the liver and released into the duodenum via the biliary tract. Approximately 95% are reabsorbed in the terminal ileum, recirculated to the liver via the portal circulation and secreted again in a process known as enterohepatic circulation [29]. The remaining primary bile acids are deconjugated by bacteria with bile salt hydrolase (BSH) activity such as *Clostridium, Enterococcus, Bifidobacterium* and *Lactobacillus*, before being absorbed or metabolised into secondary bile acids via a further gut microbiota-mediated process involving dehydroxylation (Fig. 1) [30, 31]. Secondary bile acids either return to the liver via passive absorption or are excreted in the faeces. Bile acids and the gut microbiota closely interact, and in addition to microbiota affecting bile acid metabolism, bile acids also affect microbiota composition [29–31].

There is growing evidence that disruptions in the gut–liver axis contribute to the pathogenesis of many liver diseases, including cancer [32]. A recent study by Ma et al. demonstrated that gut microbiota-mediated bile acid metabolism regulates liver cancer (hepatocellular carcinoma (HCC) and liver metastases) via accumulation of hepatic natural killer T (NKT) cells. Expression of CXCL16, a ligand on liver sinusoidal endothelial cells responsible for NKT-cell accumulation, was increased by primary bile acids and enhanced tumour inhibition, whereas increasing secondary bile acids had opposing effects [33].

Additionally, patients with BTC are at risk of developing biliary obstruction and may require interventions, including endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangiography (PTC). These necessary interventions may inadvertently introduce gut microbiota into the biliary tract. Biliary stenting has been shown to induce significant bile microbiota shifts and is associated with a higher risk of post-operative surgical-site infection [34, 35], however, the effects on oncological outcomes are less clear. A recent study by Shrader et al. treated human pancreatic cancer cells in vitro with bile samples collected during pancreaticoduodenectomy from patients (stented and non-stented) in order to observe if bacterial contamination had any effect on pancreatic cell survival. They first demonstrated that bile from patients with stents had less effect on reducing pancreatic cancer-cell survival than non-stented bile. Secondly, they found that pre-incubation of non-stented bile samples with live *Enterococcus faecalis* or *Streptococcus oralis* reduced the inhibitory effect of the bile on pancreatic cancer-cell survival [36]. These findings support the concept that introduction of gut bacteria into the biliary system through biliary stenting may alter the bile composition and its biological behaviour towards cancer cells. Further preclinical experiments and clinical studies are required to confirm this theory.

The human biliary system has often been considered to be a sterile environment in individuals without biliary pathology or prior biliary intervention, although the technical and ethical challenges of bile sample collection in 'healthy' individuals limit the evidence within this field. Molinero et al. aimed to address this limitation by collecting bile samples from the gallbladders of liver-transplant donors with no biliary pathology (control group), and comparing them with bile samples from the gallbladders of patients undergoing surgery for cholelithiasis. The results demonstrated that bacterial communities were in fact present in the bile of the control group, and the microbiota composition differed significantly from the cholelithiasis group ($p < 0.05$) [37]. Further studies are needed to improve understanding of the physiologically 'normal' bile microbiota, which will be crucial in unravelling their potential role in human disease.

Furthermore, the development of cost-effective, high-throughput next-generation sequencing (NGS) technology, which obtains 16S ribosomal ribonucleic acid (rRNA) hypervariable region or whole-genome shotgun (WGS) sequence reads for analysis of the microbiome, has resulted in a substantial increase in studies investigating the relationship between the human microbiome and cancer. This review focuses on the available

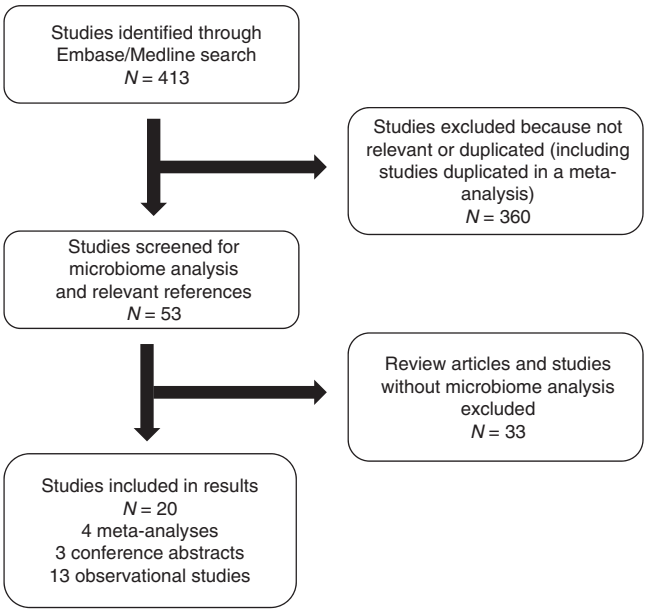

Fig. 2 Flow diagram describing identification of studies reporting on the microbiome environment in biliary tract cancer. Reasons for inclusion and exclusion are stated.

evidence on the microbiome environment in patients with BTC and potential implications this may have for therapy, including a discussion on potential directions for future research.

METHODS

A search to identify eligible studies and conference abstracts was undertaken using the Ovid Medline and Embase databases. Keywords used to identify eligible publications included (((microbio* OR microflor* OR 'gastrointestinal microbiome'/OR microbiome/ or 'bacterial microbiome'/) AND (('biliary tract' OR gallbladder OR 'ampulla of Vater' OR klatskin) ADJ3 (cancer* OR tumor* OR tumour* OR carcinoma* OR neoplas* OR malignanc*)) OR BTC OR ((perihilar OR klatskin OR hilar OR "distal bile duct" OR intrahepatic) AND cholangiocarcinoma)) OR 'biliary tract neoplasms'/OR 'biliary tract cancer'/OR exp 'bile duct carcinoma'/OR (('bile ducts, intrahepatic'/) AND (cancer* OR tumor* OR tumour* OR carcinoma* OR neoplas* OR malignanc*))). No dates of publication or language limits were applied.

Eligible studies were required to include results on microbes associated with BTC in human participants. Meta-analyses, conference abstracts, prospective studies and retrospective series were included. The references of eligible studies and relevant review articles (identified from the database search) were examined to detect other studies of interest. Exclusion due to non-relevance consisted of studies reporting on benign disease only, other malignancies, biliary tract infections, complications of biliary tract procedures or interventions, and studies reporting on antibiotic excretion, susceptibility or resistance. Eligible studies that were duplicated in a meta-analysis were also excluded.

Data collected from each of the eligible studies included author's name, country, year of publication, type of study, number of patients, disease site, sample type and handling, method of analysis and key microbiota results.

RESULTS

The database search identified 413 studies (last updated 11th March 2021): 360 ineligible studies were excluded after review of the abstracts and the remaining 53 studies were assessed firstly for results reporting microbes associated with BTC, and secondly for references detailing other studies of interest. A total of 20 eligible studies were identified: four meta-analyses, three conference abstracts and thirteen observational studies (2013–2021) (Fig. 2). One further study [38] was added following peer review, resulting in a total of 21 eligible studies.

The potential association of *Helicobacter* species and *Salmonella typhi* with BTC

Three prospective studies and two meta-analyses investigating the association of *Helicobacter* species with BTC were identified (Table 1). The meta-analyses both included ten case–control studies and six studies were duplicated across the two analyses. Xiao et al. restricted inclusion criteria to case–control studies in which control participants had no known diagnosis of cholelithiasis [39], whereas Zhou et al. included case–control studies in which inter-study control participants were with and without benign biliary pathology [40]. Both meta-analyses found a significant association between *Helicobacter* species and the presence of BTC compared with control subjects without biliary pathology, and Zhou et al. also reported significantly higher *Helicobacter* infection rates in patients with BTC compared to those with benign biliary pathology. Zhou et al. also performed a species subgroup analysis and found significantly higher rates of *H. pylori* and *H. bilis* in BTC compared with benign disease, and no significant differences in *H. hepaticus* or *H. ganmani* between groups.

Three prospective studies carried out since the meta-analyses support an association between *Helicobacter* species and BTC. Segura-López et al. [41] and Avilés-Jiménez et al. [42] identified significant associations between extra-hepatic cholangiocarcinoma and *H. bilis* or *H. pylori*, respectively, detected by polymerase chain reaction (PCR) analysis of biliary brushings at the time of scheduled ERCP. Alternatively, Murphy et al. used a *Helicobacter* species multiplex serology assay, and prospectively evaluated associations between seropositivity and BTC. The study concluded that seropositivity to *H. pylori* proteins was associated with an increased risk of developing BTC [43], however, the participants comprised entirely of Finnish male smokers and the results require validation in other populations.

Two meta-analyses investigating the association of *Salmonella typhi* and gallbladder carcinoma were identified (Table 1). Nagaraja et al. included seventeen studies [44], Koshiol et al. included 22 studies [45] and fifteen studies were duplicated across the two analyses. Both analyses included case–control and cohort studies. A variety of sample types, including bile, stool, tissue and serum from patients with and without gallbladder carcinoma, were analysed, and both meta-analyses found an association between chronic *S. typhi* carrier state and gallbladder carcinoma, based on *S. typhi* antibody levels and culture-detection methods. Nagaraja et al. also conducted a subgroup analysis of studies from South-East Asia and reported a significant association between chronic *S. typhi* carrier state and gallbladder carcinoma in this geographical distribution.

The potential association of other microbiota with BTC

The following section will highlight the reported associations of other microbiota identified with BTC.

Two studies used automated microbiology systems (Phoenix or Vitek-2) to analyse bile microbiota in patients with BTC (stage not specified). Serra et al. conducted a cross-sectional study on bile samples from females undergoing surgery for confirmed biliary tract or pancreatic cancer, and found *Pseudomonas* species to be a significant positive predictor for the presence of cholangiocarcinoma and gallbladder carcinoma [46]. Di Carlo et al. retrospectively evaluated bile samples taken at the time of scheduled ERCP in a cohort of patients with confirmed BTC, carcinoma of the head of the pancreas and benign biliary pathology, and found that

Table 1. Selected studies reporting on microbes associated with benign biliary disease and biliary tract cancer in human studies.

Author	Country	Type of study	No. of patients	Disease site	Sample type	Method of analysis	Microbiota results
Helicobacter species							
Murphy et al. [43]	Finland	Prospective	410	Biliary tract carcinoma (BTC) (89) Hepatocellular carcinoma (HCC) (97) Age-matched controls (224)	Serum	*Helicobacter* spp. Multiplex serology assay	*Helicobacter pylori* (*H. pylori*) seropositivity in 100% gallbladder cancer, 97% of extra-hepatic bile duct cancer, 96% of intrahepatic bile duct cancer and 91% of ampulla of Vater cancer. OR 5.47 (95% CI 1.17–25.65) for H. pylori seropositivity and risk of developing BTC.
Segura-López et al. [41]	Mexico	Prospective	194	Extra-hepatic cholangiocarcinoma (ECCA) (103) Control—benign biliary pathology (91)	[a]Biliary brushing	Polymerase chain reaction (PCR)	*Helicobacter bilis* (*H. bilis*) infection was significantly associated with ECCA (43% of cases) compared to benign biliary pathology (21% of cases) ($p = 0.002$). *Helicobacter hepaticus* (*H. hepaticus*) infection not significantly different between the two groups ($p = 0.82$).
Avilés-Jiménez et al. [42]	Mexico	Prospective	200	Extra-hepatic cholangiocarcinoma (100) Control—benign biliary pathology (100)	[a]Biliary brushing	PCR (all samples) 16S ribosomal ribonucleic acid (rRNA) gene sequencing (20 samples)	*H. pylori* significantly more abundant in ECCA than in control group ($p = 0.035$) Significant difference in microbiota composition between ECCA and benign biliary pathology group ($p = 0.01$). *Methylophilaceae, Fusobacterium, Prevotella, Actinomyces, Novosphingobium and H. pylori* increased in ECCA.
Zhou et al. [40]	Japan Thailand India Pakistan Germany	Meta-analysis	10 studies (726 patients)	Biliary tract cancer (190) Benign biliary pathology (434) Control—no biliary pathology (102)	Serum Bile Tissue	PCR Enzyme linked immunosorbent assay (ELISA) Culture Immunohistochemistry	Infection rate of *Helicobacter spp* significantly higher in BTC group compared to: -benign biliary pathology group (OR 3.2, 95% CI 2.15–4.77, $p = 0.0001$) -control group (OR 6.5, 95% CI 3.14–13.63, $p = 0.0001$) Higher rate of *H. pylori* (49.5% vs 33.3%, $p = 0.003$) and *H. bilis* (52.2% vs 23.7%, $p < 0.0001$) in BTC group vs benign biliary pathology group. No significant difference in rate of *H. hepaticus* and *Helicobacter ganmani* between groups.
Xiao et al. [39]	Sweden Germany Japan Thailand China	Meta-analysis	10 studies (418 patients)	Cholangiocarcinoma (CCA) Control—without cholelithiasis	Serum Bile Tissue	PCR ELISA Culture Immunohistochemistry	Significant association between *Helicobacter* spp and CCA (cumulative OR 8.88, 95% CI 3.67–21.49). Subgroup analysis based on geographic distribution: -Asia (OR 6.68, 95% CI 2.29–19.49) -Europe (OR 14.9, 95% CI 4.79–46.35)

Table 1 continued

Author	Country	Type of study	No. of patients	Disease site	Sample type	Method of analysis	Microbiota results
Salmonella typhi							
Author	Country	Type of study	No. of patients	Disease site	Sample type	Method of analysis	Microbiota results
Nagaraja et al. [44]	UK India Chile USA China Japan Bolivia Mexico	Meta-analysis	17 studies	Gallbladder carcinoma (GBC)	Serum Bile Tissue	Antibody levels Culture PCR	Subgroup analysis of studies from South-East Asia showed significant association between chronic *Salmonella typhi* (*S. typhi*) status and GBC (OR 4.13, 95% CI 2.87–5.94, $p < 0.01$). Chronic *S. typhi* carrier state significantly associated with GBC based on *S. typhi* antibody levels (OR 3.52, 95% CI: 2.48–5.00, $p < 0.01$) and culture (OR: 4.14, 95% CI 2.41–7.12, $p < 0.01$).
Koshiol et al. [45]	Chile India USA Denmark China Japan Bolivia Mexico	Meta-analysis	16 studies	Gallbladder carcinoma	Serum Bile Stool Tissue	Antibody levels Culture PCR	Elevated *S. typhi* Vi antibody seropositivity associated with GBC risk (RR 4.6, 95% CI 3.1–6.8). Positive *S. typhi* bile or stool culture associated with GBC risk (RR 5.0, 95% CI 2.7–9.3)
Other identified microbiota							
Author	Country	Type of study	No. of patients	Disease site	Sample type	Method of analysis	Microbiota results
Lenz et al. [55]	Germany	Prospective	58	Biliary tract carcinoma (24%) Chronic pancreatitis (14%) Choledocholithiasis (14%) Unclear stenosis of common bile duct (9%) Remaining 39% not specified	[a]Bile	16S rRNA gene sequencing	In all patients, the 9 most common bacterial phyla were *Pseudomonadales* (11.8%), *Enterobacteriales* (10.0 %), *Sphingomonadales* (8.3 %), *Burkholderiales* (8.1%), *Lactobacillales* (7.6%), *Caulobacterales* (6.7%), *Alteromonadales* (6.3%), *Rhizobiales* (6.0%) and *Clostridiales* (5.8%). No significant correlation between phyla and primary diagnosis ($p = 0.803$), CCA ($p = 0.664$) or malignant biliary stenosis ($p = 0.529$).
Tsuchiya et al. [48] 2018	Bolivia Chile	Prospective	37	Gallbladder carcinoma (7) Cholelithiasis (30)	[b]Bile	16S rRNA gene sequencing	*Fusobacterium nucleatum*, *Escherichia coli* (*E. coli*) and *Enterobacter* sp. were the predominant species in patients with GBC. *E. coli*, *Enterococcus gallinarum* and *Salmonella* sp. were the predominant species in patients with cholelithiasis.
Poudel et al. [49]	USA	Prospective	10	Cholangiocarcinoma (3) Ampullary carcinoma (1) Pancreatic ductal	[a]Bile	16S rRNA gene sequencing	Most reads were from phyla *Firmicutes* (57.9%) and *Proteobacteria* (14.9%). Benign specimen (pancreatitis)

Table 1 continued

Author	Country	Type of study	No. of patients	Disease site	Sample type	Method of analysis	Microbiota results
				adenocarcinoma (PDAC) (5) Gallstone pancreatitis (1)			separated clearly from the rest showing 98.9% of reads from *Clostridium sensu stricto* (phylum *Firmicutes*). Beta-diversity analysis: a cluster including 3 samples (2 with CCA, 1 with PDAC) had higher abundance of phyla *Fusobacteria* (90.6%) and *Verrucomicrobia* (92.9%)
Jia et al. [50]	China	Prospective	84	Intrahepatic cholangiocarcinoma (ICCA) (28) Hepatocellular carcinoma (28) Liver cirrhosis (16) Control—no biliary pathology (12)	Faecal	16S rRNA gene sequencing	ICCA patients showed higher prevalence rates of *Lactobacillus*, *Actinomyces*, *Peptostreptococcaceae*, and *Alloscardovia* than the other groups The family *Ruminococcaceae* was more abundant in patients with ICCA with vascular invasion (compared to those without vascular invasion)
Chng et al. [51]	Singapore Thailand Romania	Retrospective	60	Cholangiocarcinoma *Opisthorchis.viverrini* associated (28) Non *O. viverrini* associated (32)	[c]Tumour	16S rRNA gene sequencing	*Dietziaceae*, *Pseudomonadaceae* and *Oxalobacteraceae* were the major inhabitants of bile duct tissues in CCA patients. *Bifidobacteriaceae*, *Enterobacteriaceae* and *Enterococcaceae* enrichment in *O. viverrini* versus non - *O. viverrini* groups. Stenotrophomonas significantly enriched in tumour vs adjacent normal tissue in non - *O. viverrini* group ($p = 0.039$)
Lee et al. [57]	South Korea	Prospective	155	Biliary tract cancer (24) Cholecystitis/cholangitis (43) Control—no biliary pathology (88)	[d]Plasma	16S rRNA gene sequencing	Compositional differences of *Bifidobacteriaceae* family and *Oxalobacteraceae Ralstonia* found to be a significant positive marker, and the *Pseudomonadaceae* family, *Corynebacteriaceae Corynebacterium* and *Comamonadaceae Comamonas* were significant negative markers to differentiate BTC patients from control group.
Chen et al. [52]	China	Prospective	68	Distal cholangiocarcinoma (dCCA) (8) Common bile duct stones (44) Recurrent choledocholithiasis (16)	[a]Bile	16S rRNA gene sequencing	*Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* are the most dominant phyla in the bile of patients with dCCA and common bile duct stones. Significant increase in the phyla *Gemmatimonadetes*, *Nitrospirae*, *Chloroflexi*, *Latescibacteria*, and *Planctomycetes* in dCCA patients compared to common bile duct stones.

Table 1 continued

Author	Country	Type of study	No. of patients	Disease site	Sample type	Method of analysis	Microbiota results
Dangtakot et al. [53]	Thailand	Prospective	60	Cholangiocarcinoma (30) Choledocholithiasis (CDL) (30)	[a]Bile	16S rRNA gene sequencing	*Enterobacter*, *Stenotrophomonas* and *Pseudomonas* significantly more abundant in CCA compared to CDL ($p < 0.05$). *Cetobacterium*, *Pyramidobacter* and *Streptococcus* species significantly less abundant in CCA compared to CDL ($p < 0.05$).
Katsuyuki et al. [56]	USA	Prospective	95 bile (b) samples 58 faecal (f) samples	Cholangiocarcinoma (11f, 26b) Primary sclerosing cholangitis (31f, 35b) PSC co-existing with CCA (16f, 17b) Control—cholelithiasis or choledocholithiasis (17b)	[e]Bile Faecal	16S rRNA gene sequencing	CCA bile samples had significant difference of alpha diversity compared to control group, with less *Streptococcaceae* and *Desulfovibrionaceae*. An increased abundance of *Fusobacteria* was found after biliary stent placement and increased with the number of stents in the bile duct.
Zhang et al. [54]	China	Prospective	71	Cholangiocarcinoma (8) Hepatocellular carcinoma (10) Mixed-type liver cancer (6) Liver cirrhosis (24) Control—healthy (23)	Faecal	16S rRNA gene sequencing	*Enterobacter* and *Escherichia-Shigella* most significantly represented in patients with primary liver cancer (CCA, HCC, mixed-type). Relative abundance of *Enterobacter ludwigii* highest in primary liver cancer group and 100× greater than liver cirrhosis and healthy controls. Significantly decreased *Firmicutes/Bacteroidetes* ratio in primary liver cancer group compared to healthy controls ($p < 0.05$).
Song et al. [58]	China	Prospective	14	Gallbladder carcinoma (7) Chronic calculous cholecystitis (7)	[b]Tissue	Metagenomic sequencing	*Peptostreptococcus stomatis*, *Fusobacterium mortiferum*, *Acinetobacter junii* and *Enterococcus faecium* positively correlated and significantly contributed to GBC group.
Saab et al. [38]	Iran	Prospective	75	Extra-hepatic cholangiocarcinoma (28) Control—benign biliary pathology (47)	[a]Bile	16S rRNA gene sequencing	The most abundant genera in ECCA group were *Enterococcus*, *Streptococcus*, *Bacteroides*, *Klebsiella* and *Pyramidobacter*. In a subgroup analysis of patients without comorbidities (19 ECCA and 37 controls), relative abundance of *Bacteroides*, *Geobacillus*, *Meiothermus* and *Anoxybacillus* significantly higher in ECCA compared with controls ($p < 0.05$).
Serra et al. [46]	Italy	Prospective	53	Cholangiocarcinoma (20) Gallbladder carcinoma (2) Carcinoma of the head of the pancreas (31)	[b]Bile	Phoenix Automated Microbiology System	*E. coli* and *Pseudomonas* spp were significant positive predictors for presence of CCA ($p < 0.0001$). *Pseudomonas* spp only significant

Table 1 continued

Author	Country	Type of study	No. of patients	Disease site	Sample type	Method of analysis	Microbiota results
							positive predictor for presence of GBC ($p = 0.0001$).
Di Carlo et al. [47] 2019	Italy	Retrospective	152	Cholangiocarcinoma (42) Gallbladder carcinoma (5) Ampullary carcinoma (4) Carcinoma of the head of the pancreas (72) Cholelithiasis (27) Cholangitis (1) Chronic pancreatitis (1)	[c]Bile	Phoenix Automated Microbiology System or Vitek-2 System	*E. coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* were identified in the cancer population and their presence was associated with reduced survival time.

[a]Samples obtained at time of scheduled endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic biliary drainage (PTBD).
[b]Samples obtained at time of surgery.
[c]Archived samples (previous biopsy or biliary tract procedure).
[d]Bacteria-derived extracellular vesicles isolated in the plasma.
[e]Timing of sample collection not specified.

the presence of *E. coli, P. aeruginosa*, and *K. pneumoniae* was associated with reduced survival in the cancer population (death within 6 months of diagnosis) [47].

Thirteen studies were identified, which used 16S rRNA gene sequencing [42, 48–57] or shotgun metagenomics [58] to analyse the microbiome in patients with BTC (Table 1). A variety of sample types from patients with BTC were analysed: seven studies used bile, three used faecal samples, three used tissue and one study used plasma. Seven studies stated that samples were frozen at −80 °C prior to DNA extraction, one study stated that samples were stored in a freezer but did not specify the temperature and another study stated that samples were thawed on ice prior to placing them in lysing tubes. Three studies were identified from conference abstracts, which did not include details on sample storage. Eight different DNA-extraction kits were used across the studies, the most commonly used kits were the PowerSoil DNA Isolation Kit and the QIAamp DNA Easy Kit (used in two studies each). The most common variable regions of the 16S gene targeted for sequencing were V3 and V4. An overview of sample storage, DNA extraction and DNA sequencing is provided in Table 2.

Differentially abundant taxa described across the thirteen studies covered all taxonomic levels, from phylum to species. In particular, an increase in *Fusobacteria* (four studies), *Enterobacteriaceae* (three studies) and *Pseudomonadaceae* (three studies) was consistently reported in patients with BTC. The stage of BTC was not specified in nine out of ten of these studies. Of the four studies reporting on *Fusobacteria*, two studies found it to be a predominant species in different sample types (bile and tissue), specifically in patients with gallbladder carcinoma. *Enterobacteriaceae* was reported at an increased abundance in three different study populations and in two different patient sample types: cholangiocarcinoma (bile sample), gallbladder carcinoma (bile sample) and primary liver cancer, including cholangiocarcinoma, HCC and mixed-type HCC/cholangiocarcinoma (faecal sample). In two studies, *Pseudomonadaceae* was reported at an increased abundance in patients with cholangiocarcinoma in two different sample types (tissue and bile). No other bacterial taxa were consistently reported in more than two studies.

Establishing consistent relationships between specific taxa and a disease is challenging, and some taxon have been both positively and negatively associated with BTC. For example, Serra et al. reported *Pseudomonas* in bile samples as a significant positive predictor for the presence of cholangiocarcinoma [46]; however, Lee et al. analysed plasma samples and found it to be a significant negative marker for the presence of BTC [57]. Notably, the studies had similar sample sizes (22 versus 24 patients with BTC), however, they were conducted in two different geographical regions (Italy compared with South Korea), and used different methods of analysis (automated microbiology system versus 16S rRNA gene sequencing).

DISCUSSION

To some degree, the heterogeneity of the microbiota results can be attributed to the inherent intra- and inter-person variability of microbiome composition [59, 60], however, the methodological disparities between studies should also be considered. Differences in study population, sample type, sample handling and method of analysis of all of the included studies will now be discussed and assessed for their potential to confound the results.

Study population

Large differences in sample size existed between the studies, ranging from four to 103 participants with BTC and one to 224 control participants. The inability to replicate the results could in part be due to the low sample size of some studies. The control-group population is another variable to consider, and eleven

Table 2. Methodology of studies using 16S rRNA gene sequencing or shotgun metagenomics to analyse the microbiome in patients with BTC.

Author	Sample storage	DNA extraction	Selected region of 16 S gene	Sequencing platform
Lenz et al. [55]	Not stated	Not stated	V3–V4	Illumina
Tsuchiya et al. [48]	Stored at −80 °C	NucleoSpin Soil	V3–V4	Illumina Miseq
Jia et al. [50]	Stored at −80 °C	PowerSoil DNA Isolation kit	V4	Illumina Miseq
Lee et al. [57]	Stored in a freezer	PowerSoil DNA Isolation kit	V3–V4	Illumina Miseq
Chen et al. [52]	Stored at −80 °C	OMEGA DNA kit	V3–V4	Illumina Miseq
Chng et al. [51]	Thawed on ice and transferred into lysing tubes	EZ1 DNA Tissue kit	V3–V6	Illumina Hiseq 2000
Poudel et al. [49]	Not stated	PowerViral DNA Isolation kit	Not stated	Illumina
Avilés-Jiménez et al. [42]	Stored at −80 °C	QIAamp DNA easy kit	V4	Illumina Miseq
Dangtakot et al. [53]	Stored at −80 °C	QIAamp DNA easy kit	V3–V4	Illumina Hiseq 2500
Katsuyuki et al. [56]	Not stated	Not stated	V3–V5	Illumina Miseq
Zhang et al. [54]	Placed on ice and transferred to laboratory	QIAamp DNA mini kit	V3–V4	Illumina Hiseq
Song et al. [58]	Stored at −80 °C	QIAamp DNA mini kit	N/A (metagenomic study)	Illumina Hiseq ×10
Saab et al. [38]	Stored at −80 °C	QIAsymphony kit	V3-4	Illumina Miseq

studies included control participants with benign biliary pathology. This is largely due to the majority of studies reporting on the bile or biliary tissue microbiome, necessitating the choice of control group to include individuals that have a clinical indication for an invasive procedure, such as an ERCP, as opposed to including healthy subjects.

Nineteen different countries are represented across the results dataset, including the meta-analyses. Geographical location has been shown to have an effect on human gut microbiome variations [61], and may contribute to intra- or inter-study differences. Chng et al demonstrated compositional differences in the biliary tract tissue microbiota of liver fluke-related and non-related cholangiocarcinoma [51]. This supports the role of *O.viverrini* in enabling an altered microbiome, but the intra-study differences observed could in part be due to the multiple origins of the samples (Thailand, Singapore and Romania). Additionally, the two meta-analyses investigating an association between *S. typhi* and gallbladder carcinoma include studies that are mostly conducted in regions with a high incidence of typhoid fever, although Koshiol et al. found that the association remained even when stratified by geographical region [45].

Sample type, collection and storage

Inter-subject variation is dependent on sampling site [62], and beta-diversity analysis has demonstrated that the overall structure of microbiota is significantly different between different samples types [63]. The variety of sample types analysed in BTC studies may therefore contribute to the inter-study variability of microbiota results. The majority of the included studies collected and analysed bile or biliary tract tissue, whilst other studies analysed blood or faecal samples. Additionally, description of the biliary tract tissue micro-environment has typically been generalised from bile fluid analysis; however, Chng et al. found a significantly different composition between the two sample types in patients with cholangiocarcinoma (stage not specified) [51].

A selection of studies to date have identified similarities between bile or biliary tract tissue microbiota and faecal microbiota in patients with BTC. For example, Jia et al. reported higher abundances of four genera, including *Actinomyces,* in the gut microbiota of patients with intrahepatic cholangiocarcinoma [50], whilst Avilés-Jiménez et al. previously described an increased abundance of bile duct *Actinomyces* in patients with extra-hepatic cholangiocarcinoma [42]. Furthermore, Saab et al., who took methodological precautions to avoid contamination of collected bile with the duodenal mucosa, reported levels of *Proteobacteria* that were close to values previously described in the small intestine [38]. Factors influencing bacterial colonisation within the biliary tract may include gastric or duodenal contamination, altered sphincter of Oddi function or transportation through the process of enterohepatic circulation [38]. Further studies are needed to assess the comparability of microbiota results from studies reporting on different sample types, particularly comparisons between biliary and intestinal microbiota.

Additionally, studies comparing the methodology used for microbiome analysis have demonstrated that sample collection, transportation and storage all have an impact on sample quality [64, 65]. Optimising faecal microbiome studies is of particular interest, as participants often collect the sample at home, presenting logistical challenges. Ideally, collected samples should be transported as soon as possible and stored at −20 °C to −80 °C to prevent microbial composition alteration. Where this is impractical, a DNA stabiliser can be used as a preservation tool, and multiple commercial kits have been validated [66]. In this review, of the thirteen studies that used NGS techniques (Table 2), seven stored the samples at −80 °C, whereas others transported the samples on ice to the laboratory, or stored them in a freezer at an unspecified temperature. The three studies presented as conference abstracts did not report transportation or storage conditions [49, 55, 56]. Microbial shifts due to inconsistencies in sample transportation and storage conditions should therefore be considered as a potential source of inter-study differences.

Method of analysis

The traditional approach of using culture methods to identify bacteria involved in human disease has many limitations, including a bias towards bacteria that proliferate under laboratory conditions, and an underestimation of the diverse microbial population in question [67, 68]. In this review, culture methods were used in studies investigating an individual species (*Helicobacter* or *Salmonella*). Other included studies used PCR, enzyme-linked immunosorbent assay (ELISA), immunohistochemistry or multiplex serology assays, as a means of microbial detection. Arguably of more importance is the spectrum of methods used between studies, and the impact that this will have on differences in the results, for example, Zhou et al. reported detection rates of *Helicobacter* species varying from 0 to 83% in the bile, serum and biliary tissue of patients with BTC and benign biliary pathology across ten case–control studies using a range of detection methods [40].

In the following sections, important considerations regarding sample processing and analysis for studies using NGS techniques will be discussed. Firstly, the process of DNA extraction can introduce fundamental bias, especially in relation to extraction kits and the inclusion of a mechanical, as well as a chemical method for cell lysis [69]. The addition of a mechanical lysis step has been linked to a higher DNA yield, higher bacterial diversity and more efficient extraction of DNA from Gram-positive bacteria [70, 71]. Of the thirteen studies using NGS techniques to evaluate the microbiome in patients with BTC, eight different DNA-extraction kits were used, comprising different methods of chemical and/or mechanical lysis, and therefore may have contributed to the heterogeneity of the results between studies.

A variable region of the 16S rRNA gene must be selected for PCR amplification and sequencing. Importantly, the choice of hypervariable region and the design of PCR primers have an effect on phylogenetic resolution [72, 73]. All of the included studies that reported on the choice of variable region incorporated the V4 region for sequencing, therefore reducing the likelihood of bias. To a lesser extent, choice of sequencing platform can also explain inter-study differences [73], however, all of the included studies used a version of the Illumina sequencing platform, making this an unlikely contribution to variability observed.

Bioinformatic analysis involves the translation of bacterial sequences (generated using NGS techniques) into taxonomic profiles and relative abundance estimations. A study comparing three different 16S rRNA pipelines used for taxonomic assignment (QIIME1, MALT and DADA2) with the outputs of whole-metagenomic sequencing (WMS) reported that two of the pipelines (QIIME1 and DADA2) yielded results that were more consistent with WMS taxonomic assignments in comparison with the third choice of pipeline (MALT). Furthermore, the lower the abundance of a bacterial genus (<0.5%, as detected by WMS), the lower the probability of it being correctly identified by any of the three 16S rRNA pipelines [74]. These results indicate that the bioinformatic-processing pipeline should be considered as a source of analytical bias, and may have contributed to the heterogeneity of results observed between studies.

Studies of the microbiome in other cancer types

Key lessons from studies of the microbiome in other cancer populations will now be explored, and future directions for studies in BTC proposed.

Predictive and prognostic role of the microbiome

Recent studies show significant microbial contributions in select cancer types, primarily of the faecal microbiome. Significant differences in the diversity and composition of the gut microbiome have been demonstrated in patients with melanoma, non-small-cell lung cancer (NSCLC) and renal-cell carcinoma (RCC) who respond to anti-programmed cell death-1 (PD-1) immunotherapy (versus non-responders) [75–77]. Routy et al. analysed faecal samples from patients with NSCLC and RCC and found that *Akkermansia muciniphila* was significantly associated with patients who responded to immunotherapy ($p = 0.004$) [75]. Both Gopalakrishnan et al. and Matson et al. analysed faecal samples from patients with metastatic melanoma, and reported a significantly higher abundance of *Ruminococcaceae* ($p < 0.01$) and *Bifidobacteriaceae* ($p < 0.05$), respectively, in responders compared with non-responders [76, 77]. Direct comparison of differentially abundant microbiota across these studies is limited by differences in methods of analysis, as well as differences in the method of discriminating between responders and non-responders. Nonetheless, there is agreement that there is an association between the composition and diversity of the gut microbiome and anti-PD-1 efficacy, and further studies are needed to determine the composition of a 'favourable' gut microbiome.

The tumour-associated microbiome is another area of interest, and a distinctive signature (*Pseudoxanthomonas–Streptomyces–Saccharopolyspora–Bacillus clausii)* was predictive of long-term survival (>5 years after surgery) in a study analysing the tumour specimens of 43 patients with resected pancreatic ductal adenocarcinoma (PDAC) [78]. Microbiota identified in the tumour may also have a role in tumour response to chemotherapy, for example, *Gammaproteobacteria* (a common class of bacteria identified in PDAC tumours) is able to metabolise gemcitabine into its inactive form and could account for gemcitabine resistance in this patient group [79]. Additionally, an enrichment of *Fusobacterium nucleatum* has been observed in multiple patient cohorts with colon cancer across the world [80–82], and tumour *Fusobacterium* load has been significantly associated with poorer survival outcomes among patients with caecum and ascending colon tumours [83].

Furthermore, studies assessing precancerous conditions have identified associations between the microbiome and disease progression. Pereira et al. compared the bile microbiota between control subjects undergoing routine ERCP with patients diagnosed with early-stage PSC, advanced-stage PSC or biliary dysplasia/cholangiocarcinoma. The results demonstrated that the bile microbiota composition of control subjects and subjects with early-stage PSC was similar, however, the presence of members of the *Streptococcus* genus in bile was positively correlated with PSC disease progression [84]. These results underline the need to further explore the role of *Streptococcus* in PSC progression and development of cholangiocarcinoma.

Potential effects of antibiotic use on the microbiome and response to systemic treatment in patients with malignancy

Biliary obstruction arises as a result of direct tumour compression in patients with BTC, and is frequently complicated by superadded infection and a requirement for antibiotics. Antibiotics can influence the gut microbiome and may affect response to cancer therapy. Specifically, administration of antibiotics within 2 months before, or 1 month after, initiation of immunotherapy, is associated with significantly shorter PFS and OS in patients with advanced NSCLC, RCC and urothelial carcinoma [75]. The link between antibiotic use, immunotherapy efficacy and reduced OS is supported by a number of recent meta-analyses [85–87]. Furthermore, the effect of antibiotics on OS was greater, depending on time of exposure in relation to immunotherapy initiation (Lurienne et al. report −60 to +60 days [85]); however, the heterogeneity of included studies in the meta-analyses remains a limiting factor.

Alternatively, the use of antibiotics to target key constituents of the cancer microbiota has been a point of interest, and Bullman et al. demonstrated that treatment of mice, bearing a colon-cancer xenograft, with metronidazole, reduced *Fusobacterium* load, cancer-cell proliferation and overall tumour growth [83]. Further studies looking into this targeted microbiota approach are needed.

The microbiome as a therapeutic target

The role of the reinstatement of the microbiome on therapeutic response in solid tumours is also currently being investigated. Modulation of the gut microbiota by means of faecal microbial transplantation (FMT) has demonstrated promising results in preclinical cancer models in mice [75, 76], and more recently in a phase-I clinical trial [88]. Using donors who achieved complete response to PD-1 blockade, FMT and re-induction of anti-PD-1 therapy in patients with refractory metastatic melanoma is safe, feasible and potentially effective (clinical response in 3/10 patients) [88]. However, the characteristics of optimal microbiota compositions of FMT donors and recipients remain elusive and future studies in larger cohorts are needed.

There are currently seven studies listed on clinicaltrials.gov (last updated 1st April 2021) recruiting patients with cancer for FMT interventions. The majority of eligible patients are those with immunotherapy-resistant disease and the cancer types being investigated are RCC, melanoma, prostate and gastrointestinal, and one study is recruiting patients with advanced solid tumours who are being treated with immunotherapy (NCT4116775, NCT04130763, NCT04264975, NCT03353402, NCT03341143, NCT04758507 and NCT037772899). Additionally, four studies are recruiting patients to investigate the effectiveness of FMT in treating immunotherapy-induced colitis (NCT04038619, NCT03819296, NCT04721041 and NCT04163289). There are currently no recorded studies recruiting patients with BTC in this specific research area.

CONCLUSION

This review highlights accumulating evidence for an association between the microbiome and BTC; however, studies to date have not yet distinguished whether changes in the composition of microbiota have a causative role, or whether they are solely an effect of the cancer. Small sample size, a lack of methodological standardisation and non-availability of information about confounding factors limit the comparability of the obtained results. Many of the studies also lack information on characteristics such as the stage of BTC, therefore limiting the applicability of associations between specific microbes and survival outcomes. Standardisation of study protocols, as well as collection and publication of information on other confounding factors, including medication history, diet and geography, must be considered in the design of future studies.

In this review, *Fusobacteria, Enterobacteriaceae* and *Pseudomonadaceae* were the most consistently reported taxa at an increased abundance in patients with BTC. *Fusobacteria*, and more specifically *F. nucleatum*, has been frequently associated with colorectal carcinogenesis [89, 90], however, it has also been reported in oral [91, 92], oesophageal [93, 94], cervical [95] and gastric cancer [96, 97]. *Enterobacteriaceae* may also promote colon cancer [89, 98]. The biliary tract is exposed to the gastrointestinal microbiome via the gut–liver axis, and therefore microbes involved in colorectal carcinogenesis and progression, such as *Fusobacteria* and *Enterobacteriaceae*, may play a similar role in BTC. Further studies are needed to confirm this relationship.

Future prospective studies in BTC should aim to explore the prognostic and predictive ability of the microbiome, and establish if differences in the diversity and composition of the microbiome are correlated with response to treatment and survival. Furthermore, studies to investigate the dynamic nature of the microbiome in BTC should assess differences between patients with resectable versus advanced disease, as well as identifying any longitudinal changes in the microbiome, for example, pre-compared with post treatment. Additionally, emerging evidence suggests a link between antibiotic use and immunotherapy efficacy; future studies need to establish whether this link exists in patients with BTC receiving systemic treatment.

Formal diagnosis of BTC, in particular cholangiocarcinoma, is made difficult by numerous factors, including a lack of screening programmes, non-specificity and late presentation of symptoms, and technical difficulties in obtaining tissue for cytology or histopathology. The microbiome is an emerging field through which new biomarkers may be identified, and offers potential as a tool for early diagnosis of BTC, prognostication or even as a future therapeutic target. As a result, this evolving field of research warrants further investigation in both preclinical and clinical BTC studies.

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ACKNOWLEDGEMENTS
Not applicable

AUTHOR CONTRIBUTIONS
RW drafted and revised the paper. EK reviewed and approved the final version of the paper. TJ performed the literature search and reviewed and approved the final version of the paper. AL reviewed and approved the final version of the paper. RAH reviewed and approved the final version of the paper. JWV reviewed and approved the final version of the paper. MMN concept initialisation and review and approval of draft and final version of the paper.

FUNDING INFORMATION
Dr. Roseanna C Wheatley is studying for an MD, funded by the Timpson Fellowship. The salary of Dr. Angela Lamarca is in part funded by The Christie Charity and the European Union's Horizon 2020 Research and Innovation Programme [grant number 825510, ESCALON].

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
Not applicable.

CONSENT TO PUBLISH
Not applicable.

Competing interests
RCW: no competing interests to declare. EK: no competing interests to declare. TJ: no competing interests to declare. AL: received travel and educational support from Ipsen, Pfizer, Bayer, AAA, SirtEx, Novartis, Mylan and Delcath; speaker honoraria from Merck, Pfizer, Ipsen, Incyte and AAA; advisory honoraria from EISAI, Nutricia Ipsen, QED and Roche; she is a member of the Knowledge Network and NETConnect Initiatives funded by Ipsen; all outside scope of this work. RAH has served on the advisory board for Roche, BMS, Eisai, Celgene, Beigene, Ipsen and BTG. He has received speaker fees from Eisai, Ipsen, Mylan and PrimeOncology, and has received travel and educational support from Bayer, BMS and Roche; all outside of the scope of this work. JWV received honoraria from Agios, AstraZeneca, Baxter, Genoscience Pharma, Hutchison Medipharma, Imaging Equipment Ltd (AAA), Incyte, Ipsen, Mundipharma EDO, Mylan, QED, Servier, Sirtex and Zymeworks; and grants, honoraria and non-financial support from NuCana, all outside of the scope of this work. MMN received research grant support from Servier, Ipsen and NuCana. She has received travel and accommodation support from Bayer and Ipsen and speaker honoraria from Pfizer, Ipsen, NuCana and Mylan. She has served on advisory boards for Celgene, Ipsen, Sirtex, Baxalta and Incyte; all outside of the scope of this work.

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