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Original Research

Identification of mutations in circulating cell-free tumour DNA as a biomarker in hepatocellular carcinoma

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Abstract ***Background:*** Hepatocellular carcinoma (HCC) is increasing globally. Prognostic biomarkers are urgently needed to guide treatment and reduce mortality. Tumour-derived circulating cell-free DNA (ctDNA) is a novel, minimally invasive means of determining genetic alterations in cancer. We evaluate the accuracy of ctDNA as a biomarker in HCC.
Methods: Plasma cell-free DNA, matched germline DNA and HCC tissue DNA were isolated from patients with HCC (n = 51) and liver cirrhosis (n = 10). Targeted, multiplex polymerase chain reaction ultra-deep sequencing was performed using a liver cancer–specific primer panel for genes *ARID1A*, *ARID2*, *AXIN1*, *ATM*, *CTNNB1*, *HNF1A* and *TP53*. Concordance of mutations in plasma ctDNA and HCC tissue DNA was determined, and associations with

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clinical outcomes were analysed.
Results: Plasma cell-free DNA was detected in all samples. Lower plasma cell-free DNA levels were seen in Barcelona Clinic Liver Cancer (BCLC A compared with BCLC stage B/C/D (median concentration 122.89 ng/mL versus 168.21 ng/mL, p = 0.041). 29 mutations in the eight genes (21 unique mutations) were detected in 18/51 patients (35%), median 1.5 mutations per patient (interquartile range 1–2). Mutations were most frequently detected in *ARID1A* (11.7%), followed by *CTNNB1* (7.8%) and *TP53* (7.8%). In patients with matched tissue DNA, all mutations detected in plasma ctDNA detected were confirmed in HCC DNA; however, 71% of patients had mutations identified in HCC tissue DNA that were not detected in matched ctDNA.
Conclusion: ctDNA is quantifiable across all HCC stages and allows detection of mutations in key driver genes of hepatic carcinogenesis. This study demonstrates high specificity but low sensitivity of plasma ctDNA for detecting mutations in matched HCC tissue.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the third most common cause of cancer death [1,2]. Significant heterogeneity in response to therapy exists within each Barcelona Clinic Liver Cancer (BCLC) stage of disease, and this combined with a lack of prognostic biomarkers to optimise treatment allocation contribute significantly to low survival rates in HCC [2]. Given the alarming global increase in both HCC incidence and mortality, improving outcomes in HCC represents a major international public health challenge [3].

Several studies have identified genetic predictors of outcome in HCC, including somatic mutations in Wnt and transforming growth factor-β signalling pathways, whilst others have identified unique gene expression signatures predictive of survival [4,5]. However, these studies all utilise tumour tissue, a significant practical limitation in the clinic as HCC diagnosis is based on radiological criteria, and only a minority of patients require liver biopsy for histopathological diagnosis [6]. Moreover, tumour biopsy is inherently limited by intratumoural heterogeneity and is invasive, limiting longitudinal assessment of genetic alterations in HCC [7].

Circulating cell-free DNA (cfDNA) is released through apoptosis and necrosis from both healthy and malignant cells into the blood stream [8,9]. Levels are significantly elevated in inflammatory states, particularly malignancy [8]. cfDNA levels can distinguish malignancy from benign inflammatory diseases such as colon cancer in the presence of inflammatory bowel disease [10,11]. More specifically, tumour-derived cfDNA (ctDNA) can be distinguished from wild-type cfDNA by identification of somatic alterations present in the tumour of interest, but not in matched somatic tissue. Both non-tumour–specific cfDNA and ctDNA quantification and the presence of genetic and epigenetic variants within ctDNA are potential biomarkers in cancer [8,11]. The key advantage of ctDNA is that it provides dynamic information about tumour biology without the need for repeated tumour biopsies [9,12]. Moreover, ctDNA can easily be collected in the clinic to monitor both response to treatment and tumour recurrence after treatment. ctDNA has therefore been likened to a 'liquid biopsy' of cancer [13].

Recently, studies have shown that both non-cancer–specific plasma cfDNA and tumour-derived plasma ctDNA show considerable promise as prognostic biomarkers prior to treatment and for monitoring treatment response in various malignancies, including breast, ovarian and lung cancer [8,14]. Several studies have demonstrated the potential utility of cfDNA levels as a clinical biomarker in HCC [11–18], and mutation detection in ctDNA from HCC patients has also been described. In patients with HCC due to hepatitis B and aflatoxin exposure, a common *TP53* gene mutation (R249S) is frequently detectable in ctDNA [15]. Kirk *et al.* demonstrated that the Ser 249 *TP53* mutation could be detected in ctDNA from West African patients with advanced-stage HCC, and this mutation had good discriminative accuracy for distinguishing hepatitis B-related HCC from chronic hepatitis B infection and HBV-related cirrhosis [9,10]. However, to date, there have been limited studies demonstrating accurate concordance between plasma ctDNA mutations and those in matched HCC tissue DNA. Moreover, few studies describe detection of mutations in genes with potential prognostic and therapeutic utility in ctDNA. Finally, utility of ctDNA quantification and mutation detection across all BCLC stages of HCC has not been evaluated.

The aims of this study were to first describe cfDNA levels in patients with early and advanced stage HCC compared with patients with cirrhosis and, second, to establish whether commonly described carcinogenic

driver mutations found in HCC tissue are present in plasma ctDNA.

2. Methods

2.1. Study design and participants

In this cross-sectional study, 51 adult patients (over 18 years of age) with confirmed radiological diagnosis of HCC [2] were recruited from two specialist liver cancer clinics (Imperial College NHS Trust, n = 19 (37.3%), United Kingdom [UK], and University of Piemonte Orientale, Italy, n = 32, 62.8%), and 10 adult patients with liver cirrhosis but no HCC were recruited as controls from a specialist liver clinic (Imperial College NHS Trust, UK). Controls were screened for HCC with liver ultrasound within 6 months of recruitment to the study. The study was approved by the local Institutional ethics committees and was conducted in accordance with the 1975 Declaration of Helsinki.

2.2. Sample collection

A 20 mL blood sample was collected from all subjects on the day of recruitment in an ethylene diamine tetra-acetic acid tube, and 1 mL aliquots of plasma and buffy coat were stored in a minus 80°C freezer until further use (Supplementary Methods). Plasma and liver tissue samples from the UK were collected within 12 months of cfDNA analysis, whereas samples from Italy were archival (Biobank), collected more than 12 months prior to cfDNA analysis.

Demographic data (including age, gender and ethnicity), clinical data (including aetiology of underlying liver disease, presence of cirrhosis determined by Fibroscan™ or liver biopsy, Child-Turcotte-Pugh score [CTP, Supplementary Table 2], liver function tests) and HCC stage (including BCLC stage, alpha-fetoprotein [AFP] level, presence of portal venous thrombosis and metastases) were also collected on the same day as the blood sample.

2.3. DNA extraction from formalin-fixed, paraffin-embedded tissues, peripheral blood mononuclear cells and plasma specimens

cfDNA was extracted from plasma using the QIAsymphony platform (Qiagen Inc, Hilden Germany) and QIAsymphony Circulating DNA Kit according to the manufacturer's recommendations. Where available, sections of matched formalin-fixed, paraffin-embedded (FFPE) HCC tissue were cut from archival blocks and macrodissection of tumour areas for targeted DNA extraction was performed. DNA from PBMC samples and HCC tissue sections was extracted on a Maxwell platform (Promega GmbH, Mannheim, Germany) using the Maxwell DNA Blood and the Maxwell DNA FFPE Plus Tissue purification kit respectively, according to the manufacturer's instructions.

2.4. DNA quantification, library preparation and next generation sequencing

Quality control was performed, and polymerase chain reaction (PCR) amplification of a 173bp region of the *HFE* gene was used to quantify amplifiable DNA in all samples. Real-time PCR was performed in triplicate (Supplementary Methods). Primer sets of the Human Liver Cancer GeneRead DNAseq targeted panel V2 from QIAgen were used for multiplex PCR-based target enrichment, including primer sets for 2052 amplicons targeting 191 KB (up to 99% coverage) in 33 liver specific genes (Supplementary Methods). Multiplex PCR, using the GeneRead DNAseq Panel PCR Kit, was performed, and the amplicons of each sample were pooled, purified and analysed by capillary electrophoresis using the Fragment Analyser (Advanced Analytical, Heidelberg, Germany). After additional purification and size selection steps, targeted DNA was PCR enriched and library pools prepared using GeneRead DNA Library I Amp Kit (Qiagen), prepared according to the MiSeq System User Guide (Illumina, Inc. San Diego, CA). Next generation sequencing was performed on a MiSeq instrument (Illumina, Inc.) using v2 chemistry as recommended by the manufacturer (Supplementary Methods). Sample analysis was performed in triplicate to identify and reduce the impact of measurement error and contaminants.

2.5. Data analysis

Analysis of matched plasma ctDNA and HCC DNA sequences was performed using Mutect v.1 (Broad Institute, Harvard US) using matched germline PBMC DNA as a reference. This method excludes genetic variants that are present in ctDNA that are also present in the matched germline PBMC DNA. Genetic variants were identified after filtering with additional quality control steps including removal of all variants with low read coverage and samples with low GC quality (outlined in Supplementary Methods, Supplementary Fig. 1). For the eight HCC patients in whom matched HCC tissue DNA was available, concordance between variants identified in ctDNA and HCC tissue DNA, but not in matched germline DNA, was determined. ctDNA sample variants were therefore called 'blind' to mutations present in matched HCC tissue DNA: ctDNA variants that did not fulfill quality control criteria and were not detected by Mutect in comparison to germline sequence were not re-interrogated to find known HCC tissue DNA mutations. Non-coding variants were excluded to focus on variants of potential clinical significance.

For HCC patients in whom only matched plasma ctDNA and germline PBMC DNA were available, mutations identified in ctDNA, but not in matched germline DNA were determined. We then applied additional filters to remove variants to maximise specificity for clinically significant variants in ctDNA. Non-coding genetic variants, variants that were also identified in cirrhotic controls and recognised variants listed in human SNP databases (dbSNP, 1000 Genomes) were excluded from analysis, unless these variants were also listed in the COSMIC cancer mutation database (see Supplemental Methods and Supplemental Fig. 1). Finally, we focussed only on variants identified in known putative genes with frequent association with HCC, namely *ALB (frequency altered in HCC 12%), ARID1A (12%), ARID2 (altered 5%), ATM (5%), AXIN1 (10%), CTNNB1 (35%), HNF1A* (4%) and *TP53(22%)* [16].

2.6. Statistical analysis

Clinical variables were described using mean ± standard deviation or median (interquartile range [IQR]) as appropriate to distribution. Associations between cfDNA levels and clinical variables were assessed by Wilcoxon rank-sum test or Kruskall-Wallis test as appropriate.

3. Results

Matched plasma and PBMC samples were collected simultaneously from 51 HCC patients and 10 cirrhotic controls. Paraffin-embedded HCC tissue samples were available for eight patients.

The mean age of HCC patients was 70 years, and the majority were male (88%) with cirrhosis (92%) and CTP class A stage liver function (62%). Fifty percent (n = 25) had hepatitis B or C. A significant proportion of patients had early-stage HCC (39% BCLC stage A, Table 1; BCLC staging system outlined in Supplementary Table 3).

Table 1
Distribution of clinical variables among HCC patients (n = 51).

Clinical variable	Number (%)
Gender (n = 51)	
Male	45 (88.2%)
Female	6 (11.8%)
Mean age (years ± SD) (n = 52)	70.3 ± 9.3 years
Cirrhosis (n = 50)	46 (92.0%)
Hepatitis B (n = 51)	6 (12.0%)
Hepatitis C (n = 51)	19 (38.0%)
Alcohol liver disease (n = 50)	21 (42.0%)
Median number of nodules (n = 51)	2 (IQR 1–5)
Median size largest nodule (cm; n = 51)	3.5 cm (IQR 2.7–5.5 cm)
Portal vein thrombosis (n = 51)	7 (14.0%)
Extrahepatic metastases (n = 51)	6 (12.0%)
Median AFP (n = 50)	19.1 (IQR 4.0–140.2)
BCLC (n = 51)	
A	20 (39.2%)
B	11 (21.6%)
C	16 (31.4%)
D	4 (7.8%)
CTP class (n = 50)	
A	31 (62.0%)
B	15 (30.0%)
C	4 (8.0%)

AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma; BCLC, Barcelona Clinic Liver Cancer score; CTP, Child-Turcotte-Pugh.

3.1. Quantification of cfDNA and clinical associations

Plasma cfDNA (released by both malignant and non-malignant cells) was detected in all cases and controls (n = 61). However, cfDNA levels were noted to be significantly lower in plasma samples stored for more than 12 months compared with samples from patients recruited within 12 months of processing and DNA extraction. Samples collected within 12 months had a median cfDNA concentration of 136.93 ng/mL (IQR 106.87–178.90 ng/mL), compared with archival samples collected more than 12 months prior to the study (mean cfDNA concentration 15.21 ng/mL [IQR 9.47–26.99 ng/mL; $p < 0.00001$]). Given cfDNA levels were significantly lower in older samples, recently stored plasma samples were therefore used to measure associations between cfDNA levels and clinical parameters (n = 39; 10 controls and 29 HCC).

There was no significant difference in median cfDNA levels in patients with HCC compared with cirrhotic controls (141.59 ng/mL, IQR 99.44–177.19 ng/mL, compared with 133.35 ng/mL, IQR 118.75–252.88 ng/mL; p = 0.85; Fig. 1A). However, when considering only those patients with HCC, patients with BCLC stage A (n = 19) had a lower median cfDNA level compared with patients with BCLC B, C or D stage HCC (n = 10; median cfDNA level 122.89 ng/mL [IQR 57.17–154.26 ng/mL] compared with 168.21 ng/mL [IQR 135.46–202.89 ng/mL], p = 0.041; Fig. 1B).

There was no relationship between cfDNA levels and the presence of either portal venous thrombosis (p = 0.33; 177.99 ng/mL [IQR 137.28–218.64 ng/mL] compared with 141.24 ng/mL [IQR 87.75–168.21 ng/mL]; Supplementary Fig. 2) or metastases (p = 0.48; 176.14 ng/mL [IQR 133.64–218.64] compared with 141.59 ng/mL [IQR 87.75–168.21 ng/mL]; Supplementary Fig. 3). There was also no significant correlation between cfDNA levels and serum AFP level (p = 0.06, adjusted $r^2 = 0.16$, Supplementary Fig. 4) or tumour size (p = 0.43, adjusted $r^2 = 0.04$, Supplementary Fig. 5).

3.2. Concordance between mutations identified in plasma ctDNA and matched HCC tissue DNA

Having observed detectable levels of ctDNA in patients with HCC, we then determined genetic variants present

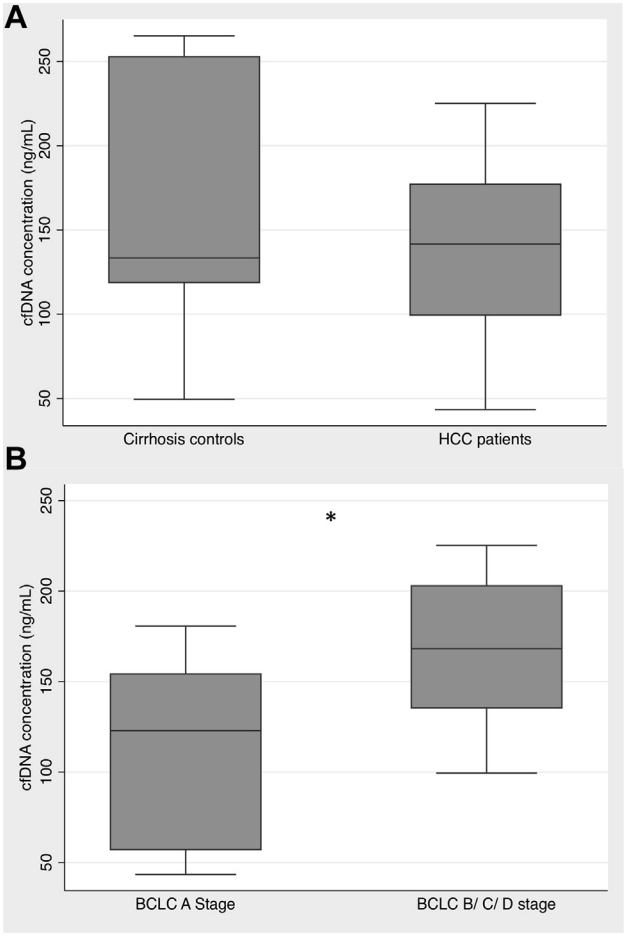

Fig. 1. (A) Comparison of circulating cell-free DNA levels between patients with HCC and patients with cirrhosis. There was no significant difference between cfDNA levels in HCC patients (median 141.59 ng/mL, IQR 99.44–177.19 ng/mL) compared with patients with cirrhosis (median 133.35 ng/mL, IQR 118.75–252.88 ng/mL). Wilcoxon rank-sum p = 0.848. (B) Circulating cell-free DNA levels stratified by BCLC stage. BCLC stage A patients had a lower median cfDNA level of 122.89 ng/mL (IQR 57.17–154.26 ng/mL), compared with patients with BCLC B, C or D stage HCC (median cfDNA level 168.21 ng/mL (IQR 135.46–202.89 ng/mL). Wilcoxon rank-sum p = 0.041.*p < 0.05. BCLC, Barcelona Clinic Liver Cancer; cfDNA, circulating cell-free DNA; HCC, hepatocellular carcinoma; IQR, interquartile range.

in ctDNA in HCC patients in comparison to controls. ctDNA samples from all HCC patients (n = 51) were analysed with a median read depth of 486 (IQR 234–797; n = 51) and median mutant allelic depth of 560 (IQR 250–705; n = 18). The median mutant allelic frequency was 0.119 (IQR 0.050–0.423; n = 18).

First, we explored the concordance between genetic variants identified in ctDNA and in matched HCC tissue samples in the subset of eight patients in whom primary tumour tissue was available for analysis. HCC tissue DNA analysis detected putative gene mutations in seven of the eight patients (88%, Table 2). Two of the seven patients in whom gene mutations were identified in HCC tissue DNA also had evidence of identical carcinogenic mutations in matched ctDNA. One patient had mutations in both the CTNNB1 (S37A; read depth 601, frequency 0.035; Table 2 and Supp Table 1) and TP53 (R248G; read depth 1151, frequency 0.063; Table 2 and Supp Table 1) genes, and one had a mutation in ARID1A (P1563S; read depth 429, frequency 0.058; Table 2 and Supp Table 1). Importantly, no additional mutations were identified in ctDNA; therefore, all the mutations identified in ctDNA were detectable and validated in matched HCC tissue DNA samples. However, five of the seven patients that had mutations identified in HCC tissue DNA did not have mutations detectable in ctDNA. Using ctDNA with targeted exome sequencing for the eight listed genes had a sensitivity of 30% but a specificity of 100% for detection of HCC in a given patient.

3.3. Mutations identified in plasma ctDNA from HCC patients without matched HCC tissue

Next, we analysed mutations in plasma ctDNA samples for whom there was no matched HCC tissue. Putative mutations in *ALB, ARID1A*, *ARID2*, *ATM*, *AXIN1*, *CTNNB1*, *HNF1A* or *TP53* were detected in plasma ctDNA in 18 of 51 patients with HCC (35%). The median number of gene mutations was 1.5 (IQR 1–2) per patient. Across the whole cohort of patients with HCC, analysis of matched plasma ctDNA and germline DNA identified 29 mutations, 21 of which were unique (Table 2). Seven of the 21 unique mutations identified (33%) were listed in the COSMIC cancer-related mutation database, 6/21 (29%) were listed in both COSMIC and dbSNP databases and 8/21 (38%) were novel mutations. The majority of genetic variants identified in ctDNA from patients with HCC were missense single-nucleotide polymorphisms (SNPs) mutations (18, 82%). 17 of the 20 protein-changing unique mutations (85%) had either a predicted or proven deleterious effect on protein function through conformational changes to key binding sites, as predicted by structural sequence analysis software (Supp Table 1; see Supplementary Methods). The most commonly mutated genes in ctDNA from HCC cases were *ARID1A* (6/51 patients, 11.7%), followed by *CTNNB1* (4, 7.8%) and *TP53* (4, 7.8%; Table 3). Nine of 18 HCC patients with detectable mutations in ctDNA (50%) had mutations in the SWI/SNF chromatin remodelling complex, and six (33%) had mutations in the Wnt-B-catenin signalling pathway (Table 3). The clinical distribution of gene mutations by BCLC stage and aetiology of underlying liver disease is presented in Fig. 2: 10 patients with BCLC stage C HCC and alcohol-related liver disease had the greatest

Table 2
Details of the putative genetic variants identified in matched plasma ctDNA and HCC tissue from patients with HCC (n = 8).

Sample ID	Gene	Position	Variant class	Exon	DNA change	Protein change	COSMIC database	Variant detected in matched plasma ctDNA
1	CTNNB1	3:41266097	Missense	3	c.94G > T	p.D32Y	COSMIC, dbSNP	No
2	No mutations in the putative genes were detected							
3	ALB	4:74286007	Missense	12	c.1372G > A	p.G458S	NOVEL	No
	ARID1A	1:27105867	Missense	20	c.5478C > G	p.D1826E	COSMIC	No
	ARID1A	1:27106918	Missense	20	c.6529G > A	p.G2177R	COSMIC	No
	ARID1A	1:27057998	Missense	3	c.1706C > T	p.A569V	NOVEL	No
	ARID1A	1:27087419	Missense	5	c.1993G > A	p.G665R	NOVEL	No
	ARID1A	1:27087889	Missense	6	c.2176C > T	p.P726S	NOVEL	No
	ARID1A	1:27088783	Missense	7	c.2392C > T	p.P798S	NOVEL	No
	ARID1A	1:27094462	Missense	11	c.3170C > T	p.S1057F	NOVEL	No
	ARID1A	1:27097752	Missense	12	c.3341C > T	p.P1114L	NOVEL	No
	ARID1A	1:27099080	Missense	13	c.3496G > A	p.A1166T	NOVEL	No
	ARID1A	1:27099096	Missense	13	c.3512G > A	p.S1171N	NOVEL	No
	ARID1A	1:27100075	Missense	16	c.3871G > A	p.E1291K	NOVEL	No
	ARID1A	1:27100853	Missense	18	c.4135C > T	p.P1379S	NOVEL	No
	ARID1A	1:27106583	Missense	20	c.6194C > T	p.A2065V	NOVEL	No
	ARID2	12:46233214	Missense	11	c.1433T > C	p.L478S	COSMIC	No
	ARID2	12:46244208	Missense	15	c.2302G > A	p.V768I	COSMIC	No
	ARID2	12:46246318	Missense	15	c.4412C > T	p.P1471L	COSMIC	No
	ARID2	12:46205247	Missense	4	c.331G > A	p.D111N	NOVEL	No
	ARID2	12:46230433	Missense	7	c.767C > T	p.S256F	NOVEL	No
	ARID2	12:46230637	Missense	8	c.886C > T	p.L296F	NOVEL	No
	ARID2	12:46243872	Missense	15	c.1966G > A	p.A656T	NOVEL	No
	ARID2	12:46243882	Missense	15	c.1976C > T	p.S659F	NOVEL	No
	ARID2	12:46244457	Missense	15	c.2551C > T	p.P851S	NOVEL	No
	ARID2	12:46244470	Missense	15	c.2564C > T	p.T855I	NOVEL	No
	ARID2	12:46245243	Missense	15	c.3337G > A	p.G1113R	NOVEL	No
	ARID2	12:46245376	Missense	15	c.3470C > T	p.S1157L	NOVEL	No
	ARID2	12:46245544	Missense	15	c.3638C > T	p.P1213L	NOVEL	No
	ARID2	12:46246458	Missense	15	c.4552C > A	p.P1518T	NOVEL	No
	ATM	11:108143312	Missense	22	c.3131A > G	p.N1044S	COSMIC	No
	ATM	11:108205823	Missense	56	c.8138G > A	p.R2713K	COSMIC	No
	ATM	11:108117849	Missense	9	c.1060C > T	p.H354Y	NOVEL	No
	ATM	11:108137962	Missense	18	c.2531G > A	p.G844E	NOVEL	No
	ATM	11:108141812	Missense	20	c.2860C > T	p.L954F	NOVEL	No
	ATM	11:108143267	Missense	22	c.3086C > T	p.T1029I	NOVEL	No
	ATM	11:108155151	Missense	27	c.3944C > T	p.A1315V	NOVEL	No
	ATM	11:108196063	Missense	47	c.6599A > G	p.E2200G	NOVEL	No
	AXIN1	16:339575	Missense	10	c.2327G > A	p.S776N	NOVEL	No
	AXIN1	16:343593	Missense	8	c.2081C > T	p.T694I	NOVEL	No
	AXIN1	16:343657	Missense	8	c.2017C > T	p.P673S	NOVEL	No
	AXIN1	16:343698	Missense	8	c.1976C > T	p.P659L	NOVEL	No
	AXIN1	16:348008	Missense	6	c.1498G > A	p.A500T	NOVEL	No
	AXIN1	16:364675	Missense	3	c.887C > T	p.S296F	NOVEL	No
	AXIN1	16:396184	Missense	2	c.842C > T	p.S281F	NOVEL	No
	AXIN1	16:396623	Missense	2	c.403G > A	p.E135K	NOVEL	Yes
	AXIN1	16:396820	Missense	2	c.206G > A	p.G69E	NOVEL	No
	CTNNB1	3:41266639	Missense	4	c.436G > A	p.A146T	NOVEL	No
	CTNNB1	3:41274868 3:41266112	Missense	8	c.1118C > T	p.P373L	NOVEL	No
	CTNNB1	12:121416767	Missense	3	c.109T > G	p.S37A	COSMIC, dbSNP	No
	HNF1A	12:121426701	Missense	1	c.196G > A	p.E66K	COSMIC	No
	HNF1A	12:121435427	Missense	2	c.392G > A	p.R131Q	COSMIC	No
	HNF1A	12:121431986	Missense	7	c.1460G > A	p.S487N	COSMIC, dbSNP	No
	HNF1A	12:121416767	Missense	4	c.733G > A	p.G245R	NOVEL	No
	TP53	17:7578188	Missense	6	c.661G > A	p.E221K	COSMIC	yes
	TP53	17:7578280	Missense	6	c.569C > T	p.P190L	COSMIC	No
	TP53	17:7579876	Missense	2	c.37C > G	p.P13A	COSMIC	
	TP53	17:7577539	Missense	7	c.742C > G	p.R248G	COSMIC, dbSNP	
	TP53	17:7579581	Missense	4	c.106C > T	p.P36S	NOVEL	
4	ARID1	1:27101405	Missense	18	c.4687C > T	P1563S	NOVEL	Yes
5	ATM	11:10813803	Missense	18	c.2572T > C	p.F858L	DBSNP + COSMIC	No
	ATM	11:10814346	Missense	23	c.3161C > G	p.P1054R	DBSNP + COSMIC	No

(*continued on next page*)

Table 2 (*continued*)

Sample ID	Gene	Position	Variant class	Exon	DNA change	Protein change	COSMIC database	Variant detected in matched plasma ctDNA
6	CTNNB1	3: 41266097	Missense	3	c.94G > A	p.D32N	DBSNP + COSMIC	No
7	CTNNB1	3:41266103	Missense	3	c.100G > A	p.G34R	DBSNP + COSMIC	No
8	TP53	17:7577534	Missense	7	c.747G > T	p.R249S	DBSNP + COSMIC	No
					Total gene variants in HCC detected in matched ctDNA			3/59 (5%)

HCC, hepatocellular carcinoma; ctDNA, tumour-derived circulating cell-free DNA.

number of mutations in ctDNA, with mutations detected across six genes.

4. Discussion

ctDNA has the potential to revolutionise the clinical management of many cancers including HCC, by obviating the need for invasive tissue biopsy and providing a dynamic insight into the mutational progression of cancer in real time for the purposes of monitoring recurrence, prognosis and treatment response. However, in contrast to other malignancies, experience with ctDNA mutation detection in HCC has been limited [11]. In this study, we have shown that cfDNA (derived from both malignant and non-malignant cells) is detectable in the plasma of patients with both early-stage and advanced HCC and that cfDNA levels correlate with BCLC stage. We have also confirmed a high concordance between mutations identified in carcinogenic driver genes in ctDNA and mutations in matched HCC tissue.

In comparison to other tumour types, HCC has a lower metastatic potential and often remains well differentiated, characteristics that may theoretically reduce ctDNA release into the bloodstream. In addition, the pathogenesis and progression of HCC is genomically heterogeneous and determined in part by liver disease aetiology (17), making targeted mutation approaches to ctDNA analysis more complex compared with tumour types where carcinogenesis is reflected in key mutational events, such as colorectal cancer. Therefore, we designed this exploratory study to investigate the feasibility and clinical utility of both cfDNA and tumour-specific ctDNA measurement in HCC across BCLC stages using matched germline DNA from PBMCs to identify tumour-specific variants, acknowledging the clinical limitations of obtaining HCC tissue samples for a cancer that has radiological diagnostic criteria and mechanisms of follow-up.

We have shown that cfDNA was detectable in all patients, including cirrhotic controls without HCC. Moreover, cfDNA levels were in keeping with those reported in other studies, despite the relatively small volume of plasma extracts used (1 mL) in the current study compared with other studies [12,13]. However, there was no significant difference in cfDNA levels between cirrhotic controls and HCC cases, in contrast to findings of some other studies [9,10,14–16]. This may reflect the fact we deliberately included patients with early-stage disease as defined by BCLC stage. Importantly, in our study, cfDNA levels were detectable in both early and late-stage HCC, confirming the potential utility of cfDNA as a biomarker across all stages of the disease. As described in other studies [12,16], cfDNA levels were higher in HCC patients with more advanced disease compared with early-stage disease, as a likely consequence of greater cfDNA release in the context of inflammation and greater tumour cell burden and necrosis [8].

We also validated the utility of ctDNA as a 'liquid biopsy' in HCC by demonstrating that genetic variants identified in ctDNA corresponded with mutations present in matched HCC tissue DNA in a subset of patients, an important finding that facilitates taking this technology further into the clinic. We observed 100% specificity, but only 30% sensitivity for detecting mutations present in HCC tissue DNA in matched ctDNA samples. These data show higher specificity and similar sensitivity for ctDNA mutation detection compared with other recent reports: in a recent study by Liao *et al.* [18], sensitivity for mutation detection was only 19.5% for mutation detection in ctDNA compared with matched HCC DNA. A recent abstract published in HCC by Labgaa *et al.* [19] also reported relatively low concordance in genetic variants between HCC DNA and ctDNA (43%). However, they were able to significantly improve sensitivity by using a panel of 58 genes to overcome the diagnostic barrier of genetic heterogeneity. In our study, we had considerable success using a limited panel of eight genes. Moreover, in these studies, a lower concentration of ctDNA detected (7–15 ng/mL) was reported compared with the mean ctDNA level in early-stage HCC patients in our study (58.2 ± 59.8 ng/mL) despite similar extraction volumes, which may explain some of the discrepancies in sensitivity. Using a 40-gene targeted sequencing panel and de-novo calling of gene variants using germline DNA, Ng et al. [20] recently reported gene variant detection in 27% of ctDNA samples from HCC patients. Interestingly, they found all patients with detectable ctDNA mutations had advanced stage HCC, though the majority of patients in the study were BCLC stage A (20). We also found a

Table 3
Frequency of putative genetic variants identified in plasma ctDNA from patients with HCC (n = 51).

Gene	Position	Exon	DNA change	Protein change	Number (%) of HCC patients with mutation (n = 51)	Number (%) HCC patients with gene affected (n = 51)
ALB	chr4:74285311	11	c.1290G > T	E430D	1 (2%)	1 (2%)
ARID1A	chr1:27087479	5	c.2503A:C	T685P	4 (8%)	5 (10%)
	chr1:27100291	17	c.e−17		1 (2%)	
	chr1:27101405	18	c.4687C > T	P1563S	1 (2%)	
ARID2	chr12:46230776	8	c.e8+2		1 (2%)	2 (4%)
	chr12:46245235	15	c.3329G > T	G1110V	1 (2%)	
ATM	chr11:108123551	13	c.1810C > T	P604S	1 (2%)	3 (6%)
	chr11:108139287	19	c.2789 T > G	L930*	1 (2%)	
	chr11:108143306	22	c.3125 T > C	L1042P	1 (2%)	
	chr11:108236237		3′UTR		1 (2%)	
AXIN1	chr16:348233	6	c.1273G > A	G425S	1 (2%)	2 (4%)
	chr16:397044		5′UTR		1 (2%)	
CTNNB1	chr3:41266110	3	c.107A > C	H36P	1 (2%)	3 (6%)
	chr3:41266112	3	c.109 T > G	S37A	2 (4%)	
	chr3:41266113	3	c.110C > A	S37Y	1 (2%)	
	chr3:41266136	3	c.133 T > C	S45P	1 (2%)	
HNF1A	chr12:121435427	7	c.1460G > A	S487N	1 (2%)	2 (4%)
	chr17:7577098	8	c.840A > T	R280S	1 (2%)	
TP53	chr17:7577539	7	c.742C > G	R248G	1 (2%)	4 (8%)
	chr17:7577543	7	c.738G > T	M246I	1 (2%)	
	chr17:7577565	7	c.716A > G	N239S	1 (2%)	
	chr17:7578257	6	c.592G > T	E198*	1 (2%)	
	chr17:7578433	5	c.497C > A	S166*	1 (2%)	

HCC, hepatocellular carcinoma; ctDNA, tumour-derived circulating cell-free DNA.
* New codon is a (premature) stop codon (ie nonsense variant).

trend towards higher mutation number in patients with advanced disease (Fig. 2), though in our study ctDNA was detectable in patients across all BCLC stages. The data by Ng et al. concur with our findings, demonstrating a low sensitivity of de-novo mutation detection using ctDNA (20). Specificity is likely to be lower in the Ng *et al.* (20) and the Labgaa *et al.* (19) studies because of the large number of genes interrogated compared with an eight-gene signature approach to analysis. Taken together, these data demonstrate improvements in gene detection methods, analysis pipelines and technology are needed to increase ctDNA detection sensitivity; however, it is very encouraging that mutations present in ctDNA had a high specificity for HCC tissue mutations to facilitate mutation-guided therapy in the future. Importantly, we detected mutations in genes shown to be key drivers of carcinogenic pathways in HCC that have been well validated in the HCC literature, namely *ALB, ARID1A, ARID2, ATM, AXIN1, CTNNB1, HNF1A* and *TP53* [5,15,16,21–25], and these were detected in ctDNA samples using a sequencing pipeline that uses comparison matched PBMC germline DNA rather than matched HCC tumour tissue to call gene variants with high accuracy, illustrating the utility of this technique at the bedside where HCC tissue is rarely available. Moreover, mutations in these key genes were found with similar frequency in ctDNA samples to what is reported in the literature: CTNNB1 mutations were present in 6% of ctDNA samples compared with reported prevalence in HCC between 11 and 30%; TP53 in 8% of ctDNA samples compared with reported frequency of 10–20% and ARID1 in 10% of ctDNA samples compared with 4–17% in the literature (16, 17).

Mutations across the eight genes studies were identified in ctDNA of 35% of HCC participants. There are several potential explanations for the relatively low rate of ctDNA mutation detection in this study. First, HCC DNA was extracted from paraffin-embedded archival blocks rather than fresh frozen tissue, which has a greater propensity for artefact when sequenced [6]. Moreover, our data showed using recently procured plasma samples with storage time <12 months yield superior cfDNA concentrations in HCC patients, suggesting that ctDNA sample quality and concentration reduces over time. This has been shown by others [8,26] and suggests that sample collection for ctDNA studies must be of high quality and dedicated for purpose, spun and stored within an hour of sampling to maximise ctDNA yield. New technologies such as ctDNA specific collection tubes also improve yield in clinical settings where timing of sample processing after procurement can be difficult to control. Third, only eight genes were considered for mutation analysis in the current study. HCC is genetically diverse and heterogeneous and the majority of even well-described carcinogenic genes in HCC are mutated in less than 10% of HCC patients (16, 17). To reduce spurious variant calls in genes that were unlikely to play a significant role in liver carcinogenesis,

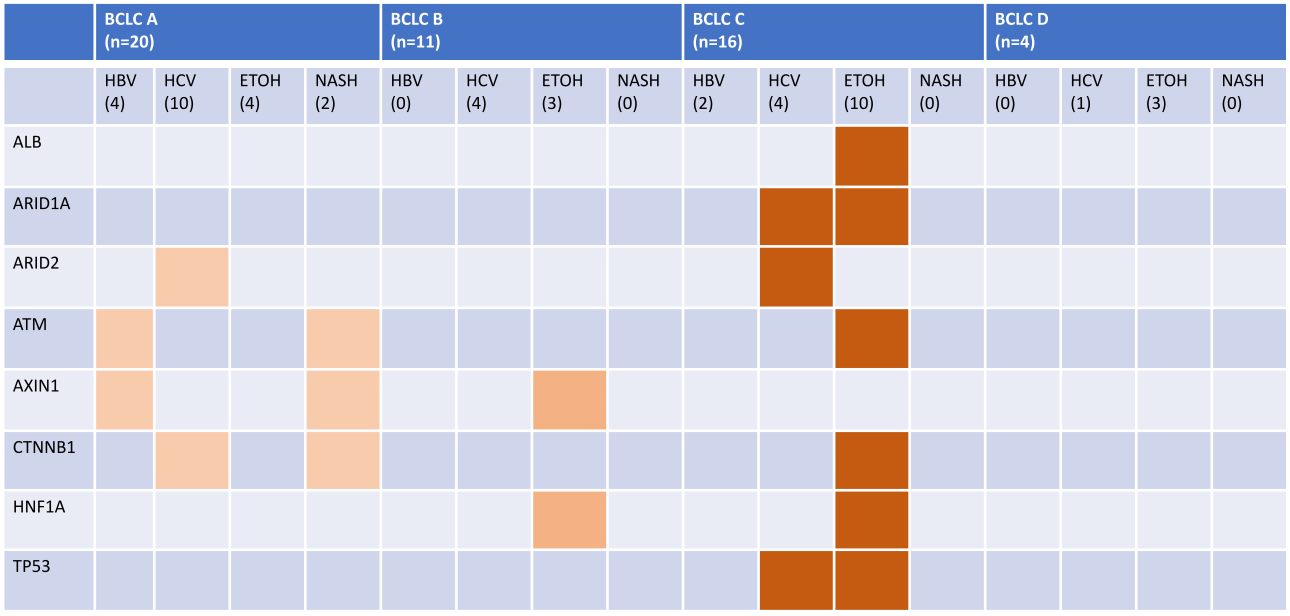

Fig. 2. Distribution of gene mutations tabulated by BCLC stage and aetiology of liver disease (n = 51). BCLC, Barcelona Clinic Liver Cancer.

we focussed on these eight genes because they are consistently reported as bearing mutations in HCC in more than 5–10% of HCC patients and because they represent key drivers of hepatic carcinogenesis [16,17]. However, *TERT* promoter mutations, one of the most common genetic alteration found in HCC [16], was not assessed in this study and would likely add greater sensitivity to HCC mutation detection in ctDNA in future studies. Finally, genetic intratumoural heterogeneity and differing mutational profiles in metastatic or necrotic HCC cells releasing ctDNA compared with malignant hepatocytes in the main tumour may also contribute to lower levels of mutation detection in ctDNA than would be expected in HCC tissue DNA. Nevertheless, these data show that ctDNA mutation detection had high specificity for mutation detection in matched HCC tissue, encouraging future larger studies to validate and optimise ctDNA use as a biomarker in HCC.

There are a number of limitations that we acknowledge in this study. Matched HCC tissue DNA was only available for a small subset of patients; this was because of the clinical limitations which are the reality in liver cancer clinics, where only a very small number of patients are suitable for resection and the minority require liver biopsy confirmation for diagnosis. We also limited our analysis to only eight of the genes covered by the liver cancer gene panel used for sequencing (see Supplementary Methods), which conceivably reduces the sensitivity of mutation detection in our study. However, despite the focussed gene analysis and small sample size, we were able to demonstrate congruity in mutations identified in ctDNA and HCC tissue DNA. ctDNA had a very high specificity but low sensitivity for mutations in HCC tissue. We did not use an orthogonal approach to confirm all mutations; however, a small selection of mutations were confirmed in an initial validation step using digital droplet targeted PCR to ensure our sequencing pipeline was accurate. We also performed all DNA processing and sequencing in triplicate to minimise measurement and sequencing errors and also applied stringent criteria for inclusion of variants in our analysis. Increasing plasma sample volumes at collection and sequencing repeat samples over time may improve sensitivity and specificity of mutation detection. Larger, well-powered longitudinal studies are needed to determine prognostic value of mutation detection in plasma ctDNA in patients with HCC, particularly those undergoing therapy, as peripheral ctDNA mutations may prove more informative than HCC tissue DNA because of tumour heterogeneity.

Finally, our small sample size limited study power to detect associations between mutations in gene pathways and survival in HCC. Nonetheless, this exploratory study provides important data demonstrating the feasibility and specificity of ctDNA as a ‘liquid biopsy’ in HCC. In particular, we have shown a significant correlation between cfDNA concentration and BCLC stage, suggesting cfDNA concentration could be used to monitor residual disease post curative therapy. The role of ctDNA variants in the stratification of patient therapy or as a marker of drug resistance has not been addressed in this study but remain important questions for future studies.

5. Conclusion

Plasma ctDNA is detectable across all stages of HCC disease and is highly specific for detection of HCC tissue mutations. Moreover, ctDNA mutation detection in HCC is feasible in the absence of HCC tissue DNA by analysis in comparison to matched germline PBMC DNA, therefore overcoming a critical practical barrier to its use in the clinic. These qualities make ctDNA an exciting potential diagnostic or prognostic biomarker in HCC, and further studies evaluating its clinical utility in large prospective cohorts of HCC patients are warranted.

Conflict of interest statement

The Institute for Pathology (University Hospital of Cologne, Germany) is an official testing institution of Qiagen Inc. (Hilden, Germany) and Reinhard Büttner is a member of the advisory board of Qiagen Inc. Furthermore, Qiagen Inc. has supported the study by providing reagents for cfDNA extraction and NGS library construction.

Author contributions

J.H. lead the study, contributed to study design, recruited UK study participants, collected UK clinical samples and data, contributed to bioinformatics pipeline development and analysis of genetic sequencing data, performed all statistical analyses and wrote the manuscript. S.A. performed the bioinformatics, contributed to the analysis of genetic sequencing data and contributed to the manuscript. D.P. provided all Italian samples and data and contributed to the manuscript. R.B., S.K. and M.O. contributed to study design and performed DNA extraction, library construction and ultra-deep targeted sequencing from all PBMC, plasma and tissue samples. R.M., M.E.B., M.L. and M.P. processed all Italian samples. S.A.K. contributed clinical data and proofread the manuscript. M.T. developed the study design and co-supervised the project. R.S. contributed to study design, provided UK samples and clinical data, contributed to the manuscript and co-supervised the project.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2019.04.014.

References

[1] Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015;136(5):E359–86.
[2] European Association for the Study of the Liver. EASL clinical practice guidelines: management of hepatocellular carcinoma. J Hepatol 2018;69(1):182–236.
[3] Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, et al. Cancer statistics. CA: A Cancer J Clin 2008;58(2):71–96.
[4] Lee JS, Chu IS, Heo J, Calvisi DF, Sun Z, Roskams T, et al. Classification and prediction of survival in hepatocellular carcinoma by gene expression profiling. Hepatology 2004;40(3):667–76.
[5] Hoshida Y, Villanueva A, Kobayashi M, Peix J, Chiang DY, Camargo A, et al. Gene expression in fixed tissues and outcome in hepatocellular carcinoma. N Engl J Med 2008;359(19):1995–2004.
[6] EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. J Hepatol 2012;56(4):908–43.
[7] Pinato DJ. Circulating-free tumour DNA and the promise of disease phenotyping in hepatocellular carcinoma. Oncogene 2018;37(34):4635–8.
[8] Alix-Panabieres C, Schwarzenbach H, Pantel K. Circulating tumor cells and circulating tumor DNA. Annu Rev Med 2012;63:199–215.
[9] Schwarzenbach H, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Canc 2011;11(6):426–37.
[10] Shapiro B, Chakrabarty M, Cohn EM, Leon SA. Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. Cancer 1983;51(11):2116–20.
[11] Howell JA, Khan SA, Knapp S, Thursz MR, Sharma R. The clinical role of circulating free tumor DNA in gastrointestinal malignancy. Transl Res 2017 May;183:137–54.
[12] Ignatiadis M, Dawson SJ. Circulating tumor cells and circulating tumor DNA for precision medicine: dream or reality? Ann Oncol 2014;25(12):2304–13.
[13] Alix-Panabieres C, Pantel K. Circulating tumor cells: liquid biopsy of cancer. Clin Chem 2013;59(1):110–8.

[14] Dawson SJ, Tsui DW, Murtaza M, Biggs H, Rueda OM, Chin SF, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med 2013;368(13):1199–209.
[15] Kirk GD, Camus-Randon AM, Mendy M, Goedert JJ, Merle P, Trepo C, et al. Ser-249 p53 mutations in plasma DNA of patients with hepatocellular carcinoma from the Gambia. J Natl Cancer Inst 2000;92(2):148–53.
[16] Schulze K, Imbeaud S, Letouze E, Alexandrov LB, Calderaro J, Rebouissou S, et al. Exome sequencing of hepatocellular carcinomas identifies new mutational signatures and potential therapeutic targets. Nat Genet 2015;47(5):505–11.
[17] Zucman-Rossi J, Villanueva A, Nault JC, Llovet JM. Genetic landscape and biomarkers of hepatocellular carcinoma. Gastroenterology 2015;149(5):1226–12239 e4.
[18] Liao W, Yang H, Xu H, Wang Y, Ge P, Ren J, et al. Noninvasive detection of tumor-associated mutations from circulating cell-free DNA in hepatocellular carcinoma patients by targeted deep sequencing. Oncotarget 2016.
[19] Labgaa I, Villacorta-Martin C, D'Avola D, Craig AJ, von Felden J, Martins-Filho SN, et al. A pilot study of ultra-deep targeted sequencing of plasma DNA identifies driver mutations in hepatocellular carcinoma. Oncogene 2018;37(27):3740–52.
[20] Ng CKY, Di Costanzo GG, Tosti N, Paradiso V, Coto-Llerena M, Roscigno G, et al. Genetic profiling using plasma-derived cell-free DNA in therapy-naive hepatocellular carcinoma patients: a pilot study. Ann Oncol 2018;29(5):1286–91.
[21] Amaddeo G, Cao Q, Ladeiro Y, Imbeaud S, Nault JC, Jaoui D, et al. Integration of tumour and viral genomic characterizations in HBV-related hepatocellular carcinomas. Gut 2015;64(5):820–9.
[22] Hoshida Y, Nijman SM, Kobayashi M, Chan JA, Brunet JP, Chiang DY, et al. Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma. Cancer Res 2009;69(18):7385–92.
[23] Zucman-Rossi J, Benhamouche S, Godard C, Boyault S, Grimber G, Balabaud C, et al. Differential effects of inactivated Axin1 and activated beta-catenin mutations in human hepatocellular carcinomas. Oncogene 2007;26(5):774–80.
[24] Taniguchi K, Roberts LR, Aderca IN, Dong X, Qian C, Murphy LM, et al. Mutational spectrum of beta-catenin, AXIN1, and AXIN2 in hepatocellular carcinomas and hepatoblastomas. Oncogene 2002;21(31):4863–71.
[25] Lu LC, Shao YY, Lee YH, Hsieh MS, Hsiao CH, Lin HH, et al. beta-catenin (CTNNB1) mutations are not associated with prognosis in advanced hepatocellular carcinoma. Oncology 2014;87(3):159–66.
[26] Sozzi G, Roz L, Conte D, Mariani L, Andriani F, Verderio P, et al. Effects of prolonged storage of whole plasma or isolated plasma DNA on the results of circulating DNA quantification assays. J Natl Cancer Inst 2005;97(24):1848–50.