The value of liquid biopsy in the diagnosis and staging of hepatocellular carcinoma: a systematic review

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Background: Blood-borne tumour markers in the form of circulating tumour cells (CTCs) are of intense research interest in the diagnostic and prognostic work-up of hepatocellular carcinoma (HCC).

Methods: This is a meta-analysis. Using a PICO strategy, adults with HCC was the population, with the individual CTCs as the intervention and comparators. The primary outcome was the sensitivity and specificity of HCC detection with tumour specific single gene methylation alteration. Secondary outcomes were the comparison using specific assay methods and the effect of early vs. late stages on CTC positivity.

We included patients with HCC who had samples taken from peripheral blood and had sufficient data to assess the outcome data. ASSIA, Cochrane library, EMBase, Medline, PubMed and the knowledge network Scotland were systematically searched with appropriate Mesh terms employed. The quality assessment of diagnostic accuracy studies (QUADAS) was used to ensure quality of data. Statistical analysis was performed using the ‘Rev Man’ meta-analysis soft ward for Windows.

Results: The review included 36 studies, with a total of 5,853 patients. Here, we found that AFP has the highest overall diagnostic performance. The average Youden index amongst all CTC was 0.46 with a mode and median of 0.5 with highest of 0.87 and lowest of 0.01.

Conclusions: The available literature provides weak evidence that there is potential in the use of CTC, however the lack of a standardised procedure in the study of CTC contribute to the lack of consensus of use. Future research should include large scaled, standardized studies for the diagnostic accuracy of CTCs.

Keywords: Hepatocellular carcinoma (HCC); tumour markers; circulating tumour cells (CTCs); liquid biopsy

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Introduction

Hepatocellular carcinoma (HCC) is responsible for approximately 90% of primary liver cancers and is the second most common cause of cancer related deaths, worldwide (1). Although it is historically associated with viral infections, the incidence of HCC in western populations is expected to rise due to the increasing prevalence of noncommunicable diseases which are linked to this malignancy. Such diseases include obesity, diabetes, non-alcoholic fatty and alcoholic liver disease (2).

There have been recent changes to the available treatment options for HCC with concomitant improvements in outcome of patients with early disease. However, the overall prognosis of HCC remains generally poor, and is correlated with presenting stage. As such, early detection of the disease has been shown to be a significant clinical challenge. A diagnostic marker which has the
capacity to detect early stage cancer is thus likely to alter
the prognosis of HCC (3,4).

The current method by which HCC is diagnosed is
primarily based on imaging methods. Whilst useful, they
have a number of limitations. Ultrasound is operator
dependent and the sensitivity may be diminished due to
body habitus. Although CT scan has demonstrated marked
increase in sensitivity and specificity in comparison to
US scan, studies have indicated an indirect correlation to
tumour size which limits its ability in diagnosis of early
cancers (5). MRI is more sensitive (6), however, these scans
are expensive, time consuming and are resource constrained.

A number of candidate markers are available which may
represent breakthroughs in future HCC diagnosis and
management. Blood borne tumour markers in the form of
circulating tumour cells (CTCs) (7) and cell free nucleic
acids (cfDNA) (8) are topics of intense research. Each may
allow for strategies to detect cancers in early stages, measure
treatment progress, and offer prognosis post treatment. CTCs have been identified as playing a large role in
metastasis and recurrence via their nature of shedding of the
primary tumour into blood, lymph and bone marrow which
allows circulation to other parts of the body (8). However,
as a tumour marker, detection of CTCs remains limited at
best due to the lack of volume sensitivity in early stage HC,
and a wide specific detection range complicated by common
hepatic diseases associated with HCC (7).

CTCs are suggested to hold exciting potentials and many
studies have been conducted to discover new possibilities.
However, the sensitivity and specificity remain unclear. To
ensure high fidelity in this novel topic, this meta-analysis
aims to determine the diagnostic accuracy of CTC in the
diagnosis of HCC.

**Methods**

This was a meta-analysis carried out according to the
PRIMA guidelines (9).

**Eligibility criteria and outcomes**

Studies eligible for the analysis were defined using the
Population, Intervention, Comparison and Outcome
strategy (PICO) (10). The study population was adults
who were diagnosed with HCC via current gold standards
methods of detection. Type of intervention was the use of
CTCs in the detection of HCC. Comparator was individual
CTC performance in the study. The primary outcome was
the overall sensitivity and specificity of HCC detection
with tumour specific single gene methylation alteration.
Secondary outcomes were the comparison using specific
assay methods, early vs. late HCC stage and specific TNM
staging and Youden index.

**Inclusion criteria**

Studies were included if they matched all of the following.
(I) All patients were diagnosed with HCC. (II) Samples
taken were from peripheral blood. (III) Sufficient data was
available to assess sensitivity and specificity of the CTC or
data were available to calculate from primary data.

Studies were excluded if insufficient data for describing
or calculating sensitivity and specificity values; sample
evaluation was not related to HCC; full papers were
unavailable or the publication type was either letters to the
editors, reviews, technical reports, case reports. Articles
written in languages other than English and non-human
studies were also excluded from the study.

**Literature search**

ASSIA, Cochrane library, EMbase, Medline, PubMed
and the knowledge network Scotland were systematically
searched. The search criteria included a combination
of Mesh and string terms, for the following searches
in each database: (I) (“liquid biopsy” OR “liquid
Neoplasms”(Mesh) AND (“cfDNA” OR “cell-free DNA”).
(III) “Liver Neoplasms”(Mesh) AND Neoplastic cells,
Circulating”(Mesh). “Liver Neoplasms”(Mesh) was
substituted with string terms “liver cancer”, “liver
neoplasm”, “liver tumor”, “hcc”, and “primary liver tumor”
in cases where the Mesh term could not be utilised.

There was no limit on the date of publication and the
search was updated till February 2019.

**Study selection and data extraction**

PT, PM, LG conducted their database search independently.
Titles and abstracts were analysed and the studies were
uploaded on to Rayyan QRCI (11) to be reviewed manually
by the authors. The abstracts were screened on Rayyan
QRCI and any disagreements on data extraction and quality
assessment of the included studies were resolved through
discussion and checked by MB. The final included studies
were uploaded to Mendeley®, Elsevier, London, UK (12).
A flow chart for the inclusions and exclusions of this study is shown in Figure 1.

Full text articles were matched in Mendeley and further examined using the criteria stated below.

The data extracted from the articles were publication year, participant demographics, experimental method, assay indicators, cut off values, CTC positivity, cancer stages and sensitivity and specificity scorings.

The data were then categorized and analysed based on four distinct subgroup. They were (I) CTCs detected in serum; (II) CTC detection methodology; (III) low stage cancers; (IV) CTC detection rates and (V) group comparing the positivity rates of low and high stage cancers.

Quality assessment

The quality score of the studies were judged based on the Quality assessment of diagnostic accuracy studies (QUADAS) (13). The quality assessment is stratified into 14 item phrased questions each with yes, no or unclear. The questions covered 4 domains patient selection, index test, reference standard, and flow and timing. Each domain is assessed in terms of risk of bias, and the first 3 domains are also assessed in terms of concerns regarding applicability. The maximum score is 14, a score of 7 or greater indicated a high-quality study, whilst less than 7 were of low quality (Table 1).

Statistical analysis

Statistical analysis was performed using the ‘Rev Man’ meta-analysis soft ward for Windows. This software managed all the data and generated all the forest plots and the Moses-Littenberg SROC curve.

The study performed the diagnostic accuracy test review by calculating the sensitivity and specificity of each test. The data was tabulated and produced a scatter plot (Figures 2,3). Using the Moses-Littenberg SROC curve on rev man a summary roc curve was produced for each data set which gives an indication for descriptive purposes (Figure 4).

Studies which compared CTC positivity in high and low
<table>
<thead>
<tr>
<th>First author</th>
<th>Year</th>
<th>QUADAS score</th>
<th>Patients</th>
<th>M/F</th>
<th>Sample</th>
<th>Assay method</th>
<th>Assay indicators</th>
<th>Cutoff</th>
<th>TP</th>
<th>FN</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Youden index</th>
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<tbody>
<tr>
<td>Aselmam et al. (14)</td>
<td>2001</td>
<td>6</td>
<td>66</td>
<td>NA</td>
<td>Blood</td>
<td>RT-PCR</td>
<td>Methylation (AFP)</td>
<td>14 ng/mL</td>
<td>5</td>
<td>13</td>
<td>0.45</td>
<td>0.55</td>
<td>0.50</td>
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<tr>
<td>Bahnassy et al. (15)</td>
<td>2014</td>
<td>6</td>
<td>183</td>
<td>121/12</td>
<td>Blood</td>
<td>RT-PCR</td>
<td>Methylation (AFP)</td>
<td>7.5 ng/mL</td>
<td>115</td>
<td>91</td>
<td>0.87</td>
<td>0.87</td>
<td>0.87</td>
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<tr>
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<td>2008</td>
<td>6</td>
<td>37</td>
<td>NA</td>
<td>Plasma</td>
<td>MS-PCR</td>
<td>Methylation (APC)</td>
<td>N/A</td>
<td>14</td>
<td>62</td>
<td>0.49</td>
<td>0.49</td>
<td>0.49</td>
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<tr>
<td>Chen et al. (17)</td>
<td>2012</td>
<td>9</td>
<td>210</td>
<td>144/86</td>
<td>Blood</td>
<td>qPCR</td>
<td>Methylation (cDNA)</td>
<td>213.8 ng/mL</td>
<td>69</td>
<td>64</td>
<td>0.54</td>
<td>0.54</td>
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<td>7</td>
<td>69</td>
<td>45/24</td>
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<td>Chemiluminescence</td>
<td>Methylation (cDNA)</td>
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<td>7</td>
<td>1647</td>
<td>NA</td>
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<td>ELISA</td>
<td>Methylation (Hsp90a)</td>
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<td>221/72</td>
<td>Blood</td>
<td>MS-PCR</td>
<td>Methylation (AFP)</td>
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<td>75</td>
<td>67/18</td>
<td>Blood samples</td>
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<td>254 ng/mL</td>
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<td>109</td>
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<td>Pyrosequencing</td>
<td>Methylation (P16)</td>
<td>5%</td>
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<td>0.38</td>
<td>0.38</td>
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<td>150</td>
<td>61/11</td>
<td>Plasma</td>
<td>RO-PCR</td>
<td>Quantitative analysis of cDNA</td>
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<td>65</td>
<td>90</td>
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<td>0.19</td>
<td>0.19</td>
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<tr>
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<td>8</td>
<td>162</td>
<td>NA</td>
<td>Plasma</td>
<td>Nested-PCR</td>
<td>Methylation (249ser P53)</td>
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<td>6</td>
<td>100</td>
<td>0.43</td>
<td>0.43</td>
<td>0.43</td>
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<tr>
<td>Iizuka et al. (27)</td>
<td>2006</td>
<td>9</td>
<td>82</td>
<td>65/35</td>
<td>Serum</td>
<td>Real-time PCR</td>
<td>Quantitative analysis cDNA</td>
<td>73 ng</td>
<td>36</td>
<td>93</td>
<td>0.63</td>
<td>0.63</td>
<td>0.63</td>
</tr>
<tr>
<td>Jeng et al. (28)</td>
<td>2010</td>
<td>11</td>
<td>258</td>
<td>123/135</td>
<td>Serum</td>
<td>MS-PCR</td>
<td>Methylation (APC)</td>
<td>N/A</td>
<td>4</td>
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<td>0.50</td>
<td>0.50</td>
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<tr>
<td>Jeng et al. (29)</td>
<td>2004</td>
<td>7</td>
<td>111</td>
<td>57/54</td>
<td>Blood</td>
<td>RT-PCR</td>
<td>Methylation (AFP mRNA)</td>
<td>1,000 ng/mL</td>
<td>61</td>
<td>75</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Table 1 (continued)
The stages of cancer were plotted on a graph and compared (Figure 5).

**Results**

In this review, 240 studies were initially identified in the literature search. After analysing titles and abstracts, there were 181 studies excluded and 59 potential studies which were further reviewed. Of the 59 studies, 24 were excluded as they did not meet the inclusion criteria (Figure 1). Finally, 35 studies were compliant with the inclusion criteria and were eligible for the meta-analysis (Table 1).

There were a total of 5,945 patients, of whom 2,344 were male. All patients involved were diagnosed with HCC. Samples were taken from patient blood in 13 groups, serum in 12, plasma in 7 in and 4 had samples taken from both serum and plasma.

All studies were published from 1994 onward. The flow chart of inclusion and exclusion studies is presented in the figure below. The average quadas score was 7.8 with lowest at 5 and highest 12.

From the forty one [41] studies, twenty four [24] evaluated the use of a tumour specific single gene methylation. Eleven trials assessed patients of the same cancer stage comparing the positivity rates of each CTC demonstrated in graphical form (Figure 4).

In addition, the method of CTC detection was analysed. There were 5 methods of analysis including chemiluminescence [variation of the standard enzyme immunoassay (EIA), which is a biochemical technique used in immunochemistry], ELISA, MS-PCR and Real-time polymerase chain reaction (RT PCR). These methods of analysis had 6 studies, 3 studies, 15 studies and 15 studies respectively.

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### Table 1

<table>
<thead>
<tr>
<th>First author</th>
<th>Year</th>
<th>QUADAS score</th>
<th>Patients</th>
<th>M/F</th>
<th>Sample</th>
<th>Assay method</th>
<th>Assay indicators</th>
<th>Cutoff</th>
<th>TP</th>
<th>FP</th>
<th>TN</th>
<th>FN</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jia et al. [30]</td>
<td>2014</td>
<td>9</td>
<td>289</td>
<td>100/21</td>
<td>Serum</td>
<td>MS-PCR</td>
<td>Methylation (MT1G)</td>
<td>NA</td>
<td>85</td>
<td>36</td>
<td>8</td>
<td>62</td>
<td>70</td>
<td>91</td>
<td>0.50</td>
</tr>
<tr>
<td>Jiang et al. [31]</td>
<td>2015</td>
<td>8</td>
<td>205</td>
<td>NA</td>
<td>Serum/plasma</td>
<td>RT-PCR</td>
<td>Methylation (HBB)</td>
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<td>76</td>
<td>14</td>
<td>32</td>
<td>83</td>
<td>80</td>
<td>94</td>
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<tr>
<td>Julich-Haertel et al. [32]</td>
<td>2017</td>
<td>8</td>
<td>517</td>
<td>310/145</td>
<td>Serum/plasma</td>
<td>Fluorescence</td>
<td>Methylation (βmRNA)</td>
<td>9.3</td>
<td>63</td>
<td>23</td>
<td>355</td>
<td>76</td>
<td>73</td>
<td>82</td>
<td>0.50</td>
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<tr>
<td>Korkut et al. [33]</td>
<td>2008</td>
<td>8</td>
<td>119</td>
<td>97/22</td>
<td>Blood</td>
<td>PCR</td>
<td>Methylation (243uar P53)</td>
<td>NA</td>
<td>19</td>
<td>5</td>
<td>61</td>
<td>34</td>
<td>36</td>
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<td>Ko et al. [34]</td>
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<td>MS-PCR</td>
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<td>7</td>
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<td>2012</td>
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<td>100</td>
<td>70/30</td>
<td>Serum</td>
<td>Real-time PCR</td>
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<td>8</td>
<td>91</td>
<td>12-Jul</td>
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<td>PCR</td>
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<td>12</td>
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<td>10</td>
<td>142</td>
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<td>91</td>
<td>43</td>
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<td>8</td>
<td>119</td>
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<td>Plasma</td>
<td>Transilluminator</td>
<td>Quantitative analysis CFNA</td>
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<td>41</td>
<td>38</td>
<td>9</td>
<td>31</td>
<td>52</td>
<td>95</td>
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<td>78</td>
<td>60/18</td>
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<td>201</td>
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<td>24</td>
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Sensitivity and specificity of different circulating tumor cells

Four studies observed the 249 ser p53 CTC, which all have high specificity $>0.8$ but moderate to low sensitivity which is indicated on part of the roc curve. Alpha feto protein (AFP) has 8 studies all of which have moderate to high sensitivity and a high specificity $>0.6$. Circulating free DNA (cfDNA) has 4 studies with high the highest sensitivity and specificity, most of the studies above 0.6. cfDNA show promising result in comparison to the other liquid biopsy. P16 and Ras association domain-containing protein 1 (RASSF1A) had the lowest sensitivity and specificity. The SROC curves were incomplete denoting uncertainty. The observed data is presented graphically on the forest plot (Figure 3) and SROC curve (Figure 5). Further analysis demonstrated that CD133, CK19, CD90 could be used to provide discriminatory values between the early and late...
<table>
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Figure 4 Forest plots of comparing the specificity and sensitivity of different circulating tumour cells at different cut off (21,24).

Figure 5 SROC curve for different circulating tumour cells.
stages as shown in Figure 6.

**Sensitivity and specificity for different assay methodology**

Reverse transcription polymerase chain reaction had the highest sensitivity for AFP with sensitivity of 97% whilst MS PCR had the highest specificity of 99%. Chemiluminescence and Elisa had moderate sensitivity 70%, 67% and specificity 57% and 75% respectively. Most of the other CTC studies used RT-PCR, a total of 15 studies were identified. RT PCR shown to produce a high specificity above 0.6 but a moderate sensitivity.

**Positivity of early vs. late stages cancers in CTC detection**

AFP mRNA, cytokeratin-19 (Ck19), cluster differentiation (CD90), cfDNA and tissue factor pathway inhibitor-2 (TFPI2) all indicated CTC show a higher percentage of positivity in later stages in comparison to earlier stages. There was a difference of 6%, 44%, 27%, 5% and 13% respectively. However other CTC such as cfDNA show no difference. Interesting MT1G shows higher positivity in lower stages in comparison to higher stages.

**Sensitivity and specificity for the different cutoff values**

Han et al. (21) and Huang et al. (24) explored the effect of various cut off to the sensitivity and specificity of the diagnostic test. In the study of AFP, a higher cut off levels (400 ng/mL) was found to be more specific (99%) to lower levels (20 ng/mL) (65%), whilst being less sensitive (24% to 58%). Similar results were seen in TRG5+ AFP (specificity of 90% to 65% and sensitivity of 65% to 81%) and P16 INK4A (specificity of 98% to 81% and sensitivity of 27% to 74%) (Figure 4).

**Youden index**

Youden index is summary statistic of the roc curve used in the interpretation and evaluation of biomarkers. A value of zero indicates the diagnostic test gives a positive result for those with or without the disease and a value of 1 indicates no false positive or false negative. An acceptably benchmark is 0.50 (50). In this study, the highest index of 0.87 was produced by Bahnassy et al. (15) who studied AFP. In contrast, Iizuka et al. (28) analyzed SPINT2 that was found to have the lowest index of 0.01. The average index amongst all CTC was 0.46 with a mode and median of 0.5. In comparing, 5 most common CTC that is AFP, 249serP54, P16, RASSF1A and cfDNA, CfDNA had the highest average index of 0.53 followed by RASSF1A (0.41), P16 (0.40). Both AFP and 249serP54 had the worst overall index of 0.28 (Table 1).

**Discussion**

Blood based biomarkers could have promising value in early
diagnosis of HCC and therefore allow prompt treatment (51). It could be used a less-invasive alternative to current approach in diagnosis. However despite the range of CTC currently under investigation (52), there is variation in the reported diagnostic accuracy and the lack of standardized technical approach has contributed to the lack of consensus.

In a recent update in the Cochrane methods of screening and diagnostics tests, the current statistical model used in meta-analysis of diagnostic accuracy is SROC curves and the use of pooled sensitivity and specificity is considered an accurate method of reporting of such data (53).

The following criteria was used to evaluate the pooled sensitivity and specificity: high (0.6–1), moderate (0.4–0.59), low (<0.4) (51). In this meta-analysis we found that AFP has the highest overall diagnostic performance. The most common CTCs currently studied (249ser P53, P16, cfDNA and RASSF1A) have low to average Youden Index 0.28 to 0.56. Interestingly whilst Bahnassy et al. (15) demonstrated the highest overall Youden index using AFP, the average index of AFP was 0.28. This may result from different cutoff used or assay method. Further studies are needed to better understand this.

From our available statistical analysis, the study demonstrated that liquid biopsies have a high sensitivity/specificity however there is several limitations. This study has identified several heterogenous variables such as the follows: First demographic data (age, sex and race), sample size and etiology of HCC which was missing in the data. In addition, the underlying etiology of HCC was variable among and within studies.

There were inconsistencies in cut off values used for individual CTC's, therefore the sensitivity and specificity could have been over or underestimated as shown in Figure 4. Unsurprisingly, the higher the cutoff value the higher the specificity but lower sensitivity due to higher rates of false negatives. For future studies, a singles cutoff value should be determined for each CTC to reduce outcome bias.

In addition, assay methods for CTC detection have shown to produce different results for the same type of liquid biopsy. A different cutoff value and varied experimental set up may account for these findings, however from our results we can take into consideration that different CTC detection methods of the same CTC may potentially create bias. Currently the standard for CTC detection immunocytochemistry (ICC) and reverse transcriptase polymerase chain reaction (RT-PCR).

We were unable to identify complete data sets. True positive, false positive, True Negative or false negative of various articles were calculated using the available sensitivity and specificity.

Finally our study sample size per CTC was too small which limited our ability to complete a full SROC curve thus the analysis from the SROC curve provided in this study was descriptive.

Overall, there is potential in the use of CTC however the lack of a standardized procedure in the study of CTC contributes to the lack of consensus of its use.

Future research should include large scaled, standardized studies for the diagnostic accuracy of CTC. Only when such a challenge is met should it be translated these promising results to clinical practice.

**Conclusions**

The CTC markers have variable sensitivity and specificity for HCC. CD133 and CK19 could potentially be used to differentiate early versus late stages irrespective to the morphology. Further studies are required to establish it use as an isolated test for detection. Therefore, in the current clinical context, CTCs must be used with other clinical investigations.

**Acknowledgments**

None.

**Footnote**

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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