The evidence base for circulating tumour DNA blood-based biomarkers for the early detection of cancer: a systematic mapping review

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Abstract

Background: The presence of circulating cell-free DNA from tumours in blood (ctDNA) is of major importance to those interested in early cancer detection, as well as to those wishing to monitor tumour progression or diagnose the presence of activating mutations to guide treatment. In 2014, the UK Early Cancer Detection Consortium undertook a systematic mapping review of the literature to identify blood-based biomarkers with potential for the development of a non-invasive blood test for cancer screening, and which identified this as a major area of interest. This review builds on the mapping review to expand the ctDNA dataset to examine the best options for the detection of multiple cancer types.

Methods: The original mapping review was based on comprehensive searches of the electronic databases Medline, Embase, CINAHL, the Cochrane library, and Biosis to obtain relevant literature on blood-based biomarkers for cancer detection in humans (PROSPERO no. CRD42014010827). The abstracts for each paper were reviewed to determine whether validation data were reported, and then examined in full. Publications concentrating on monitoring of disease burden or mutations were excluded.

Results: The search identified 94 ctDNA studies meeting the criteria for review. All but 5 studies examined one cancer type, with breast, colorectal and lung cancers representing 60% of studies. The size and design of the studies varied widely. Controls were included in 77% of publications. The largest study included 640 patients, but the median study size was 65 cases and 35 controls, and the bulk of studies (71%) included less than 100 patients. Studies either estimated cfDNA levels non-specifically or tested for cancer-specific mutations or methylation changes (the majority using PCR-based methods).

Conclusion: We have systematically reviewed ctDNA blood biomarkers for the early detection of cancer. Pre-analytical, analytical, and post-analytical considerations were identified which need to be addressed before such biomarkers enter clinical practice. The value of small studies with no comparison between methods, or even the inclusion of controls is highly questionable, and larger validation studies will be required before such methods can be considered for early cancer detection.

Keywords: cfDNA, ctDNA, Cancer, Detection, Diagnosis, Liquid biopsy
The early detection of cancers before they metastasise to other organs allows definitive local treatment, resulting in excellent survival rates. This is particularly true for breast cancer, but also others, including lung and colorectal cancer [1]. Early detection and diagnosis has therefore been a major goal of cancer research for many years, and the concept of early detection from a blood sample has been the focus of considerable effort. However, to date no blood biomarkers have had sufficient sensitivity and specificity to warrant their clinical use for early cancer detection, and their potential remains un-realised [2]. Hanahan and Weinberg [3] identified the major biological attributes of cancer, and it is apparent that most if not all of these biological processes give rise to biomarkers present in blood [4]. Circulating cell free DNA produced from cancers is known as circulating tumour DNA (ctDNA), and represents a subset of the circulating DNA (cfDNA) normally present at low levels in the blood of healthy individuals.

Since the first description of circulating cfDNA in blood [5, 6], it has become clear that total ctDNA levels rise in a number of disorders in addition to cancer including myocardial infarction [7], serious infections, and inflammatory conditions [8], as well as pregnancy where it can be used for prenatal diagnosis [9]. The source of this DNA appears to be mainly the result of cell death – either by necrosis or apoptosis [5, 9–11]. A raised ctDNA level is therefore non-specific, but may indicate the presence of serious disease. In blood, ctDNA is always present as small fragments, which makes assay design challenging [12]. Nevertheless, many analytical methods are available to measure ctDNA, and the field is rapidly maturing to the point where it may be clinically relevant to many patients.

In 2014, the UK Early Cancer Detection Consortium (ECDC) conducted a rapid mapping review of blood biomarkers of potential interest for cancer screening [13], and identified 814 biomarkers, including 39 ctDNA biomarkers. This paper uses the list generated from the mapping review, updated with relevant publications published since its completion to discuss the candidacy of ctDNA markers for early detection of cancer.

**Methods**

Our mapping review [13] conducted comprehensive searches of the electronic databases Medline, Embase, CINAHL, the Cochrane library, and Biosis to obtain relevant literature on blood-based biomarkers for cancer detection in humans (PROSPERO no. CRD42014010827). The search period finished in July 2014, therefore the searches have been updated to December 2016 using the same search terms. The abstracts of the publications retrieved were reviewed to identify those with validation data (usually indicated by case-control design) and to determine what ctDNA biomarkers had been measured in serum or plasma. Full details of the methods used are published elsewhere [13], and described briefly here. English language publications of any sample size were eligible and the full eligibility criteria used are provided in Table 1.

The search strategy was deliberately inclusive, using keywords and subject headings as follows, to provide a comprehensive list of those ctDNA candidate biomarkers that had been used to identify cancers from blood samples. The search terms included ‘cancer’ ‘diagnosis’, ‘markers’, ‘blood’, and ‘screening’ with ‘DNA’, ‘cfDNA’, or ‘ctDNA’. Keywords and subject headings were determined by members of the ECDC working with the review team at the University of Sheffield. The results of the searches were collated in an Endnote database and results tabulated, with references, size of study, and methods used. To avoid bias, two reviewers conducted screening; references identified by either as relevant were included for further inspection. Those featuring ctDNA with data related to diagnosis or detection of three or more types of cancer were identified and retained for closer scrutiny to determine their potential utility.

| Table 1 Search criteria for ctDNA publications |
|-----------------------------------------------|
| **Inclusion Criteria** | **Exclusion Criteria** |
| English language studies | Studies published in non-English language |
| Studies within last seven years (2010–2016) | Studies published in 2009 or earlier |
| Controlled studies | Citation titles without abstracts |
| Validation Studies (comparison with controls) | Parallel publications and reviews based on the same or overlapping patient populations* |
| Cancer detection/ diagnosis/screening | Prognosis or prediction (treatment response) associated markers |
| Biomarkers measured in blood plasma or serum (markers or biomarkers) | Tissue, blood cells, or other bodily fluid samples |
| DNA (including cfDNA and ctDNA) | Abstracts of panels which do not state which biomarkers are studied |
| Human DNA | Viral and microbial DNA |

*Reviews and meta-analyses are cited, but not considered as evidence, but studies were included if they appeared to contain new data.
Results

Following the updated searches and study selection, a total of 84 ctDNA markers were identified from 94 individual publications (Table 2 and Fig. 1).

The ctDNA biomarkers divided naturally into two groups:

I. those with potential specificity for neoplasia (ctDNA - usually mutations or DNA alterations such as methylation), and

II. those designed to measure DNA levels, which may not be specific to neoplasia.

Figure 2 shows the distribution of studies by cancer type, including two publications on amplification [12, 14], and one on clonality [15]. One of the amplification papers looked at HER2 [14], while the other examined multiple targets by NGS [12].

Of the 94 publications included, 72 publications (77%) were case-control design diagnostic validation studies, and 22 were case series. The size and design of the studies varied widely. The largest study included 640 cancer patients [16]. The median study size was 65 cases, with a mean of 98 cases (range 12–640 cancer patients), indicating that the bulk of studies (67/94, 71%) included <100 patients (Fig. 3).

Most publications were focussed on ctDNA in plasma (n = 67) rather than serum (n = 25) with 2 comparing both. Plasma was used for 38 markers, and serum for 28 markers, and either for 18 markers (Fig. 4). Two comparative studies of serum and plasma were conducted: one for BRAF mutations, and the other for PIK3CA mutations [17, 18].

The target of ctDNA studies and the methods used to measure these targets varied considerably (Figs. 5 and 6 respectively). Non-specific total ctDNA levels (quantitation) were usually estimated by size distribution assays based on repeats: LINE1, and ALU were used in 3 [19–21] and 6 publications respectively [20–25]. However, some single genes were also used to measure DNA levels – particularly GAPDH in a series of 4 publications on breast cancer [26–29], and hTERT in 4 publications [30–33]. The majority of publications examined gene methylation markers (n = 49), though most examined methylation of multiple target genes for a particular tumour type (Fig. 5). Genes commonly mutated in cancer were also markers of interest, namely APC, BRAF, EGFR, HER2, GNAQ, GNA11, KRAS, P53, and PIK3CA. Only one gene, APC, was studied for both methylation and mutation. Few markers were used to identify particular tumour types, but some are particularly likely to occur in certain tumour types. GNAQ and GNA11 mutations have been identified in the plasma of uveal melanoma patients and are rare in other tumour types [34]. Other mutations are not tumour type-specific, and mutations in 6 of the 9 genes listed above were reported in multiple tumour types.

Discussion

The number of publications on ctDNA is increasing rapidly [35, 36], and a recent review emphasises the potential of the field [37]. Most (71%) are small case control studies with less than 100 patients, and in our view very few studies meet the requirements of analytical validation allowing their use within accredited (ISO:15,189) clinical laboratories, though some may have unpublished commercially-held analytical validation data. The stage and size of the tumours included is variable, and few studies are large enough to give robust subgroup assessments. Larger tumours produce more ctDNA, though tumour type also has an impact [16]. The value of small studies with no comparison between methods, or even the inclusion of controls is highly questionable. Most include a statement that ‘larger studies are required,’ but larger trials rarely result due to the necessary cost implications. Unless well-designed prospective studies based on sample size calculations are performed, there is little likelihood of such methods reaching clinical practice for the detection of cancer at an early stage. There is also a likelihood of bias in that negative results for these markers are rarely if ever reported, and unlike clinical trials, there is no requirement for the registration of diagnostic validation studies. The use of ctDNA for early cancer detection comes under existing molecular pathology guidance, which emphasises the requirements for careful pre-analytical preparation, analysis, and reporting of results [38]. It is important that studies adhere to the Standards for Reporting of Diagnostic Accuracy Studies (STARD) guidance [39], and regional guidance (e.g. US Food and Drug Adminstration (FDA); UK National Institute for Health and Care Excellence (NICE); Clinical & Laboratory Standards Institute (CLSI)). It is hardly surprising then that, to date, no ctDNA markers have made it into screening programmes, due in part to the economic feasibility of completing the necessary stages of validation [40]. Nevertheless, there is encouraging evidence that ctDNA can be used to detect cancers of many types [16], and the poor quality of many studies should not detract from this fact.

A plethora of methods are available for ctDNA measurement, which have been well reviewed elsewhere [41]. BEAMing, PCR clamping methods, and deep sequencing using NGS are now the most commonly used [42, 43] and are widely regarded as the most sensitive methods currently available. A recent report of copy number variation (CNV) in breast cancer is not surprising given the ability of this method to detect such changes in pregnancy [15]. However, it should be noted that many of
| No | Biomarker | Acronym | Cancer | DNA alteration | Assay type (qPCR, ddPCR, BEAMing, NGS, Other) | Size Cases (controls) | Plasma or Serum | Refs |
|----|-----------|---------|--------|----------------|--------------------------------|----------------------|-----------------|------|
| 1  | 14–3-3 sigma | 14–3-3 s | Breast | Methylation | qPCR | 106 (74) | Serum | [48] |
| 2  | absent in melanoma 1 | AIM1; Beta/gamma crystallin domain-containing protein 1 | Lung | Methylation | qPCR | 76 (30) | Serum | [62] |
| 3  | ADAM: metallopeptidase with thrombospondin type 1 motif, 1 | ADAMTS1 | Pancreatic | Methylation | qPCR | 42 | Serum | [63] |
| 4  | Adenomatous Polyposis Coli | APC | Lung | Methylation | qPCR | 76 (30) | Serum | [62] |
|    |           |         | CRC | Mutation | qPCR | 33 (10) | Plasma | [64] |
|    |           |         | Testicular | Methylation | qPCR | 73 (35) | Serum | [47] |
|    |           |         | CRC | Mutation | qPCR | 191 | Plasma | [65] |
|    |           |         | CRC | Methylation | qPCR | 33 | Serum | [53] |
|    |           |         | CRC | Mutation | PCR | 104 | Serum | [86] |
|    |           |         | Ovarian | Methylation | qPCR | 87 (62) | Serum | [67] |
|    |           |         | Renal | Methylation | PCR | 35 (54) | Serum | [68] |
|    |           |         | Breast | Methylation | qPCR | 36 (30) | Plasma | [69] |
|    |           |         | Lung | Methylation | qPCR | 110 (50) | Plasma | [70] |
|    |           |         | Renal | Methylation | qPCR | 27 (15) | Plasma | [71] |
|    |           |         | CRC | Methylation | PCR | 60 (100) | Plasma | [72] |
| 5  | ALU repeat | Alu 115 bp | Breast | NA | qPCR | 39 (49) | Plasma | [22] |
|    |           | Alu 247 bp | Pancreatic | NA | qPCR | 73 (43) | Plasma | [73] |
|    |           |         | CRC | NA | qPCR | 50 (35) | Plasma | [20] |
|    |           |         | Breast | NA | qPCR | 293 (100) | Plasma | [19] |
|    |           |         | Thyroid | NA | qPCR | 176 (19) | Plasma | [24] |
|    |           |         | CRC | NA | qPCR | 104 (173) | Serum | [23] |
| 6  | Basonuclin 1 | BNC1 | Pancreatic | Methylation | qPCR | 42 | Serum | [63] |
| 7  | BIN1 | BIN1 | Breast | Methylation | qPCR | 76 (30) | Serum | [62] |
| 8  | BLU | BLU | Lung | Methylation | qPCR | 63 (36) | Plasma | [74] |
| 9  | BRAF | BRAF (V600E) | Melanoma | Mutation | qPCR | 221 | Both | [17] |
|    |           |         | Lung | Mutation | NGS | 68 (107) | Plasma | [75] |
|    |           |         | LCH | Mutation | qPCR | 30 | Plasma | [76] |
|    |           |         | CRC | Mutation | qPCR | 106 | Plasma | [77] |
|    |           |         | Thyroid | Mutation | qPCR | 77 | Plasma | [78] |
|    |           |         | CRC | Mutation | BEAMing | 503 | Plasma | [21] |
|    |           |         | CRC | Mutation | qPCR | 191 | Plasma | [65] |
| No | Biomarker | Acronym | Cancer | DNA alteration | Assay type (qPCR, ddPCR, BEAMing, NGS, Other) | Size Cases (controls) | Plasma or Serum | Refs |
|----|-----------|---------|--------|----------------|---------------------------------------------|----------------------|----------------|------|
| 10 | BRCA1     | BRCA1   | Breast | Methylation    | qPCR                                        | 89 Serum             | [79]           |      |
| 11 | CALCA     | CALCA   | Ovarian| Methylation    | PCR                                         | 30 (30) Plasma       | [82]           |      |
| 12 | CDH1      | CDH1    | Ovarian| Methylation    | PCR                                         | 87 (62) Serum        | [67]           |      |
| 13 | CDH13     | CDH13   | Lung   | Methylation    | qPCR                                        | 63 (36) Plasma       | [74]           |      |
| 14 | CD01      | CD01    | Various| Methylation    | PCR                                         | 150 (60) Plasma      | [83]           |      |
| 15 | CHD1      | CHD1    | Lung   | Methylation    | qPCR                                        | 76 (30) Serum        | [62]           |      |
| 16 | CST6      | CST6    | Breast | Methylation    | qPCR                                        | 196 (37) Plasma      | [84]           |      |
| 17 | CHRM2     | CHRM2   | Gastric| Methylation    | qPCR                                        | 58 (30) Serum        | [85]           |      |
| 18 | CYCD2     | CYCD2   | CRC    | Methylation    | PCR                                         | 30 (30) Plasma       | [86]           |      |
| 19 | DAPK1     | DAPK1   | HNSCC  | Methylation    | PCR                                         | 40 (41) Serum        | [87]           |      |
| 20 | DCC       | DCC     | Lung   | Methylation    | PCR                                         | 76 (30) Serum        | [62]           |      |
| 21 | DCLK1     | DCLK1   | Lung   | Methylation    | qPCR                                        | 65 (95) Plasma       | [88]           |      |
| 22 | DKK3      | DKK3    | Breast | Methylation    | qPCR                                        | 604 (59) Serum       | [90]           |      |
| 23 | DLEC1     | DLEC1   | Lung   | Methylation    | qPCR                                        | 110 (50) Plasma      | [70]           |      |
| 24 | DNA (NOS) | DNA     |        |                | qPCR v Seq                                   | 30 (26) Plasma       | [91]           |      |
| 25 | e-cadherin| e-cadherin|        |                |                                             | Various No NGS       | [45]           |      |
| 26 | EGFR      | EGFR    | Lung   | Mutation       | NGS                                         | 68 (107) Plasma      | [75]           |      |
| 27 | EP300     | EP300   | Ovarian| Methylation    | PCR                                         | 30 (30) Plasma       | [82]           |      |
| 28 | ERBB2     | HER2    | Lung   | Mutation       | NGS                                         | 68 (107) Plasma      | [75]           |      |
|    |           |         | Breast | Amplification  | qPCR                                        | 120 (98) Plasma      | [14]           |      |
|    |           |         |        |                | Oesophageal Amplification qPCR               | 41 (34) Plasma       | [94]           |      |
| No | Biomarker | Acronym | Cancer    | DNA alteration | Assay type (qPCR, ddPCR, BEAMing, NGS, Other) | Size Cases (controls) | Plasma or Serum | Refs |
|----|-----------|---------|-----------|----------------|---------------------------------------------|-----------------------|----------------|------|
| 29 | ESR       | ESR     | Breast    | Methylation    | qPCR                                        | 106 (74) Serum        |                | [48] |
| 30 | FAM5C     | FAM5C   | Gastric   | Methylation    | qPCR                                        | 58 (30) Serum         |                | [85] |
| 31 | FHIT      | FHIT    | Lung      | Methylation    | qPCR                                        | 63 (36) Plasma        |                | [74] |
| 32 | Glyceraldehyde-3-phosphate dehydrogenase | GAPDH | Breast    | NA             | qPCR                                        | 200 (100) Serum       |                | [26] |
|     |           |         | Breast    | NA             | qPCR                                        | 33 (50) Serum         |                | [27] |
|     |           |         | Breast    | NA             | qPCR                                        | 27 (32) Serum         |                | [28] |
|     |           |         | Breast    | NA             | qPCR                                        | 33 (32) Serum         |                | [29] |
| 33 | GNA11     | GNA11   | Uveal     | Mutation       | NGS                                         | 28 Plasma             |                | [34] |
| 34 | GNAQ      | GNAQ    | Uveal     | Mutation       | NGS                                         | 28 Plasma             |                | [34] |
| 35 | GPC3      | GPC3    | Pancreatic | Methylation    | qPCR                                        | 30 (30) Plasma        |                | [86] |
| 36 | GSTP1     | GSTP1   | Breast    | Methylation    | qPCR                                        | 89 Serum              |                | [79] |
|     |           |         | Breast    | Methylation    | qPCR                                        | 36 (30) Plasma        |                | [69] |
|     |           |         | Prostate  | Methylation    | PCR                                         | 12 (10) Plasma        |                | [95] |
|     |           |         | Prostate  | Methylation    | qPCR                                        | 31 (44) Plasma        |                | [96] |
|     |           |         | Testicular| Methylation    | qPCR                                        | 73 (35) Serum         |                | [47] |
|     |           |         | Renal     | Methylation    | PCR                                         | 35 (54) Serum         |                | [68] |
|     |           |         | Prostate  | Methylation    | PCR                                         | 31 (34) Serum         |                | [97] |
| 37 | HIC1      | HIC1    | CRC       | Methylation    | PCR                                         | 30 (30) Plasma        |                | [98] |
|     |           |         | CRC       | Methylation    | qPCR                                        | 30 (30) Plasma        |                | [86] |
| 38 | HOXA7     | HOXA7   | Various   | Methylation    | qPCR                                        | 150 (60) Plasma       |                | [83] |
| 39 | HOXA9     | HOXA9   | Various   | Methylation    | qPCR                                        | 150 (60) Plasma       |                | [83] |
| 40 | HOXD13    | HOXD13  | Breast    | Methylation    | qPCR                                        | 253 (434) Serum       |                | [39] |
| 41 | IgH       | FR3AMLJH| Lymphoma  | Clonality      | NGS                                         | 75 Plasma             |                | [43] |
| 42 | IL1H5     |         | Breast    | Methylation    | qPCR                                        | 604 (59) Serum        |                | [90] |
| 43 | INK4A     | INK4A   | HCC       | Methylation    | Seq                                         | 66 (43) Plasma        |                | [100]|
| 44 | KLK10     | KLK10   | Lung      | Methylation    | qPCR                                        | 110 (50) Plasma       |                | [70] |
| 45 | KRAS      | KRAS    | Lung      | Mutation       | NGS                                         | 68 (107) Plasma       |                | [75] |
|     |           |         | CRC       | Mutation       | qPCR                                        | 52 Plasma             |                | [101]|
| No | Biomarker | Acronym | Cancer | DNA alteration | Assay type (qPCR, ddPCR, BEAMing, NGS, Other) | Size | Cases (controls) | Plasma or Serum | Refs |
|----|-----------|---------|--------|----------------|---------------------------------------------|------|-----------------|----------------|-----|
| 46 | LINE1 Repeat | LINE1 79 bp | CRC | Mutation | qPCR | 50 (35) | Plasma | [20] |
| 46 | LINE1 Repeat | LINE1 300 bp | CRC | NA | qPCR | 503 | Plasma | [21] |
| 47 | MDG1 | MDG1 | CRC | Methylation | PCR | 30 (30) | Plasma | [98] |
| 48 | Microsatellite alterations | FHIT LoH | Lung | NA | PCR | 87 (14) | Plasma | [104] |
| 48 | Microsatellite alterations | FHIT LoH | Lung | NA | PCR | 32 (10) | Serum | [105] |
| 49 | mitochondrial DNA | mtDNA | Breast | NA | qPCR | 60 (51) | Plasma | [108] |
| 50 | MLH1 | hMLH1 | Breast | Methylation | qPCR | 253 (434) | Serum | [99] |
| 51 | MYC | MYC | Neuroblastoma | Amplification | ddPCR | 44 | Plasma | [42] |
| 52 | MYF3 | MYF3 | Pancreatic | Methylation | qPCR | 30 (30) | Plasma | [86] |
| 53 | MYLK | MYLK | Gastric | Methylation | qPCR | 58 (30) | Serum | [85] |
| 54 | O(6)-methylguanine-DNA methyltransferase (MGMT) | MGMT | CRC | Methylation | qPCR | 76 | Serum | [62] |
| 55 | OPCML | OPCML | Ovarian | Methylation | qPCR | 87 (62) | Serum | [67] |
| 56 | P14 ARF tumor suppressor protein gene | P14 | Testicular | Methylation | qPCR | 73 (35) | Serum | [47] |
| 56 | P14 ARF tumor suppressor protein gene | P14 | Renal | Methylation | PCR | 35 (54) | Serum | [68] |
| 57 | P16 cyclin-dependent kinase inhibitor 2A (CDKN2A) | P16, CDKN2A | Testicular | Methylation | qPCR | 73 (35) | Serum | [47] |
| 57 | P16 cyclin-dependent kinase inhibitor 2A (CDKN2A) | P16, CDKN2A | Renal | Methylation | PCR | 35 (54) | Serum | [68] |
| 57 | P16 cyclin-dependent kinase inhibitor 2A (CDKN2A) | P16, CDKN2A | Breast | Methylation | qPCR | 36 (30) | Plasma | [69] |
| 57 | P16 cyclin-dependent kinase inhibitor 2A (CDKN2A) | P16, CDKN2A | Lung | Methylation | qPCR | 63 (36) | Plasma | [74] |
| 57 | P16 cyclin-dependent kinase inhibitor 2A (CDKN2A) | P16, CDKN2A | Breast | Methylation | qPCR | 253 (434) | Serum | [99] |
| No | Biomarker | Acronym | Cancer | DNA alteration | Assay type (qPCR, ddPCR, BEAMing, NGS, Other) | Size Cases (controls) | Plasma or Serum | Refs |
|----|-----------|---------|--------|----------------|---------------------------------------------|-----------------------|-----------------|------|
| 58 | P21       | P21     | Breast | Methylation    | qPCR                                        | 40 (41) Serum         | [87]            |
| 59 | P53       |         | Various | Mutation       | qPCR                                        | 20 (16) Plasma        | [109]           |
| 60 | PCDHGB7   | PCDHGB7 | Breast | Methylation    | qPCR                                        | 253 (434) Serum       | [99]            |
| 61 | Peptidylprolyl isomerase A | cyclophilin A, gCYC, PPIA | Various | Mutation       | qPCR                                        | 229 (100) Plasma     | [102]           |
| 62 | PIK3CA    | PIK3CA  | Breast | Methylation    | qPCR                                        | 76 Both              | [18]            |
| 63 | Prostaglandin-endoperoxid synthase 2 | PTGS2 | Renal   | Methylation    | PCR                                         | 35 (54) Serum        | [68]            |
| 64 | Protocadherin 10 | PCDH10 | Testicular | Methylation | qPCR                                        | 73 (35) Serum        | [47]            |
| 65 | Retinoid-acid-receptor-beta gene | RARBeta2 | Breast | Methylation    | PCR                                         | 20 (25) Plasma        | [112]           |
| 66 | RASSF1A   | RASSF1A | Breast | Methylation    | PCR                                         | 93 (76) Plasma        | [113]           |

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| No | Biomarker | Acronym | Cancer | DNA alteration | Assay type (qPCR, ddPCR, BEAMing, NGS, Other) | Size Cases (controls) | Plasma or Serum | Refs |
|----|-----------|---------|--------|----------------|---------------------------------------------|----------------------|-----------------|------|
| 67 | RUNX3     | RUNX3   | Ovarian| Methylation   | PCR                                         | 87 (62)              | Serum           | [67] |
| 68 | Septin 9  | Septin 9| CRC    | Methylation   | qPCR                                        | 97 (172)             | Plasma          | [119]|
| 69 | SFN       | SFN     | Breast | Methylation   | qPCR                                        | 253 (434)            | Serum           | [99] |
| 70 | SFRP5     | SFRP5   | Ovarian| Methylation   | qPCR                                        | 87 (62)              | Serum           | [67] |
| 71 | SHOX2     | SHOX2   | Lung   | Methylation   | qPCR                                        | 188 (153)            | Plasma          | [125]|
| 72 | SOX17     | SOX17   | Breast | Methylation   | qPCR                                        | 114 (66)             | Plasma          | [127]|
| 73 | SLC26A4   | SLC26A4 | Various| Methylation   | qPCR                                        | 150 (60)             | Plasma          | [83] |
| 74 | SLC5A8    | SLC5A8  | Thyroid| Methylation   | qPCR                                        | 176 (19)             | Plasma          | [24] |
| 75 | SRBC      | SRBC    | Pancreatic| Methylation | qPCR                                        | 30 (30)              | Plasma          | [86] |
| 76 | TAC1      | TAC1    | Various| Methylation   | qPCR                                        | 150 (60)             | Plasma          | [83] |
| 77 | human telomerase reverse transcriptase DNA | hTERT | CRC    | Methylation   | NA                                          | 35 (135)             | Plasma          | [30] |
|    |           |         | HCC    | Methylation   | NA                                          | 70 (30)              | Plasma          | [31] |
|    |           |         | HCC    | Methylation   | NA                                          | 60 (29)              | Plasma          | [32] |
|    |           |         | HNSCC  | Methylation   | NA                                          | 200                  | Plasma          | [33] |
| No | Biomarker | Acronym | Cancer | DNA alteration | Assay type (qPCR, ddPCR, BEAMing, NGS, Other) | Size Cases (controls) | Plasma or Serum | Refs |
|----|-----------|---------|--------|----------------|---------------------------------------------|----------------------|----------------|------|
| 78 | TFPI2     | TFPI2   | Ovarian | Methylation    | PCR                                         | 87 (62)              | Serum          | [67] |
| 79 | THBD-M    | THBD-M  | CRC    | Methylation    | qPCR                                        | 107 (98)             | Plasma & Serum | [128]|
| 80 | TIMP3     | TIMP3   | Renal  | Methylation    | PCR                                         | 35 (54)              | Serum          | [68] |
| 81 | TMS       | TMS     | Pancreatic | Methylation | qPCR                                      | 30 (30)              | Plasma         | [86] |
| 82 | UCHL1     | UCHL1   | HNSCC  | Methylation    | PCR                                         | 40 (41)              | Serum          | [87] |
| 83 | Von Hippel Lindau gene | VHL     | CRC    | Methylation    | qPCR                                        | 30 (30)              | Plasma         | [86] |
| 84 | ZFP42     | ZFP42   | Various | Methylation    | qPCR                                        | 150 (60)             | Plasma         | [83] |

CRC, colorectal cancer; HNSCC, head and neck squamous cell carcinoma; HCC, hepatocellular carcinoma; LCH, Langerhans cell histiocytosis; SCLC, small cell lung cancer.
Fig. 1 PRISMA diagram

Fig. 2 Number of targets and publications by tumour type, showing the expected concentration of studies on common cancer types. CRC, colorectal cancer; HNSCC, head and neck squamous cell carcinoma; HCC, hepatocellular carcinoma.
these methods are expensive. The development of highly sensitive NGS methods for ctDNA may prove necessary to obtain the best results [44], but large blood samples (> 10 ml may be needed as the number of DNA molecules present in small samples is often low) [45]. This may be at odds with the key requirement of cost effectiveness for screening programmes, and in our view this represents a real challenge for ctDNA. The problem is probably not insuperable if automation allows the integration of such methods into large blood sciences laboratories, but this is not as yet the case.

As ctDNA is composed largely of short fragments, short amplicons are required for maximum sensitivity of PCR reactions, particularly if mutations are being detected [46]. This is compounded by DNA loss in some reactions, particularly bisulphite modification of DNA, and it may be preferable to use nuclease protection assays [47, 48]. Methylation of key genes involved in carcinogenesis can be found in ctDNA, and has been studied by many groups, but it should be noted that substantial numbers of normal controls also have methylation of ctDNA for these genes [49].

It is clear that high sensitivity methods will be needed if ctDNA is to be used for early cancer detection. Several factors affect the sensitivity of ctDNA measurement. The first is the extraction method, and there are as yet too few studies which have compared the different options available, which now include automated instruments as well as manual extraction systems [50, 51]. The proportion of tumour derived DNA (ctDNA) in total cfDNA is greater in plasma than serum, and the higher ctDNA levels in serum are due to leakage from leukocytes during clotting [17]. The dilution effect for ctDNA in serum results in a reduced ability to detect mutations,
particularly by methods with low analytical sensitivity [50]. Most groups working in the field realise this, and the majority of publications now look at plasma rather than serum.

Several publications were noteworthy, including one influential study which did not include healthy controls [16]. However, the comparison of DNA levels and multiple mutations in plasma from many different tumour types is helpful [44], and makes it clear that some tumours (e.g. gliomas) do not have high ctDNA levels in plasma, as previously found when comparing CSF with plasma [52]. This is also one of several publications that examines early stage disease, and shows that patients with localised disease have lower ctDNA levels [16]. Few publications have examined the ability of ctDNA to detect smaller tumours, though all agree that ctDNA levels increase as tumours enlarge [42].

Choice of target also influences results: the use of LINE1 and ALU repeats allows quantitative size distribution of DNA to be measured. Several publications suggest that this can distinguish cancer, and even precancerous conditions from controls [30]. The size distribution of CRC appears to be different from other tumours due to first pass hepatic metabolism [20, 53]. Absolute quantitation by single gene methods such as GAPDH or hTERT will result in lower estimates of DNA content, and it is likely that this is due to the higher sensitivity of the ALU and LINE1 assays [30].

The use of mutations common within cancers is attractive, and the use of ctDNA to provide companion diagnostic information in patients whom biopsy material is not available is now entering practice [54]. However, it should be noted that such mutations in P53 can occur in the blood of healthy controls, and could give rise to substantial numbers of false positive results [55].

Septin 9 methylation is often regarded as a model for future work [56, 57], and it is notable that there are some large studies [58] within the evidence base for the use of this marker in colorectal cancer, often used in addition to other markers, such as faecal occult blood testing (FoBT) or faecal immunohistochemical testing (FIT). Pre-analytical factors have been examined for this marker [59], including diurnal variation [60]. Plasma methylation of Septin 9 is now available as a commercial test (Epi proColon 2.0; Epigenomics AG, Berlin, Germany) which has recently obtained FDA approval for colorectal cancer screening (April 2016). This is the first blood test to be approved for cancer screening, and represents an encouraging milestone.

Other methylation targets have been studied in depth and show considerable promise. These include APC for colorectal cancer, with a large number of studies (Table 2), and SHOX3, for which a recent meta-analysis suggests that it could have an important role in the diagnosis of lung cancer [61].

There is an encouraging trend towards larger, more ambitious studies, supported by the commercial sector (e.g. (https://clinicaltrials.gov/ct2/show/NCT02889978, and https://clinicaltrials.gov/ct2/show/NCT03085888). Case control studies (particularly retrospective ones) can give biased results, and prospective studies in at-risk cohorts would be more useful in examining the predictive capability of these markers. Such prospective studies should include controls proven not to have cancer. The comparison of new with existing methods (e.g. tumour markers, radiology), and competing technologies, is recommended, and often required by regulators. This has cost implications for funding bodies, but is essential if the field is to progress rapidly.

Conclusions
While ctDNA analysis may provide a viable option for the early detection of cancers, not all cancers are detectable using current methods. However, improvements in technology are rapidly overcoming some of the issues of analytical sensitivity, and it is likely that mutation and methylation analysis of ctDNA will improve specificity for the diagnosis of cancer.

Abbreviations
14–3–3 sigma or tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein theta; ADAM: metallopeptidase with thrombospondin type 1 motif 1; AML1: absent in melanoma 1; ALU: Alu repeat/element 9e; APC: Adenomatous Polyposis Coli; ARF: alternate reading frame; BIN1: bridging integrator 1; BLU: zinc finger MYND-type containing 10; BM: biomarker; BNC1: basonuclin 1; bp: base pair; BRAF: B-Raf proto-oncogene, serine/threonine kinase; BRCA1: breast cancer 1, DNA repair associated; BRANPS: BMP/Retinoic Acid Inducible Neural Specific 3; CALCA: calcitonin related polypeptide alpha; CDH1: cadherin 1; CDH13: cadherin 13; CDO1: cysteine dioxygenase type 1; cDNA: circulating cell-free DNA; CHD1: chromodomain helicase DNA binding protein 1; CHRM2: cholinergic receptor muscarinic 2; CINAH: Cumulative Index to Nursing and Allied Health Literature; CLSI: Clinical & Laboratory Standards Institute; CRC: colorectal carcinoma; CST6: cystatin 6; cDNA: circulating tumour DNA; CYCD2: cyclin D2; DAPK1: death-associated protein kinase 1; DCC: DCC. Netrin 1 receptor; DCLK1: doublecortin like kinase 1; dPCR: digital droplet polymerase chain reaction; DKKS: Dickkopf WNT signaling pathway inhibitor 3; DLEC1: deleted in lung and esophageal cancer 1; DNA: deoxyribonucleic acid; ECDC: UK Early Cancer Detection Consortium;
EGFR epidermal growth factor receptor (HER1); EP300: E1A binding protein P300; ERBB2: erb-B2 receptor tyrosine kinase 2 (HER2); ESR: estrogen receptor 1; FAM5C: BMP/retinoic acid inducible neural specific 3 (BRINP3); FDA: US Food and Drug Administration; FHIT: fragile histidine triad; FIT: faecal immunohistochemical testing; FoBT: faecal occult blood testing; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GYPC: cycloglobin A; GNA11: G protein subunit alpha 11; GNAQ: G protein subunit alpha Q; GPC3: glypican 3; GSTP1: glutathione S-transferase pi 1; HCC: hepatocellular carcinoma; HER1: human epidermal growth factor receptor 1; HER2: human epidermal growth factor receptor 2; HIC1: HIC ZBTB transcriptional repressor 1; HNSEC: head and neck squamous cell carcinoma; HOXA7: Homeobox A7; HOXA9: Homeobox A9; HOXD13: Homeobox D13; HTERT: human telomerase reverse transcriptase; ICH: humomoglobulin heavy locus; INK4A: cyclin dependent kinase inhibitor 2A (CDKN2A/P16); ISO: International Standardization Organization; ITHS: inter-alpha-trypsin inhibitor heavy chain family member 5; KLK10: kallikrein related peptide 10; KRAS: KRAS Proto-Oncogene, GTPase; LCH: Langerhans cell histiocytosis; LINE1: long interspersed nuclear element 1; LoH: loss of heterozygosity; MDM1: microvascular endothelial differentiation gene 1; MGMT: O(6)-methyl-guanine-DNA methyltransferase; Min: minimum; MLH1: Mutl Homolog 1; mTDNA: mitochondrial DNA; MYC: MYC proto-oncogene; MYF3: myogenic differentiation 1 (MYOD1); MYLK: myosin light chain kinase; NGS: next generation sequencing; NICE: UK National Institute for Health and Care Excellence; NOS: not otherwise specified; OPGCML: opioid binding protein/cell adhesion molecule like; P14: P14 ARF tumor suppressor protein gene; P16: P16 cyclin-dependent kinase inhibitor 2A (CDKN2A); P21: cyclin dependent kinase inhibitor 1A; P53: tumor protein P53; PCDH10: Protocadherin 10; PCDHG8: protocadherin gamma subfamily B7; PCR: polymerase chain reaction; PhK3CA: phosphatidylinositol-4,5-bisphosphate-3-kinase catalytic subunit alpha; PP2A: Pp2a beta regulatory subunit; PTGS2: Prostaglandin-endoperoxid synthase 2; qPCR: quantitative polymerase chain reaction; RARBeta2: Retinoid acid-receptor-beta gene; RASSF1A: Ras association domain family member 1; RUNX3: runt related transcription factor 3; SFN: StatrinF; SRP56: secreted frizzled related protein 5; SHOX2: short stature homeobox 2; SLC26A4: solute carrier family 26 member 4; SLC5A8: solute carrier family 5 member 8; SOX17: SRY-Box 17; SRBC: serum deprivation; STARD: Standards for Reporting of Diagnostic Accuracy Studies; TAC1: tachykinin precursor 1; TFF2: tissue factor pathway inhibitor 2; THBD: thrombomodulin; TIMP3: tissue inhibitor of metalloproteinase 3; TIMS: tumor differentially expressed protein 1; UCHL1: Ubiquitin C-Terminal Hydrolyase L1; V600E: Mutation resulting in an amino acid substitution at position 600 in BRAF, from a valine (V) to a glutamic acid (E); VHL: Von Hippel Lindau gene; ZFPM2: ZFPM2 Zinc Finger Protein

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Availability of data and materials

The papers quoted are publicly available from the publishers, and many are now open access.

Authors’ contributions

IC, SH, BW, and STP designed the study. Searches were performed by HBW. LU and HBW performed the mapping review with input from the ECDC. HK and IC scanned the resulting publications relating to ctDNA. The draft manuscript was prepared by IC with input from MM, AC, DT, OS, AR, HK, HBW, BW and JS. All authors agreed the final version. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

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