



Circulating tumour DNA — looking beyond the blood

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Abstract | Over the past decade, various liquid biopsy techniques have emerged as viable alternatives to the analysis of traditional tissue biopsy samples. Such surrogate ‘biopsies’ offer numerous advantages, including the relative ease of obtaining serial samples and overcoming the issues of interpreting one or more small tissue samples that might not reflect the entire tumour burden. To date, the majority of research in the area of liquid biopsies has focused on blood-based biomarkers, predominantly using plasma-derived circulating tumour DNA (ctDNA). However, ctDNA can also be obtained from various non-blood sources and these might offer unique advantages over plasma ctDNA. In this Review, we discuss advances in the analysis of ctDNA from non-blood sources, focusing on urine, cerebrospinal fluid, and pleural or peritoneal fluid, but also consider other sources of ctDNA. We discuss how these alternative sources can have a distinct yet complementary role to that of blood ctDNA analysis and consider various technical aspects of non-blood ctDNA assay development. We also reflect on the settings in which non-blood ctDNA can offer distinct advantages over plasma ctDNA and explore some of the challenges associated with translating these alternative assays from academia into clinical use.

The past decades have witnessed an explosion in therapeutic options for both early stage and advanced-stage malignancies. This increase in available therapies has been accompanied by growing interest in developing more personalized treatment approaches, thus increasing the demand for molecular profiling strategies that enable the successful delivery of precision medicine.

The term ‘liquid biopsies’ refers to biomarkers found within biological fluids, traditionally blood, that can be sampled to provide clinically valuable information on both the patient and their underlying malignancy¹. This approach offers an alternative to traditional needle-based biopsy sampling methods that enables minimally invasive repeat biopsy sampling, even for patients with tumours that are anatomically difficult to sample directly. Liquid biopsy approaches can also overcome issues such as a lack of representativeness, which is associated with the analysis of small tissue biopsy samples². To date, the majority of liquid biopsy research has focused on blood-based biomarkers, including circulating tumour DNA (ctDNA), circulating tumour cells (CTCs) and circulating microRNAs³. However, data published over the past decades indicate that these cancer-derived cells and molecules can also be found within non-blood biological fluids, including, among others, cerebrospinal fluid (CSF), urine, pleural

fluid and peritoneal fluid^{4–10}. This expanding interest in liquid biopsies from a wider range of sources is supported by the improved availability of ctDNA detection and analysis technologies. In this Review, we discuss the role of non-blood biological fluids as sources of ctDNA and their potentially distinct and/or complementary roles in oncology (BOX 1) as well as exploring some of the challenges associated with translating these alternative biomarker assays from the laboratory to the clinic.

Definitions and assay considerations

The term cell-free DNA (cfDNA) refers to fragments of DNA that are present outside of cells that can be detected within bodily fluids. In blood plasma, cfDNA typically consists of double-stranded DNA fragments of around 140–170 base pairs (bp) in length that mostly originate from leukocytes^{11,12}. ctDNA refers to the portion of cfDNA derived from cancer cells, which typically comprises strands of <145 bp in length and is responsible for the substantially higher plasma cfDNA concentrations often seen in patients with cancer^{11,13–15} (BOX 2). Various studies have confirmed a high level of concordance between genomic alterations detected in plasma ctDNA and those found in tumour tissues^{16,17}. Nonetheless, the mechanisms of ctDNA release are still poorly defined and various hypotheses include tumour cell apoptosis, direct release from tumour cells, secretion from

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Key points

- Plasma-based circulating tumour DNA (ctDNA) assays can provide invaluable information on the status of a patient's cancer; however, numerous alternative sources of ctDNA are available that might offer unique advantages in certain settings.
- Non-blood sources of ctDNA include urine, cerebrospinal fluid, pleural or peritoneal fluid, saliva, stool, and seminal fluid, among others.
- Analysing ctDNA from non-blood sources might provide a more sensitive method than plasma-based ctDNA assays for particular tumour types or anatomical locations.
- Non-blood ctDNA assays might be complementary to plasma assays when used for the detection of driver alterations or mechanisms of resistance.
- Challenges to the clinical implementation of non-blood-based ctDNA assays include difficulties relating to standardization of pre-analytical factors, lack of commercially available assays and the invasive procedures required to obtain certain sample types.

macrophages following phagocytosis of tumour cells and, to a lesser degree, release from CTCs¹³.

ctDNA can also arise from direct contact with tumour tissues in non-blood biological fluids, and these fluids might therefore contain a higher proportion of ctDNA than plasma. Furthermore, the comparative absence of cfDNA originating from haematopoietic cells in non-blood sources means that ctDNA might be present at higher relative concentrations and at higher variant allele frequencies (VAFs) compared with ctDNA isolated from blood¹⁸. This relative lack of cfDNA can be advantageous when quantifying ctDNA and when attempting to detect alterations present at low relative frequencies owing to a lack of the background noise created by clonal haematopoiesis, which typically increases with advancing age and/or previous radiotherapy¹⁹. Furthermore, ctDNA from non-blood sources might be more reflective of the primary tumour (as seen in pleural fluid samples obtained from patients with

advanced-stage thoracic malignancies) or, alternatively, might enable the characterization of a genetically distinct population of tumour cells, such as those located in the CSF in patients with intracranial disease^{18,20}.

A variety of assay types can be used to measure ctDNA and these have been extensively reviewed elsewhere^{1,21}. Assay choice is influenced by the sample type^{1,21}. Another key consideration is the relative amount of ctDNA present within a sample, which dictates the required assay sensitivity. ctDNA assays include those designed to detect specific preselected mutations using digital PCR (dPCR)-based techniques, targeted next-generation sequencing (NGS) panels (comprising up to 1,000 genes), or even broader approaches involving whole-exome sequencing or the analysis of global methylation status²². Considerations regarding the choice of assay apply equally to ctDNA derived from plasma and from non-blood sources. However, the high ctDNA to cfDNA ratio within most non-blood samples relative to plasma suggests that these samples might be less susceptible to dilution by non-tumour-derived DNA. Obtaining larger sample volumes, enabling both a greater quantity of ctDNA to be extracted and analysed and an improved level of sensitivity, is also easier when sampling certain fluids such as urine²³.

A plethora of alternative sources of ctDNA are available, and several roles for these samples are now emerging (FIG. 1 and BOX 1). Each has advantages and disadvantages when compared to ctDNA from blood plasma (BOX 3). In the following sections, we discuss existing evidence supporting the utility of ctDNA obtained from non-blood sources and highlight how the information provided from these samples might augment blood-based analysis. Of note, we use the term ctDNA to refer more broadly to these non-blood sources of tumour-derived cfDNA.

Box 1 | Roles of non-blood ctDNA at various stages of the cancer clinical pathway

Screening programmes

- Urine or seminal fluid for prostate cancer.
- Stool ctDNA for colorectal cancer.

Initial diagnosis

- Sampling of CSF might avoid the need for brain tumour biopsy sampling and/or enable diagnosis prior to resection.
- Analysis of pleural fluid might provide results with a faster turnaround than typically achieved with tumour tissue analysis.

Therapeutic decisions

- Provides an additional companion diagnostic that increases sensitivity for the detection of targetable alterations and identifies intracranial-specific populations in the CSF with faster turnaround than typically achieved using tissue analysis.

Minimal residual disease monitoring

- Urinary ctDNA potentially enables the entirely non-invasive repeat monitoring

of both urological and non-urological cancers.

- CSF monitoring for possible central nervous system relapse in patients with breast cancer following surgery.
- Saliva for patients with oral cancers following radical chemoradiotherapy.

Response monitoring

- Urine/saliva enables entirely non-invasive monitoring and is thus amenable to serial sampling.

Detecting resistance

- Urine can act as an additional source for the detection of resistance mutations such as *EGFR*^{T790M}.
- Analysis of CSF can provide evidence of intracranial-specific resistance.

Trial enrolment

- Can act as an additional source of ctDNA to assist in stratification prior to clinical trial enrolment.

CSF, cerebrospinal fluid; ctDNA, circulating tumour DNA.

Urinary ctDNA

Transrenal tumour DNA. The presence of DNA within urine and its potential clinical use have been recognized since the early 2000s. Urine provides a promising source of ctDNA that can be sampled entirely non-invasively^{9,24}. ctDNA within the urine is comprised of two distinct fractions⁹. The first is transrenal tumour DNA (trtDNA), which originates from plasma and enters the urine through glomerular filtration and is therefore limited in size (typically <250 bp)²⁵. The low concentration of trtDNA has previously restricted the analysis of this type of ctDNA, although these issues have been partially overcome using modern analysis approaches such as NGS²³. The second fraction originates from tumour cells shedding directly from the urinary tract and, by virtue of not undergoing renal filtration, can be of larger molecular weight²⁶.

The most notable advantage of analysing urinary ctDNA is the ability to obtain samples entirely non-invasively, without the need for venesection or for a health-care professional to be present during sampling²⁷. This advantage makes serial sampling much simpler and enables samples to be obtained within the patient's home, which is of particular interest given the focus on 'virtual' care following the COVID-19 pandemic²⁸.

Urine samples are potentially also less susceptible to dilution of ctDNA with cfDNA from leukocyte lysis²⁹ although cfDNA shed from the urinary tract epithelia might have a similar effect³⁰. Nonetheless, urine has the unique benefit of enabling the direct assessment of DNA released from urological cancers.

Despite these theoretical advantages, urinary ctDNA assays are substantially less developed than their plasma-based alternatives and certain disadvantages exist and should be highlighted. Firstly, trtDNA must pass through the glomerulus, which limits the molecular weight of the fragments to <250 bp. Secondly, the glomerular filtration rate controls the rate of urinary trtDNA accumulation and can be highly variable, particularly in patients receiving systemic anticancer therapy. Indeed, an analysis of urine samples obtained at various times of day suggests substantially lower trtDNA yields from samples obtained <1.5 h after a previous void²⁷. The use of preservatives and optimized storage and transport temperatures is also crucial given the short half-life of unpreserved DNA^{27,31}. A further potential drawback is the often large volume of voided urine and the dilutional effects this will have on ctDNA. Processing of large-volume samples using cfDNA isolation protocols is more technically challenging and, while some studies describe the detection of ctDNA in small volumes of urine (entry volumes of 1–2 ml), others used larger volumes and added a sample concentration step to the workflow^{30,32,33}.

Most research into clinical uses of trtDNA is from patients with non-small-cell lung cancer (NSCLC), testing for alterations in *EGFR*, including the T790M mutation, and *KRAS*^{23,34,35}. *EGFR* mutation status was examined through dPCR and NGS analysis of urine samples from patients receiving third-generation *EGFR* inhibitors in clinical trials²³. In a cohort of 63 patients from the Tiger-X trial²³, comparisons with tissue analysis demonstrated that trtDNA had a detection sensitivity of 67–75% depending on the specific mutation subtype, which increased to 80–93% when larger volumes of urine

were available (compared with 87–100% for plasma samples)²³. Combined analysis of urinary and plasma ctDNA identified 89% of all T790M mutations compared with 75% following tissue-only analyses²³. These data highlight a potential synergistic effect of combining different liquid biopsy methods and provide early evidence of concordance between trtDNA and tissue *EGFR* status. This approach also enables the identification of *EGFR* alterations that were not detected in tumour samples and could be used to monitor both treatment response and the development of acquired resistance. Similarly, analysis of the presence of *KRAS* mutations in trtDNA has shown high levels of concordance of around 70–77% relative to tissue sampling, which correlates favourably with plasma ctDNA³⁶, as well as providing prognostic information for patients with elevated *KRAS*-mutant trtDNA at baseline^{35,37}.

trtDNA analysis has also shown utility across several other cancer types, including gastric, colorectal, pancreatic and ovarian cancers, as well as central nervous system (CNS) lymphomas^{38–40}. dPCR-based analysis of *KRAS* and *BRAF* mutations in ctDNA from plasma and urine samples from patients with colorectal cancer (CRC) revealed sensitivity and specificity values of 85.5% and 100%, respectively, for urinary ctDNA, with overall agreement with tissue analysis of 91%, which was similar to plasma ctDNA (sensitivity 87.3%, specificity 100%, overall agreement 92%)⁴¹. Whole-exome sequencing has also been shown to be a feasible method of analysing trtDNA in urine samples obtained from patients with CRC; however, in a study involving 24 patients, only 4 had levels of trtDNA sufficient for analysis, thus highlighting the current challenges associated with this approach³⁰. Nonetheless, liquid biopsy of trtDNA is garnering increasing interest as an early detection method^{42,43}. For example, oncogenic *KRAS* mutations can be detected in urine samples from a subset of patients with stage I CRC despite the lower levels of ctDNA inherent to early stage disease reducing the sensitivity of the method, suggesting a potential role in CRC diagnosis⁴³.

Owing to the entirely non-invasive sampling process, the utility of trtDNA has been investigated as a method of monitoring disease status following curative-intent treatment. trtDNA levels have been found to decline following surgery in women with early stage breast cancer (2.1-fold reduction within 6 months of surgery), with both a higher absolute concentration at 6 months (HR 1.71, 95% CI 1.28–2.28) and greater change from baseline (HR 2.42, 95% CI 1.88–3.33) found to be predictive of disease relapse within 3 years⁴⁴. These results were similar to those obtained with plasma ctDNA analysis, although the authors observed that urinary DNA might be a slightly better risk-stratification method. Similarly, trtDNA has been detected as early as 9 months prior to radiological evidence of disease recurrence in patients with hepatocellular carcinoma following tumour ablation with a curative intent⁴⁵. Therefore, regular urine sampling (which can be performed at home, with the samples returned by post) following curative treatment could enable early detection of disease recurrence with a greater level of compliance compared with strategies that involve regular clinic visits.

Box 2 | Advantages and disadvantages of plasma ctDNA^{2,3}

Advantages

- Easily obtainable using standardized processes.
- Familiar to an increasing number of laboratories.
- Minimally invasive collection enables longitudinal sampling at multiple timepoints.
- Enables early detection of acquired resistance.
- Might enable tumour heterogeneity to be better captured than with tissue biopsy sampling.

Disadvantages

- Low circulating tumour DNA (ctDNA) to cell-free DNA (cfDNA) ratio owing to predominance of clonal haematopoiesis.
- Only certain cell subtypes might release ctDNA into the circulation.
- Poorly representative of CNS disease.
- Poorly representative of tumours restricted to certain anatomical locations (such as early stage non-small-cell lung cancers and sarcomas).

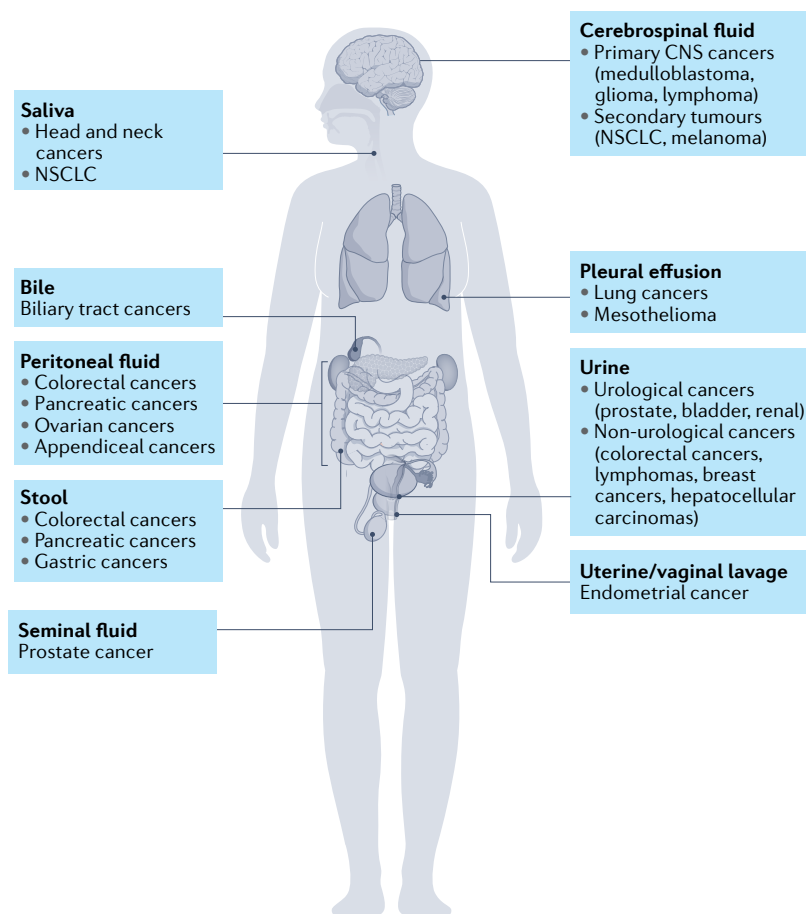


Fig. 1 | Examples of different cancers that can be investigated using non-blood sources of ctDNA. Owing to the advantages of direct contact with one or more tumour types, non-blood sources of circulating tumour DNA (ctDNA) have certain advantages that could either supplant or complement plasma ctDNA. CNS, central nervous system; NSCLC, non-small-cell lung cancer.

Urinary cell-free DNA. Bladder, prostate and renal cancers, which occur within the urinary tract, can release DNA fragments directly into urine. Research by various groups has demonstrated higher concentrations of urinary cfDNA (ucfDNA) in patients with bladder cancer, especially in those with advanced-stage disease compared to those without cancer or with benign urological conditions^{46–48}. Indeed, an analysis of ucfDNA using NGS identified a diagnostic model based on the detection of mutations in five genes (*TERT*, *FGFR3*, *TP53*, *PIK3CA* and *KRAS*) that had excellent diagnostic accuracy for urothelial carcinoma in individuals with haematuria (AUC 0.94), although this method might be best deployed in combination with other methods owing to a number of false-negative findings⁴⁹.

The use of ucfDNA for disease monitoring and prediction of relapse in patients with urothelial carcinoma has also been investigated^{50,51}. In one of these studies, higher concentrations of ucfDNA were found in patients with disease progression even when plasma ctDNA was undetectable⁵¹. High concentrations of ucfDNA were also detected several months before clinical progression⁵¹. Urine can also be used for genomic analysis of bladder cancer^{52,53}. In one prospective study, 59 patients with

suspected urothelial carcinoma were identified using cystoscopy, and comparisons between tumour DNA from the cystoscopy specimen, ctDNA from plasma and ucfDNA revealed robust concordance (specificity 99.3%, sensitivity 86.7%) between tumour DNA and ucfDNA but not ctDNA (sensitivity 10.3%)²⁹. These data highlight two key benefits of ucfDNA testing. Firstly, the authors identified a much lower frequency of urothelial carcinoma-associated genomic alterations (such as *TERT* promoter mutations and those in *TP53*, *KDM6A*, *FGFR3*, *PIK3CA* and *ARID1A*) and a disproportionately larger number of clonal haematopoiesis-related mutations (such as those involving *CHEK2*, *DNMT3A*, *TET2* and *ASXL1*) in plasma samples compared with urine. Secondly, the urine samples could consistently be successfully analysed after being collected at home and stored in preservative containers.

The non-invasive nature of ucfDNA analysis, in addition to the early evidence that detection is feasible, has increased the level of interest in its use for the early detection of urological cancers. UroSEEK, a specific multiplex PCR-based assay (ten-gene multiplex, *TERT* singleplex and aneuploidy) has been developed specifically for the early detection of urothelial carcinoma and tested in a cohort of 570 patients with symptoms suggestive of this disease, such as haematuria or lower urinary tract symptoms⁵⁴. UroSEEK alone identified 83% of patients who went on to be diagnosed with bladder cancer, this sensitivity increasing to 95% when combined with urinary cytology, with a specificity of 93%. UroSEEK positivity preceded a clinical diagnosis of urothelial carcinoma by an average of 2.3 months and by >1 year in eight patients. In addition to UroSEEK, urinary CAPPseq-based techniques have also demonstrated potential for disease monitoring in patients at risk of disease recurrence^{54,55}. In one study, surveillance following local therapy revealed detectable tumour DNA in urine samples from 91% of patients who would go on to have disease recurrence⁵⁵. The detection of ctDNA in urine preceded clinical disease recurrence in 92% of patients (median 2.7 months), which has the potential to provide considerable clinical benefit. In summary, ucfDNA provides a promising method of early cancer detection and/or monitoring for disease progression or recurrence in patients with urothelial carcinomas.

Renal cell carcinomas (RCCs) are known to shed comparatively less ctDNA than most other tumour types⁵⁶. Studies comparing plasma and urine samples from patients with RCC have found only limited overlap between the ctDNA content of both fluids (owing to few patients having detectable ctDNA in both plasma and urine); however, similar to plasma ctDNA, ucfDNA can also overcome the issues of tumour heterogeneity associated with analysis of a single tumour biopsy sample⁵⁷. Furthermore, analysis of the DNA methylome of ucfDNA derived from patients with RCC has been shown to enable the detection of RCC with a high level of sensitivity (AUC 0.86) although lower than that for the equivalent analysis of blood plasma (AUC 0.99)⁵⁸. In another study, levels of *STOX1* detected in ucfDNA from patients with RCC decreased substantially following nephrectomy, highlighting possible prognostic implications⁵⁹.

Box 3 | Advantages and disadvantages of ctDNA from non-blood sources

Urine^{22,25,26,60}

Advantages

- Entirely non-invasive sampling enables longitudinal sampling at multiple timepoints.
- Could have a role in screening analogous to that of serum prostate-specific antigen in men with prostate cancer.
- No restrictions on sampling location owing to ease of sampling.
- Potentially high sensitivity for renal cell and urothelial carcinomas owing to direct contact with urine.
- Higher circulating tumour DNA (ctDNA) to cell-free DNA (cfDNA) ratio than plasma.
- Provides results that are potentially complementary to those obtained from plasma ctDNA.

Disadvantages

- Transrenal ctDNA content limited by glomerular filtration.
- ctDNA yield can vary by time since previous void.
- No standardized methods of preservation or analysis available.

Cerebrospinal fluid^{5,70–72,87}

Advantages

- Provides a diagnostic alternative to CNS biopsy sampling for primary CNS neoplasms.
- Enables variant detection at high variant allele frequencies, owing to limited interference from clonal haematopoiesis, and the detection of CNS-restricted disease.
- Permits the diagnosis of leptomeningeal disease.
- Higher ctDNA to cfDNA ratio than plasma.
- Provides information on risks of CNS relapse following definitive therapy.

Disadvantages

- Invasive relative to other liquid biopsies.
- Sampling requires specifically trained medical personnel.
- Difficult to obtain large numbers of samples or repeat samples.
- Challenging to standardize sample collection and/or processing.

Pleural fluid^{18,101}

Advantages

- Physical proximity to pleural/lung parenchymal disease.
- Higher ctDNA to cfDNA ratio than plasma.
- Quick turnaround, increased genomic yield complementing low-content lung/pleural biopsies.
- Easy to collect large sample volumes.

Disadvantages

- Invasive sampling procedure that might not be suitable for serial sampling.
- Unclear at present whether effusion sediment or supernatant should be used for analysis.

Peritoneal fluid^{107–109}

Advantages

- Physical proximity to peritoneal disease.
- Higher ctDNA to cfDNA ratio than plasma.
- More sensitive than plasma ctDNA in cancers with predominantly peritoneal metastases.
- Potential for sampling both before and after surgery in order to assess recurrence risk.

Disadvantages

- Invasive sampling procedure that is only indicated in certain settings.

Saliva^{114,115,119}

Advantages

- Direct contact with oropharyngeal cancers.
- Entirely non-invasive sampling enables longitudinal sampling at multiple timepoints.
- No restrictions on sampling location owing to ease of sampling.

Disadvantages

- Typically yields only low ctDNA concentrations.
- Best used in combination with other techniques for analysing tumours of the oropharynx, hypopharynx and larynx.
- May require highly specific techniques, such as electric field-induced release and measurement for non-oropharyngeal disease, owing to the limited fragment size.

Seminal fluid^{6,137}

Advantages

- Non-invasive sampling enables longitudinal sampling at multiple timepoints.
- Contains very high DNA concentrations, including large DNA fragments.
- Produced in part by the prostate gland, implying direct contact with prostate cancers.
- DNase activity reduced by components of semen.

Disadvantages

- Sample collection following therapy can be challenging owing to the removal of key organs and/or ongoing androgen-deprivation therapy.
- Relevance limited to men with prostate cancer.

Stool^{124,129,142}

Advantages

- Physical proximity to colorectal disease.
- Analysis with next-generation sequencing techniques is feasible.
- Stool already used for colorectal cancer screening.

Disadvantages

- ctDNA extraction can be challenging.
- Low ctDNA component owing to the presence of microbial DNA.
- Patient aversion.

Bile^{4,6,137}

Advantages

- Higher biliary tract ctDNA concentrations than in plasma.
- Provides an alternative to tissue biopsy sampling, which can be challenging in biliary tract cancers.
- Might be obtainable during therapeutic procedures (stenting, cholecystostomy).

Disadvantages

- Invasive procedure and pre-analytical factors can be difficult to standardize.

Uterine lavage/vaginal blood^{10,14}

Advantages

- Less invasive than hysteroscopy and biopsy sampling and can be done in an outpatient or clinic setting.
- Vaginal tampons permit home-based sampling.

Disadvantages

- Patient aversion.
- Uterine lavage can be invasive relative to other liquid biopsy methods.

Serum prostate-specific antigen (PSA) is routinely used for both screening and disease monitoring in men with suspected or known prostate cancer; however, this biomarker comes with a high risk of overdiagnosis, especially when used in isolation, and ucfDNA might provide a more accurate alternative⁶⁰. A real-time PCR-based assay designed to detect >250 bp fragments of the genes encoding MYC, HER2 and AR showed promising levels of sensitivity (0.79) and specificity (0.84) in a pilot study comparing ucfDNA from 29 patients with prostate cancer with that obtained from 25 volunteers without cancer; however, the diagnostic performance of this assay was lower than that of serum PSA when tested in a larger cohort that included patients with benign urogenital conditions^{33,61}. These results might be explained by a number of patients in the control group having conditions, such as calculi or renal tract inflammation, that might have led to increased cfDNA shedding into urine, leading to false-positive results. Emerging interest exists in the analysis of epigenetic alterations, including DNA methylation, in ucfDNA obtained from men with prostate cancer⁶².

In summary, ucfDNA analysis has been demonstrated to be feasible and to provide results that are highly concordant with those obtained from tissue and plasma ctDNA sequencing. These observations suggest clinical validity in cancer detection, personalized treatment-related decision-making, serial monitoring and/or following curative therapy in a variety of cancer subtypes⁶³. Analyses focusing on ucfDNA have several advantages: similar to plasma DNA, the effects of tumour heterogeneity on the accuracy of a single tissue biopsy sample can be avoided; furthermore, the ease of serial monitoring without the need for hospital visits provides a unique benefit for patients with urological cancers. The main challenges to the implementation of ucfDNA-based assays are analytical, with differences in processing methodologies and collection timings limiting the widespread adoption of these methods. Utilizing these techniques within the clinic would require extensive prospective testing, which is currently lacking.

CSF ctDNA

For patients with extracranial malignancies, the aim of a liquid biopsy is generally not to supplant the gold standard of diagnosis based on tumour histology but rather to provide information on treatment response or disease relapse or to identify targetable alterations. However, obtaining tumour tissue samples from patients with CNS malignancies is both considerably more challenging and potentially dangerous⁶⁴. Both the extent of surgical intervention and the overall treatment strategy are dependent on prognosis (and therefore tumour type), which potentially necessitates an intraoperative histological diagnosis, thereby both delaying and potentially complicating any surgical procedures⁶⁵. In these scenarios, liquid biopsy sampling could have a valuable role in both the diagnosis of primary CNS disease and in confirming possible secondary leptomeningeal spread.

Plasma ctDNA has been shown to be present in only a minority (<10–45% depending on primary histology) of patients with both primary CNS malignancies and

isolated CNS metastases and is typically detected at lower concentrations than those in patients with non-CNS solid tumours, likely owing to the blood–brain barrier limiting the translocation of both ctDNA and CTCs^{66–69}. By contrast, CSF circulates around the brain parenchyma within the intracerebral ventricles, sub-arachnoid cisterns, sulci, and spinal canal and is, therefore, an excellent source of ctDNA from CNS tumours. Furthermore, the CSF generally contains fewer circulating immune cells relative to blood plasma, which might reduce the overall cfDNA content of the CSF, thus permitting the more sensitive detection of cancer-specific mutations present at low VAFs⁷⁰.

The comparative abundance of ctDNA in the CSF compared with plasma (given the relative ease in obtaining plasma samples) is a key consideration for the use of liquid biopsy sampling in patients with CNS-restricted disease. Alterations present in ctDNA can consistently be detected at lower VAFs in the CSF compared with plasma and, when assessed longitudinally, changes in the abundance of ctDNA in the CSF are also more representative of tumour burden^{8,71,72}. For example, in one study involving 19 patients with gliomas with detectable ctDNA present in the CSF (as determined by NGS), tumour-associated mutations were only detected in plasma samples from three patients (16%). In these three patients, the VAF of the alteration was substantially lower in plasma relative to CSF (mean VAF 0.58% versus 23.96%)⁷³.

CSF ctDNA was also more abundant in patients with meningeal involvement or in those with parenchymal tumours that were in close contact with the CSF reservoir^{66,72}. Fragmentation patterns in CSF ctDNA obtained from patients with glioma also demonstrated a relative enrichment of shorter DNA fragments (~145 bp) similar to that seen in plasma ctDNA from patients with a high systemic tumour burden^{14,74}.

Several established molecular markers have been integrated into the classification of gliomas, including 1p/19q codeletions and alterations in *IDH1/2*, *H3F3A*, *HIST1H3B/C*, *ATRX*, *RELA*, *TP53*, *TERT* and *BRAF*⁷⁵. Alterations in a number of these genes have been studied in CSF ctDNA in cohorts of patients with diffuse gliomas using dPCR-based approaches^{76,77}. These studies demonstrated that analysis of CSF ctDNA enables tumours to be successfully classified into molecular subtypes and provides prognostic information. Indeed, enrolment in an ongoing clinical trial testing the tyrosine-kinase inhibitor anlotinib in patients with glioblastoma (NCT04004975) is restricted to patients with mutations in one of four target genes (*VEGFR*, *KIT*, *PDGFR* or *FGFR*) detected in both CSF ctDNA and a pathological tumour specimen.

Diffuse midline gliomas are associated with a particularly dismal prognosis. These tumours are frequently associated with the histone mutation *H3*^{K27M}, which is often used as a criterion for clinical trial enrolment and has shown promise as a potential CSF biomarker^{76–79}. This approach to stratification has potential utility both in offering diagnostic confirmation when biopsy sampling is not feasible and in enriching the cohort with patients who are more likely to respond to a specific targeted therapy. Substantial reductions in *H3*^{K27M} in CSF

ctDNA were concordant with radiological responses in 10 of 12 patients according to longitudinal analysis⁷⁸.

Research interest in the role of CSF ctDNA in medulloblastoma has also increased over the past few years^{71,80}. Liu et al.⁸⁰ collected serial CSF samples from a total of 123 patients from surgical resection and throughout the course of adjuvant therapy in a prospective study. Copy number variants in CSF matching those detected in the primary tumour were used as a marker of minimal residual disease (MRD). Baseline MRD status correlated strongly with metastatic status (85% of patients with metastatic disease had detectable MRD versus 54% with localized disease) and serial ctDNA measurements revealed detectable MRD ≥ 3 months prior to radiological progression in 16 of 32 patients who had a complete response and subsequent disease relapse. A robust association with inferior post-radiotherapy progression-free survival in patients with MRD was also observed (HR 6.53, 95% CI 3.22–13.26; $P < 0.0001$). A particular feature of medulloblastomas that might make these tumours amenable to CSF cfDNA assays is that patients often present with hydrocephalus requiring therapeutic drainage prior to surgery and might also require the insertion of further drainage devices for therapeutic purposes, thereby allaying some of the concerns around the invasiveness of repeat diagnostic lumbar punctures⁷¹.

Interest also exists in the potential of a ctDNA-based CSF biomarker for patients with primary CNS lymphomas (PCNSLs), both for diagnostic purposes in patients with equivocal disease histology or in whom tumour material is unobtainable and for monitoring of response and/or detection of disease relapse. CSF sampling is performed routinely in all patients with suspected CNS lymphoma, both for prognostic and staging purposes. However, CSF cytology is normally only positive in patients with leptomeningeal involvement or in whom the disease is connecting with the ventricular system.

The majority of studies involving patients with PCNSLs have used dPCR-based approaches for analysis, with driver mutations selected based on tissue genome or exome analyses or using other preselected target genes. Of these, *MYD88* is the most commonly studied genomic feature of PCNSLs. Nonetheless, the sensitivity of CSF-detected *MYD88* mutations varies considerably between studies, ranging from 25% to 81% in unselected patients with PCNSL^{40,81–83}.

A subset of patients with PCNSLs have a good initial radiological response to induction chemotherapy but subsequently have very early disease relapse after consolidative autologous stem cell transplantation. If CSF ctDNA can be used to identify this group before the emergence of radiological evidence of disease relapse, an alternative consolidation therapy, such as whole-brain radiotherapy (for presumed chemoresistant disease), could be offered earlier in the course of disease⁸⁴. Biomarker analyses performed in the context of a phase Ib trial using an NGS panel designed specifically for patients with haematological malignancies were able to demonstrate a positive correlation between a radiological complete response and disappearance of CSF ctDNA, with CSF ctDNA recurrence preceding radiological relapse in one patient⁸³. In another study using

variant-specific digital-droplet PCR, investigators were able to detect CSF ctDNA as early as 8 months prior to CNS relapse being confirmed radiologically⁸⁵.

Contrasting results have been observed when PCNSL ctDNA has been examined in paired plasma and CSF samples. In one study, ctDNA was detectable in the CSF but not in plasma from patients with CNS-restricted PCNSLs⁸⁵. By contrast, *MYD88*^{L265P} was detected in samples obtained from the CSF, plasma and urine of patients with PCNSL in a separate study⁴⁰. These investigators were able to detect this alteration at 100% sensitivity (based on tissue positivity for *MYD88*^{L265P}) in both plasma and CSF samples and, intriguingly, at 88% sensitivity (in 8 of 9 patients) in urine, although mutant copies were present at higher fractional abundances in CSF samples than in either plasma or urine⁴⁰. These contrasting results might be related to the timing of sampling in relation to primary surgery (either biopsy sampling or resection).

ctDNA could have a valuable role in the detection, monitoring and characterization of specific resistance mechanisms unique to the intracranial lesions in patients with CNS metastases from extracranial primary tumours. Discrepancies in response to therapy between intracranial and extracranial lesions are not uncommon (even in patients receiving drugs with good CNS penetration) and this observation might reflect the existence of one or more subclonal populations harbouring different mechanisms of resistance^{8,86}. In this setting, biopsy sampling of CNS disease is unlikely to be justifiable and many of these alterations are likely to be difficult to detect in ctDNA obtained from plasma samples⁸⁷.

Leptomeningeal metastases are often particularly difficult to diagnose and are associated with a poor prognosis. Studies using NGS-based methods to assess the utility of CSF ctDNA for this purpose have attempted to characterize the mutational landscape of leptomeningeal metastases in patients with primary NSCLC^{88,89}. The ctDNA detection rate and VAFs of detected alleles were both higher in CSF samples than in plasma (sensitivity 81.5% and 62.5% for CSF and plasma, respectively). The maximum allelic fraction was 43.6% for CSF versus 4.6% for plasma and a unique genomic profile was observed in the CSF, primarily owing to the presence of CSF-specific copy number variants^{20,88,90}. In patients with NSCLC, analysis of CSF ctDNA might enable more sensitive detection of alterations than analysis of plasma given that *EGFR* alterations are typically detected at a sensitivity of 70–90% in plasma samples from patients with advanced-stage *EGFR*-mutant disease^{91–95}. Analysis of CSF ctDNA has also been demonstrated to provide insights into mechanisms of acquired resistance that might be confined to the CNS and might better represent the genomic landscape of leptomeningeal disease^{20,96,97}. A number of case reports describing patients with melanoma suggest that CSF ctDNA might provide a useful method of monitoring intracranial disease response or identifying CNS-restricted targetable alterations, although these findings have not been validated in larger cohorts thus far^{89,98,99}.

Certain practical elements require further consideration in order for CSF ctDNA-based assays to be used

more widely. Lumbar punctures are routinely performed in certain settings, such as in patients with leptomeningeal disease and in those with haematological cancers with CNS involvement, although they are not usually mandated for those with secondary brain parenchymal metastases. Owing to the invasive nature of a lumbar puncture, obtaining samples for translational research (in the absence of a therapeutic indication such as hydrocephalus) might be ethically challenging. Other limitations to the development of ctDNA-based assays for the analysis of CSF include the small sample sizes, biases created by the exclusion of patients for whom a lumbar puncture is contraindicated and technical issues such as contamination with blood during the procedure and variability in sample processing times.

Pleural and peritoneal fluid ctDNA

Pleural and peritoneal fluids provide a thin lubricating layer that reduces friction between pleural linings, abdominal organs and the peritoneum. They are ultrafiltrates of the blood that are relatively enriched in non-haematopoietic cfDNA owing to an absence of peripheral blood cells. Malignant pleural effusions (MPEs) and ascites involve tumour cells that infiltrate the pleura and/or peritoneum via direct, haematogenous or lymphatic spread. The fluid is therefore in close proximity to tumour tissue and might contain ctDNA from tumour cells¹⁰⁰.

Even when cytology is negative, MPEs can have a high cfDNA content and can be more amenable to genomic analysis than both tissue and plasma samples^{18,101}. The most sensitive platforms for plasma ctDNA profiling have only 70–80% sensitivity for samples obtained from patients with advanced-stage NSCLC and <50% for those with early stage disease¹⁰². Negative results therefore necessitate tissue biopsy confirmation, which can be challenging in those with early stage disease who have a lower tumour burden and possibly poorly accessible primary lesions. An advantage of analysing pleural ctDNA is that samples (obtained via pleural effusion) are immediately available for genetic testing, whereas tissue samples usually require conventional histopathological processing as per standard-of-care diagnostic workflows. Parallel histological analysis of tissue samples and molecular profiling of targetable mutations in ctDNA from pleural effusions might enable earlier detection of targetable alterations and thus permit earlier selection of the most appropriate therapy¹⁰³. One study used an NGS-based panel of 416 genes to analyse pleural fluid, plasma, and tissue samples and reported higher VAFs and mutation detection rates in pleural fluid cfDNA (98.4% versus 87% in plasma ctDNA)¹⁸. Furthermore, 93% of tissue-determined driver alterations were detectable in pleural fluid ctDNA compared with 62% in plasma¹⁸. In another study, actionable variants were identified in 87% of MPE samples versus only 48% of paired plasma samples¹⁰³.

A number of studies have investigated the utility of ctDNA in peritoneal fluid, either obtained from ascites that have developed as a consequence of malignancy or from peritoneal lavage during surgical resection^{104,105}. This approach might be particularly useful for the detection and monitoring of peritoneal disease^{106–108}. A study involving 20 patients with CRC and isolated

peritoneal disease demonstrated that peritoneal ctDNA was detectable in all patients, whereas only 20% of patients had detectable plasma ctDNA, with significantly higher VAFs in peritoneal cfDNA (16.4% versus 0.28%; $P=0.0019$)¹⁰⁸. Smaller-cohort studies have investigated ctDNA obtained from presurgical or postsurgical peritoneal lavage as a predictor of disease recurrence, with promising initial results reported^{107,109}.

Similar considerations regarding the feasibility of sample collection apply to both pleural or peritoneal aspiration and CSF sampling: pleural and/or peritoneal aspiration is a moderately invasive procedure requiring a trained operator. Aspiration for diagnostic or therapeutic purposes at initial presentation is the standard-of-care approach in patients with unexplained effusions/ascites and might also be appropriate if effusion occurs on disease relapse or progression; however pre-planned repeat sampling to monitor treatment response might be less practical.

Salivary ctDNA

Saliva is another body fluid that can be non-invasively obtained without requiring the presence of a health-care professional¹¹⁰. Saliva contains cells, proteins and nucleic acids, but is considerably less complex than blood¹¹¹. The ability to detect germline DNA in saliva has been used in forensic science for decades as well as for certain medical indications: for example, germline *BRCA1/2* mutations can be identified in saliva¹¹². Nonetheless, the lower concentrations of ctDNA relative to germline DNA require more advanced detection technologies¹¹³. Similar to other non-blood forms of ctDNA, salivary ctDNA (sctDNA) is ideally placed (owing to direct tumour contact via cellular exfoliation or apoptosis) to inform on the status of local tumours such as head and neck cancers. Furthermore, sctDNA can also originate from more distal malignancies either through passive diffusion, active transport or ultrafiltration of ctDNA from the blood across the mucosal membranes¹¹⁴.

Analysis of sctDNA has the ability to differentiate patients with head and neck squamous cell carcinomas (HNSCCs) from those without this disease⁵. HNSCC is commonly associated with human papillomavirus (HPV) infection. In a PCR-based analysis of plasma and saliva samples from patients with oropharyngeal squamous cell carcinomas, HPV16 DNA was detected prior to treatment with a sensitivity of 52.8% in saliva (67.2% in plasma) and a specificity of 100%¹¹⁵. Furthermore, detectable HPV DNA and the concentration of HPV DNA in saliva following treatment were both associated with a significantly higher risk of disease recurrence (HR 10.7, 95% CI 2.36–48.50; $P=0.002$) and inferior OS (HR for death 25.9, 95% CI 3.23–208; $P=0.002$)¹¹⁵ and, in a separate study, were demonstrated to correlate with tumour burden and to be predictive of treatment response¹¹⁶. Results from pretreatment and post-treatment samples suggest saliva alone is the ideal sample for the assessment of malignancies of the oral cavity, although this should be used in combination with plasma ctDNA for assessments of tumours located in the oropharynx, hypopharynx and larynx, all of which have less direct exposure to saliva¹¹⁷.

The analysis of sctDNA is limited by several technical challenges¹¹⁸. In patients with NSCLC, dPCR-based analysis of *EGFR* mutations in plasma and saliva samples revealed high levels of concordance, although *EGFR* alterations were not detected in saliva DNA samples from several patients with detectable *EGFR* alterations in plasma. This observation might be explained by the low concentrations of ctDNA entering the saliva from plasma¹¹⁸. Other research suggests that sctDNA fragments are ultrashort (40–60 bp) and might not be amplifiable using conventional PCR techniques; this observation led to the development of an electric field-induced release and measurement (EFIRM) assay to detect *EGFR* alterations in saliva^{119–121}. Direct comparisons suggest that EFIRM is a more sensitive method of detection than dPCR, and might therefore be the optimal method of assessing saliva samples from patients with malignancies other than HNSCC¹²².

Stool ctDNA

Human DNA is hypothesized to enter the stool via a combination of cellular shedding and colonocyte apoptosis¹²³. Nonetheless, owing to the large and often diverse gastrointestinal microbiome, human DNA accounts for only around 0.01% of the total DNA content of stool, with the remainder derived from bacteria present in the gastrointestinal tract and/or diet^{123,124}.

Screening for CRC is an important current focus of research into the role of stool DNA. This aspect is one of the most advanced areas where non-blood ctDNA testing is being translated into clinical use. The rationale is that early stage colorectal lesions develop predominantly within the mucosa with epithelial shedding of DNA into the lumen of the colon. Analyses of stool DNA samples using sequencing panels comprising 3 mutations in *KRAS*, 8 in *TP53*, 10 in *APC*, *BAT-26* and a marker of elongated DNA strands have demonstrated increased sensitivity for detection of CRC compared to faecal immunochemistry and occult blood testing, albeit with an increased risk of false-positive results^{7,125}. This assay has subsequently been developed into a standalone FDA-approved screening test (Cologuard), with evidence of increased uptake among individuals who were previously not compliant with stool screening tests^{126,127}. However, this technique is less cost-effective than the alternatives and therefore might not be appropriate for use in large-scale screening programmes¹²⁸. The Cologuard assay is also being studied as an alternative to colonoscopic surveillance post removal of polyps/adenomas, which could reduce both costs and burden on patients¹²⁹.

The utility of stool DNA analysis has also been investigated in patients with other tumour types. Pancreatic cancer is notorious for its usually late diagnosis and poor prognosis; therefore, substantial efforts are currently focused on early diagnosis. Pancreatic secretions directly enter the gastrointestinal tract and the feasibility of detecting *KRAS* mutations in stool samples obtained from patients with pancreatic adenocarcinomas or cholangiocarcinomas has been studied; five of the six mutations found in stool were identical to those present in the resected carcinomas¹³⁰. Mutations in

KRAS and DNA methylation markers were detected in stool samples from patients with pancreatic cancer using dPCR, although only 19% of patients enrolled in this study agreed to provide a stool sample, highlighting compliance as a limitation¹³¹. Indeed, several studies have identified participant aversion to stool-based sampling, which might hinder the adoption of such methods^{132,133}.

Outside of screening, NGS-based analysis of stool DNA samples has been successful in the detection of a range of hotspot mutations in established cancer-associated genes in samples from patients with gastrointestinal cancers, thus highlighting a role for stool DNA analysis in precision medicine and prognostication¹³⁴. Analysis of stool DNA might also have utility beyond the detection of tumour DNA: analysis of 16S ribosomal DNA in stool samples from patients with NSCLC suggests that the composition of the microbiome can be associated with both responsiveness and toxicities in patients with immune-checkpoint inhibitors and could act as a predictive biomarker¹³⁵.

In summary, stool DNA has the potential to improve outcomes via analysis as part of cancer screening programmes and to provide information on the genomic profiles of individual tumours, thus informing precision medicine approaches. Current challenges include the rarity of detectable tumour DNA within stool, an insufficient understanding of the stool microbiome and patient aversion to providing faecal samples.

Seminal fluid ctDNA

Seminal fluid, originating from the seminal vesicles and contributing to the liquid component of the male ejaculate, is another bodily fluid that can be used to obtain ctDNA for analysis. Cell-free seminal fluid DNA (cfsDNA) generally exists at higher concentrations relative to cfDNA in other bodily fluids; this relative abundance of cfsDNA might be explained by a combination of sexual abstinence (3–5 days) before providing a sample (permitting secreted DNA to accumulate) and reduced DNase activity owing to the effects of other semen content¹³⁶. DNA fragments detected in the seminal fluid range from 180 bp to 15 kbp in length¹³⁶. Owing to the abundance of prostate secretions in the seminal fluid, growing research interest exists in the utility of cfsDNA in men with prostate cancer. Comparisons of cfsDNA from men with prostate cancer to that from men without indicate significantly higher median concentrations in the former (428.45 ng/ml versus 25.4 ng/ml; $P < 0.001$), with those with benign prostatic hypertrophy having an intermediate median concentration (77.4 ng/ml)^{6,137}. Notably, men with prostate cancer can have median cfsDNA concentrations of up to 2,243 ng/ml, which is approximately 100-fold higher than blood cfDNA concentrations (180 ng/ml) reported for patients with cancer¹¹. More comprehensive analyses of cfsDNA from men with prostate cancer (such as those involving NGS) are warranted given the high concentration of DNA available within this fluid, especially as this fluid can also be obtained non-invasively.

Limitations of the clinical utility of cfsDNA-based assays currently include the lack of defined protocols

for the collection and analysis of samples; these issues would need to be addressed before widespread adoption. Furthermore, difficulties might emerge in obtaining seminal fluid samples from patients who have developed erectile dysfunction following androgen deprivation or in whom semen volume is reduced following brachytherapy and/or radiotherapy¹³⁸. The utility of this approach is also potentially abrogated in those who have undergone radical prostatectomy with seminal vesicle removal.

Emerging sources of cfDNA

Beyond the fluids already discussed, several other possible sources of cfDNA are available. cfDNA has been identified in bile from patients with biliary tract cancers and was shown to be superior to plasma ctDNA for the detection of cancer-related somatic mutations^{4,139,140}. Uterine lavage fluid has been studied as a source of cfDNA in a cohort of 107 women undergoing hysteroscopy and curettage following post-menopausal bleeding. Mutations in cancer-associated genes were identified in all seven women subsequently diagnosed with endometrial malignancies (six of whom were diagnosed with stage IA disease), suggesting potential as a relatively non-invasive screening method¹⁴¹. However, almost half of patients ($n = 51$) had cancer-associated mutations at detectable allele frequencies despite no histological evidence of cancer, suggesting a high risk of false-positive findings. Similarly, patterns of hypermethylation typically associated with endometrial cancer can be detected in vaginal fluid samples collected from tampons from women with endometrial carcinoma and also, to a lesser extent, from those with benign conditions¹⁰. These examples highlight the potential scope of novel approaches involving the application of ctDNA technologies that are already used to analyse plasma ctDNA to other bodily fluids.

Challenges and opportunities

Several challenges must be overcome in attempts to translate the analysis of non-blood ctDNA into the clinic. Some of these challenges are specific to certain sample types and others apply more broadly, including to plasma (BOX 1). The half-life of ctDNA is relatively short (approximately 2 h)¹⁴² indicating a need for rapid processing, which presents a challenge for samples obtained using more-invasive procedures, and there remains a need to streamline the sampling process from collection to processing. Preservative-containing stabilization tubes are available and can extend the processing time for ctDNA present in plasma samples to up to 48 h without compromising the sensitivity of detection^{143,144}. However, the effects of these preservatives on ctDNA from non-blood sources have not been established. A number of other pre-analytical variables relating to transport and storage conditions that might be harder to standardize for samples from non-blood sources also remain unaddressed. The incorporation of invasive sampling procedures, such as pleural aspiration and lumbar puncture, into clinical trial protocols might also be difficult to justify, especially before the assays are fully validated. Conversely, samples of urine and saliva can be

obtained entirely non-invasively and repeated over many timepoints with minimal risks to patients.

The majority of studies discussed in this Review were conducted in academic settings and used 'in-house' sequencing platforms. The lack of standardized analytical approaches currently limits the use of non-blood ctDNA to specific research purposes and/or within the context of a clinical trial. If such assays are to be implemented clinically, the chosen assay will first require robust analytical and clinical validation. Access to accredited laboratories (ISO 17025/FDA accredited) is usually required for analytical validation; these are rarely present in universities and contracting to commercial research laboratories is costly. Furthermore, a separate process would be needed for validation of each sample type, although consideration should also be given as to whether these processes could be streamlined. For example, the same NGS assay could potentially be validated for the analysis of cfDNA from multiple sample types in parallel. The past few years have seen an explosion in the number of biotech companies offering a wide range of plasma liquid biopsy tests (such as FoundationOne and Guardant360)^{145,146}. Despite this, commercial tests enabling the analysis of ctDNA from non-blood sources are limited to Cologuard, which is approved for stool-based CRC screening¹²⁹. Further clinical validation in clinical trials in which non-blood ctDNA testing is used to determine either treatment eligibility, timing or choice of therapy will be required.

Analyses of ctDNA obtained from a number of the non-blood sources discussed in this manuscript have demonstrated levels of sensitivity comparable to those achieved with plasma ctDNA (Supplemental Table 1). However, the collection of ctDNA from multiple biological fluids might be complementary and could be used to increase overall sensitivity compared with the analysis of plasma ctDNA alone as well as offering greater insight into tumour characteristics across different anatomical sites and times. A challenge for the development of plasma ctDNA-based assays has been defining the gold standard against which to assess sensitivity and specificity given the underlying rationale that liquid biopsies might reveal mutations that are not detected in a single tumour biopsy sample¹⁴³. Adding further sample types might achieve increased sensitivity but care must also be taken to avoid a concomitant increase in false-positive findings. Recognized standards for assessing the performance of newly developed assays in the wide range of available non-blood sample types will need to be developed.

Clonal haematopoiesis is common in patients with cancer and especially in those of advancing age or who previously received radiotherapy. This feature can confound genomic analysis, especially the specificity of plasma ctDNA¹⁹. The reduced influence of clonal haematopoiesis on non-blood sample types might therefore offer advantages in the context of screening programmes and early detection (in which the relevant allele might be present at very low frequencies and be more restricted to the primary site and its immediate milieu). Indeed, the sensitivity of plasma ctDNA for the detection of early stage malignancies (the target of screening programmes)

can be lower than for advanced-stage disease, which highlights where assays involving biofluids that are in closer proximity to the primary tumour might be advantageous^{7,29,137,147,148}. Such an approach would clearly be limited to particular tumour types (prostate, bladder, endometrial and/or colorectal cancers) compared with plasma, which might offer the ability to screen multiple cancer types using a single blood test^{149,150}. This lower specificity would likely be less disadvantageous in the context of later-stage disease monitoring or in the detection of specific resistance mutations²³.

All liquid biopsies, including those involving ctDNA from blood and non-blood sources, are vulnerable to the effects of varying anatomical disease distribution on ctDNA concentrations. For example, in patients with extrathoracic metastases from NSCLC, the concordance of results obtained from analysis of plasma ctDNA is substantially higher than in those without such metastases¹⁵¹. Similarly, in patients with CRC, plasma ctDNA can be detected at substantially lower levels in those with isolated peritoneal disease than in those with liver metastases (in contrast to peritoneal cfDNA)¹⁰⁸. Thus, identifying sources of genetic material beyond plasma or tissue might help circumvent these effects of anatomical variations, although crucially, the disease distribution in a particular patient will need to be considered carefully when deciding which assays are

likely to yield the most useful results and how best to interpret those results.

The majority of published data available at this point have used mutation-based approaches to study ctDNA and this is reflected in the scope of our Review. The most obvious direct clinical application of these methods currently seems to be the detection of targetable driver or resistance mutations. However, growing research interest exists in the detection of DNA methylation and fragmentation in ctDNA; evidence of the ability to apply such methods to the various biofluids discussed in this Review is still emerging^{58,62,148,152}.

Conclusions

In summary, the analysis of ctDNA obtained from non-blood bodily fluids has several potential benefits, from enabling exploratory research through to routine clinical use, and provides unique benefits such as enabling more detailed analysis of specific tumour types in certain anatomical locations and the potential for entirely non-invasive serial sampling. However, well-designed clinical trials in which the results are used to inform treatment decisions and that demonstrate meaningful benefits to patients will be necessary before non-blood ctDNA assays are to be broadly implemented clinically.

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Author contributions

A.T., M.C. and N.C. researched data for article and wrote the manuscript. All authors contributed to discussions of content and reviewed and/or edited the manuscript before submission.

Competing interests

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