European Journal of Cancer 168 (2022) 1–11

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejcancer.com

Original Research

Osteosarcoma: Novel prognostic biomarkers using circulating and cell-free tumour DNA

Iben Lyskjær [a,b], Neesha Kara [b], Solange De Noon [a,c], Christopher Davies [a,c], Ana Maia Rocha [a,c], Anna-Christina Strobl [c], Inga Usher [a], Craig Gerrand [d], Sandra J. Strauss [e], Daniel Schrimpf [f], Andreas von Deimling [f], Stephan Beck [b,1], Adrienne M. Flanagan [a,c,*,1]

[a] *Research Department of Pathology, University College London, London, UK*
[b] *Medical Genomics Research Group, University College London, London, UK*
[c] *Department of Histopathology, Royal National Orthopaedic Hospital, Stanmore, HA7 4LP, UK*
[d] *Bone Tumour Unit, Royal National Orthopaedic Hospital, Stanmore, HA7 4LP, UK*
[e] *Department of Oncology, University College London, London, UK*
[f] *Department of Neuropathology, Institute of Pathology, University Hospital Heidelberg, and CCU Neuropathology, German Cancer Institute, Heidelberg, Germany*

Received 3 January 2022; received in revised form 2 March 2022; accepted 7 March 2022
Available online 14 April 2022

KEYWORDS
Osteosarcoma;
DNA methylation;
Epigenetics;
Epigenetic biomarkers;
Liquid biopsy;
Prognosis;
Circulating tumour DNA

Abstract ***Aim:*** Osteosarcoma (OS) is the most common primary bone tumour in children and adolescents. Circulating free (cfDNA) and circulating tumour DNA (ctDNA) are promising biomarkers for disease surveillance and prognostication in several cancer types; however, few such studies are reported for OS. The purpose of this study was to discover and validate methylation-based biomarkers to detect plasma ctDNA in patients with OS and explore their utility as prognostic markers.
Methods: Candidate CpG markers were selected through analysis of methylation array data for OS, non-OS tumours and germline samples. Candidates were validated in two independent OS datasets (n = 162, n = 107) and the four top-performing markers were selected. Methylation-specific digital droplet PCR (ddPCR) assays were designed and experimentally validated in OS tumour samples (n = 20) and control plasma samples. Finally, ddPCR assays were applied to pre-operative plasma and where available post-operative plasma from 72 patients with OS, and findings correlated with outcome.

* *Corresponding author*. Department of Pathology Research, UCL Cancer Institute, Rockefeller Building, 21 University Street, London, WC1E 6DE, UK.
E-mail address: a.flanagan@ucl.ac.uk (A.M. Flanagan).
[1] These authors contributed equally.

https://doi.org/10.1016/j.ejca.2022.03.002

Results: Custom ddPCR assays detected ctDNA in 69% and 40% of pre-operative plasma samples (n = 72), based on thresholds of one or two positive markers respectively. ctDNA was detected in 5/17 (29%) post-operative plasma samples from patients, which in four cases were associated with or preceded disease relapse. Both pre-operative cfDNA levels and ctDNA detection independently correlated with overall survival (p = 0.0015 and p = 0.0096, respectively).

Conclusion: Our findings illustrate the potential of mutation-independent methylation-based ctDNA assays for OS. This study lays the foundation for multi-institutional collaborative studies to explore the utility of plasma-derived biomarkers in the management of OS.

1. Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumour in childhood and adolescents, generally arising in the metaphysis of long bones [1]. Approximately 80–90% of all OS tumours are high grade, and despite toxic multimodal therapies, survival for the majority of patients with OS has not improved over the last 40 years [2], with 30–40% of patients with OS dying of their disease [3]. Although response to chemotherapy is an indicator of survival [4], it is currently not possible to predict which patients are likely to respond to chemotherapy.

Studies have demonstrated the value of minimally invasive assays in cancer management in assessing treatment efficacy through detection of residual tumour following surgery, as predictive biomarkers of response to targeted therapies, and as effective tools for surveillance of disease relapse [5]. Measurement of total circulating free DNA (cfDNA) and circulating tumour DNA (ctDNA), the latter representing DNA shed into the bloodstream by tumour cells, has been shown to correlate with important clinical endpoints (*e.g.* progression-free survival and or overall survival) in several cancer types, as reviewed in Refs. [6–8]. In 2010, McBride *et al.* published the first ctDNA study in OS from a single patient and known genomic alterations from the tumour [9]. Eight years later, Barris *et al.* applied targeted sequencing of genetic alterations detected in the tumours from 10 OS patients (28 samples) [10] and found that ctDNA was detected in approximately half of the plasma samples (46%) [10]. Shulman *et al.* used ultra-low-pass whole-genome sequencing (ULP-WGS) and detected ctDNA in 57% (41/72 OS patients) of localised OS prior to treatment, and reported that detection of ctDNA was associated with inferior survival but the results were not statistically significant [11]. Klega *et al.* detected ctDNA in 9/10 OS patients when looking at copy number changes using ULP-WGS [12], but did not correlate their findings to clinical outcome. miRNA methylation studies have also shown promising results (reviewed in Ref. [13]) although this is not a focus of this current study.

In the human genome, DNA methylation predominantly occurs at CpG dinucleotides, the site where a cytosine residue precedes a guanine residue in the 5′-3′ direction. In the healthy human genome, the majority of CpGs are methylated, except at CpG islands [14]. Conversely, a cancer genome is often characterised by global hypomethylation and focal hypermethylation at CpG islands, leading to both genomic instability and transcriptional repression [15]. This feature of cancer has been exploited as a highly sensitive and specific means of detecting cancers [16]. To date, only a few studies have investigated genome-wide methylation patterns in samples from OS patients and correlated with patient outcomes [17–20]. The use of DNA methylation as a marker of ctDNA in OS is attractive because this tumour is characterised by complex genomes and high inter-tumour heterogeneity rather than recurrent genetic alterations [21–23], making the selection of a universal genetic ctDNA marker challenging. Furthermore, as changes in methylation represent an early event in cancer and are considered to be retained through tumour evolution [24], they could be exploited as powerful biomarkers and would be expected to be only moderately confounded by tumour genetic heterogeneity [25]. For this reason, this approach has potential advantages over those employed by previous ctDNA studies in patients with OS in which genomic alterations were used for marker development [10,11].

It has been established previoulsy that high levels of DNA in the circulation (cfDNA) have been linked to inferior outcomes [26–28] in patients with cancer. However, in contrast to ctDNA which represents a tumour-specific assay, cfDNA levels can be influenced by other factors including physical activity, age and rate of clearance. For these reasons, cfDNA is considered a less reliable biomarker, particularly when measured shortly after surgery [6]. Despite this, the clinical utility of cfDNA as a prognostic marker has been proven in a wide range of cancers [6].

The aim of this study was to identify and validate methylation-based biomarkers for the purpose of detecting ctDNA in patients with OS, and then to explore their utility as prognostic markers.

2. Materials and methods

2.1. Ethical approval

The Royal National Orthopaedic Hospital (RNOH) Biobank is a satellite of the UCL/UCLH Biobank for Health and Diseaseand which is approved by the National Research Ethics Committee of the Health Research Committee (reference: Integrated Research Application System (IRAS) project identifier: 272816). This project (EC17.14) was approved by the National Research Ethics Committee UCL/UCLH Biobank Ethics Committee.

2.2. Training methylation cohort

Our *in-house* dataset consisted of 750 samples of various sarcoma subtypes (Supplementary Table 1) (EMBL-EBI, under accession number E-MTAB-9875) previously published [29]. Infinium 450K methylation data were downloaded from TCGA (n = 8730; https://www.cancer.gov/tcg) using TCGAbiolinks [30] and from Marmal-aid [31] (kindly provided by Dr Robert Lowe, UCL, QMUL, UK) (n = 2885; Supplementary Table 2).

2.3. External validation cohorts for identified methylation markers

Validation Set I consisted of 450K and EPIC methylation raw IDAT files from 162 OS from Heidelberg, Germany (provided by DS and AVD [32], GSE140686) and 699 peripheral blood leucocytes (PBLs) from non-cancer patients (GSE125105 [33]). Validation Set II consisted of 21 publicly available OS samples (GSE58770 [34]), 86 OS samples from the TARGET-OS (Children's Oncology Group and The Hospital for Sick Children in Toronto, Canada, dbGaP accession phs000218.v21.p7, http://ocg.cancer.gov/programs/target and 732 healthy blood samples (GSE87571 [35]).

2.4. Biomarker discovery and digital droplet PCR assay design

To avoid false-positive results in the plasma samples, we first excluded all CpG sites that showed signs of DNA methylation (β-value > 0.2) in more than 1% of all peripheral blood leucocytes (PBLs) samples (TCGA/Marmal-aid cohorts). Next, we excluded a) CpGs that were hypomethylated (β < 0.5) in more than 20% of all OS cases, b) methylated CpGs (β > 0.5) that were detected in more than 50% of other cancers and c) methylated CpGs (β > 0.2) detected in more than >30% of healthy tissues (Supplementary Figure 1B).

The 17 identified candidate methylation markers (CpGs) were annotated using the *COHCAP* [36] R package (using the hg19 genome build). Methylation-specific digital droplet PCR (ddPCR) assays were designed using Primer3Plus [37] and MethPrimer [38], and checked using BiSearch [39]. Receiver operating characteristics (ROC) analyses were performed using the pROC R package [40] to determine the sensitivity and specificity of the probes both individually and in combination (where at least two of the markers were positive). A β-value of 0.5 was chosen as the threshold for determining if a sample should be classified as positive. To be able to use a ddPCR assay with the available plasma (1–2 mL), we reduced the number of markers from 17 to four. This was achieved by first calculating the sensitivities and specificities in the Training and Validation sets (Supplementary Table 3) and then identifying regions for which ddPCR assays could be designed. This led us to selecting the following CpGs for experimental validation; cg02169391, cg22082800, cg25680486 and cg26100986. All of these markers are located on chromosome (chr) 7: cg22082800 (chr7: 100203350), cg25680486 (chr7: 100203114) and cg26100986 (chr7: 100202882). cg02169391 is located in the promoter of the MEST gene, while the remaining CpGs are sited in the intragenic region of procollagen C-endopeptidase enhancer 1 (PCOLCE). Primer and probes sequence for these CpGs are provided in Supplementary Table 4.

2.5. Clinical samples for experimental validation

Three clinical sample sets were available for experimental validation: 1) high-grade OS tumour samples from 20 patients (Supplementary Table 5), 2) control plasma samples obtained from 47 healthy volunteers (2.5 mL plasma) and 69 individuals with benign bone disease (see below for list) and degenerative joint disease (referred to hereafter as non-cancer patients; 4.5 mL plasma, Supplementary Table 6), and 3) samples from 72 patients with OS; these included blood for germline DNA and their pre-operative, pre-treatment plasma samples, in addition to 17 post-OP plasma samples (a total of 89 plasma samples with a median of 4.5 mL, range 3–4.5 mL (1–2 mL were used for ddPCR analysis), overview in Supplementary Table 7).

The diagnoses of patients with benign bone disease mentioned above included simple bone cysts, osteoarthritis, osteoblastoma, fibrous dysplasia, osteoid osteoma, osteochondroma, tenosynovial giant cell tumour, synovial chondromatosis; the degenerative joint disease

cohort comprised patients undergoing hip or knee replacements (Supplementary Table 8).

2.6. Digital droplet PCR analysis of tumour and plasma samples

The reaction mix (total volume = 22 µl) consisted of 18 mM of forward and reverse primer, 0.05 mM probe, 2x Supermix for probes (no UTP) (Cat.No 186–3023, Bio-Rad, USA) and with 2–9 µl input material. Droplets were generated on an automatic droplet generator QX200 AutoDG Droplet Digital PCR System (Bio-Rad) or on a QX200 Droplet Generator and PCR amplified in a Mastercycler Nexus GSX1 (Eppendorf) using the program: 95 °C for 10 min, 45 cycles of 95 °C for 30 s, X°C for 1 min (X = assay-specific, see Supplementary Table 4), one cycle of 98 °C for 10 min and cooled to 4 °C and left for 2h to rehydrate the droplets. Droplets were read using the QX100 reader (Bio-Rad), and the generated data analysed using Quantasoft v1.6.6 software (Bio-Rad).

2.7. Determining limit of blank and limit of detection

For each assay, 20 negative control samples (10 ng germline PBLs DNA) were profiled to establish the limit of blank (LOB) and detection (LOD) values [41]. To refine further the LOD we used 47 non-cancer control plasma samples controls. Each CpG pair (cg02169391 and cg26100986 or cg22082800 and cg25680486) was run on 1 mL of plasma. Firstly, 20/47 plasma samples were assessed, which revealed very low methylation levels in all samples; the average methylation levels were 0.005, 0.002, 0.001, and 0.002 for cg02169391, cg22082800, cg25680486, and cg26100986, respectively (Supplementary Table 6). Using the original LOD thresholds resulted in 18 correctly classified negative samples and two false-positive samples. Based upon these data, the thresholds were adjusted using ROC analyses to identify thresholds which provided minimal false-positive results. The new adjusted thresholds or optimal LODs are provided in Supplementary Table 4. Using the optimal LODs provided no false positives in the cohort of 20 non-cancer plasma samples and remained at a 100% sensitivity and specificity for the previous 20 tumour and germline blood samples. The remaining 27 non-cancer plasma samples were then run with assays and the new optimal LODs were applied, thereby correctly classifying all plasma as negative, thus achieving a 100% specificity. These LOD thresholds were applied subsequently to all analyses.

A list of all ddPCR assays and LOB/LOD levels are provided in Supplementary Table 4. In each ddPCR run three controls were included: a positive control (Universal methylated Human DNA, Cat.No. D5011, Nordic Biosite), a negative control (un-methylated control DNA, Human HCT116 DKO non-methylated DNA, Cat.No. D5014-1, Nordic Biosite) and a non-template control (NTC, H_2O).

2.8. Clinical correlates

OS patients were identified from an anonymised research database of patients diagnosed and managed within the London Sarcoma Service and included paediatric and adult patients. Accompanying clinical annotations were available for the majority of cases, including demographic data, pathological information, treatment history and outcome data (Supplementary Table 9).

2.9. Statistical analysis

All statistical analysis was performed in R (version 3.6.1) [42]. A p-value <5% was deemed statistically significant. A ctDNA-positive sample was defined as a sample with a least two of the four methylation markers above the limit of detection (LOD). Survival analysis was performed using the Kaplan–Meier estimator with all-cause mortality as the end point. The multivariate analysis was a Cox proportional hazards regression model with time from diagnosis to death or censorship (time to last follow-up) as an end point.

2.10. Data availability

In-house raw methylation array data have previously been published [29] and are available from the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress), accession number E-MTAB-9875.

3. Results

3.1. In silico identification and validation of methylation markers through large scale bioinformatic analyses

Our first step in the identification of novel CpG sites as candidates for ctDNA assays involved downloading and interrogating the publicly available methylation data from 8730 and 2885 human tissue samples from the TCGA [43] and Marmal-Aid [31], respectively (Supplementary Table 2 and Fig. 1 for study outline). 10,766 samples were eligible for further analysis after filtering for incomplete data (Methods, Supplementary Figure 1A), and were analysed alongside 750 *in-house* sarcoma methylation profiles. Data from the remaining 10,766 samples were used for bioinformatic analysis together with 750 of our *in-house* sarcoma samples [29]. Hence, the final discovery group included methylome data from 11,516 samples, including 1578 blood samples, 202 normal tissues, 9565 non-OS cancer samples and 171 OS tumour samples (Supplementary Figure 1A, Supplementary Tables 1 and 2).

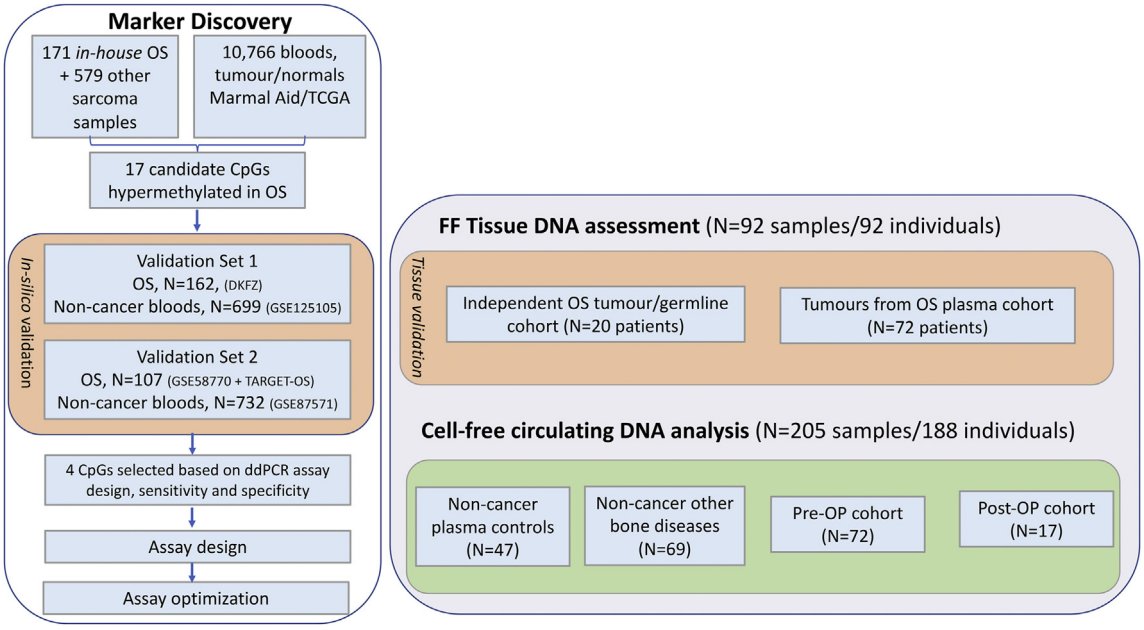

Fig. 1. **Overview of the combined biomarker and validation study.** 17 OS-specific candidate methylation markers were identified through a stepwise approach for selecting CpG sites uniquely methylated in OS (see Methods). Four markers were chosen for methylation-specific ddPCR analysis. The markers were subsequently validated both *in silico* and experimentally in tissue, before being used for ctDNA assessment of plasma samples from OS patients. 'Other sarcoma' denotes various other non-OS soft tissue and bone sarcoma subtypes (Supplementary Table 1). FF: fresh frozen tumour tissue.

3.1.1. Osteosarcoma-specific DNA methylation markers

We next took a stepwise approach to identify DNA methylation markers (CpG sites), which were uniquely methylated in OS (Supplementary Figure 1B). Using our screening pipeline (Supplementary Figure 1B), we identified 17 candidate methylated markers for OS (Supplementary Figure 2). To obtain an estimate of their combined sensitivity and specificity, we performed ROC curve analysis on 171 OS and 1578 non-cancer PBLs (Training Set). The mean area under the curve (AUC) was 0.994 and included all 171 OS and only 11/1578 (0.7%) non-cancer PBLs samples (Supplementary Figure 3A).

3.1.2. Candidate DNA methylation markers show robust performance in independent OS datasets

Two independent rounds of *in silico* validation of these candidate methylation markers were performed using Validation Set I comprising 162 OS tumour and 699 non-cancer PBLs, and Validation Set II consisting of 107 OS tumour and 732 PBL samples (Materials and Methods). By including all 17 candidate markers with a β-value cut-off ≥ 0.5, all OS in both validation sample sets were correctly classified. None of the 699 PBLs were methylated ($\beta \geq 0.5$) at any of the 17 candidate markers in Validation Set I, and only two of 732 PBLs (0.27%) from non-cancer patients were misclassified as OS in Validation Set 2. ROC analyses gave an AUC = 1.00 for Validation Set 1 and AUC = 0.99 for Validation Set 2 (Supplementary Fig. 3B and 3C).

3.1.3. Selection of four DNA-methylation markers for experimental validation

ddPCR-based assays were chosen as the method for ctDNA measurement as this represents a widely employed and highly sensitive, specific, and cost-effective methodology. The amount of plasma available from patients and the number of markers that can be multiplexed represent limitations of this approach and therefore from the 17 candidate markers, we selected four CpGs (cg02169391, cg22082800, cg25680486 and cg26100986) to take forward (Methods). Methylation of all four markers correctly identified 422/440 of all OS samples studied (95.9%) and misclassified only 6/3009 (false positives = 0.2%) non-cancer PBLs as OS *in silico* (Training and the two Validation Sets) (Supplementary Table 3, Supplementary Figure 4).

3.2. Experimental validation of methylation markers in clinical OS cohorts

3.2.1. Candidate markers are highly sensitive and specific for OS tumour tissue

To validate further the sensitivity and specificity of the four markers, we tested these experimentally using two duplex ddPCR assays on samples from another set of 20 high-grade OS and matching germline blood DNA (Supplementary Table 5). Methylation at these CpG

Table 1
Correlation of clinical outcome with pre-treatment ctDNA. PR: poor responder; GR: good responder, NCG: No chemo given, ctDNA: circulating tumour DNA.

	Pre-operative ctDNA negative (n = 43)	Pre-operative ctDNA positive (n = 29)
Metastasis at diagnosis (n = 19)	7 (16%)	12 (41%)
No metastases at diagnosis (n = 53)	36 (68%)	17 (32%)
All-cause mortality (n = 32)	14 (33%)	18 (62%)
Disease-related event (n = 39)	18 (42%)	21 (72%)
Response to chemotherapy	PR: 28 (65%) GR: 9 (21%) NCG:6 (14%)	PR: 11 (38%) GR: 8 (28%) NCG:4 (14%) Unknown/NA: 5

sites was confirmed in each of the tumours and in none of the germline samples (Supplementary Figure 5).

To refine the limit of detection (LOD, Materials and Methods, and Supplementary Methods), we also applied the four assays (two duplexed assays) to 47 control plasma samples from patients with non-neoplastic conditions (Supplementary Table 7, Supplementary Figure 5) and plasma from 69 individuals with benign bone disease (Methods, Supplementary Table 8). 3/116 samples gave a false-positive result (cases NCND_63 and 64 (osteochondromas) and NCND_65 (healthy volunteer), with an overall specificity of 97.4%.

3.2.2. Circulating tumour DNA detection correlates with disease progression

Finally, we measured the four methylation markers in matched tumour, PBLs and pre-treatment plasma samples from 72 patients with OS (Supplementary Tables 6 and 9). This cohort comprised 19 patients with metastatic disease at diagnosis and 53 patients with only localised disease at diagnosis. Multivariate analysis confirmed published reports that the presence of metastases at diagnosis is an independent prognostic factor in our cohort (Table 2) [44]. At least two of our four assays were positive in all 72 tumour samples. We first ran one duplexed assay on each plasma sample based on the probe with the highest methylation level in the matched tumour tissue. If neither or only one of the methylation markers was detected, we ran the other duplex assay. Although we observed methylation of at least one marker in 50/72 (69%) samples, we set a minimum of at least two markers to be detected above the LOD for a sample to be classified as 'ctDNA positive' and employed this criterion hereafter.

ctDNA assays were positive in 29/72 (40%) pre-operative samples ($ctDNA^{pre+}$, Table 1, Supplementary table 4). Amongst the patients with metastatic disease at diagnosis, ctDNA assays were positive in 12/19 (63%) compared with 17/53 (32%) $ctDNA^{pre+}$ patients with localised disease. Maximum tumour dimension was the only notable difference between those patients presenting with metastatic disease who were $ctDNA^{pre+}$ and $ctDNA^{pre-}$. Specifically, in 16 patients whose primary tumour size was available, median values for maximum tumour size in $ctDNA^{pre+}$ cases was 142.5 mm versus a median of 92.5 mm for $ctDNA^{pre-}$ cases ($p = 0.009$, Supplementary Table 9). Nevertheless, both group medians correspond to a tumour stage pT2+ (pT1b + for tumours of the pelvis; AJCC 8th edition) and tumour maximum dimension is a crude approximation of tumour burden. Looking at patients with localised

Table 2
Uni-and multivariate analysis of clinical factors and their relation to survival. cfDNA: circulating free DNA; ctDNA: circulating tumour DNA; pre-OP: pre-operatively.

SURVIVAL	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Pre-OP cfDNA (n = 72)						
<75th centile (n = 53)	0.34	0.38	**0.005**	0.56	0.43	0.18
>75th centile (n = 19)	REFERENCE					
Pre-OP ctDNA (n = 72)						
Negative (n = 43)	REFERENCE					
Positive (n = 29)	2.51	0.36	**0.01**	1.48	0.42	0.36
Metastasis at presentation (n = 72)						
Yes (n = 19)	5	0.37	**1.2*10−5**	3.78	0.0.40	**0.0096**
No (n = 53)	REFERENCE					
Gender (n = 72)						
Male (n = 38)	REFERENCE					
Female (n = 34)	1.16	0.36	0.68			
Age (n = 72)						
<30 years (n = 62)	REFERENCE					
=>30 years (n = 10)	1.14	0.54	0.81			
Max tumour size (n = 64)						
≤80 mm (n = 14)	REFERENCE					
>80 mm (n = 50)	1.38	0.5	0.51			

disease, ten (59%) of the 17 ctDNA^{pre+} patients subsequently suffered a disease-related event (death, n = 8; or metastasis, n = 2), while the remaining seven were alive without disease at the last follow-up (median follow-up = 5.3 years, range 0.9–11.3 years, Supplementary Table 9). Overall, the detection of pre-treatment ctDNA correlated with inferior survival outcomes ($p < 0.01$, Fig. 2A, Table 1) and with a disease-related event ($p = 0.01$, chi-square test), but not with tumour size ($p = 0.14$, Student's t-test), gender (0.53) or age at presentation ($p = 0.92$). Within the localised disease group, however, no significant association with outcome was observed (likelihood ratio $p = 0.19$). ctDNA positivity did not have a significant association with outcome on multivariate analysis (Table 2), but it should be noted that this model also included metastatic disease status at diagnosis which is known to be linked to increased tumour burden [44,45], and thus higher probability of ctDNA detection.

Post-operative plasma was available from 17 of the 72 patients in our plasma validation cohort, including five ctDNA^{pre+} patients. Five of 17 post-operative samples were positive for ctDNA (29%, median days from surgery 18, range: 7–30 days, Supplementary Table 9), including two subjects with ctDNA detected in both pre- and post-operative samples (OS_22 & OS_59). Two of five ctDNA^{post+} patients were also noted to have R1 resection margins (OS_11 & OS_59). A review of follow-up data showed that four of the five ctDNA^{post+} patients had a disease-related event at the time of plasma sampling (n = 2) or subsequently (n = 2) (Supplementary Table 9). The remaining patient (OS_66, postoperative ctDNA detected day 30 post-surgery) was clinically disease-free at seven years. Detection of ctDNA post-operatively was not associated with the presence of metastasis at diagnosis ($p = 0.45$, Student's t-test).

3.2.3. Total circulating free DNA levels correlate with outcome

Finally, we measured the total amount of cfDNA in patients' pre-operative plasma samples, the median level of which was 1848 copies/mL plasma (range 231-15,180 copies/mL plasma). This correlated with survival (cfDNA^{high} cutoff = 75th centile, $p = 0.003$, Fig. 2B), but not with tumour size (rho = 0.63, Spearman rank test), metastasis at diagnosis ($p = 0.18$, Kruskal–Wallis rank-sum test) or anatomical site (femur versus tibia; $p = 0.63$, Kruskal–Wallis rank-sum test). Twelve out of nineteen (63%) cfDNA^{high} patients were also ctDNA^{pre+}; of the remaining 7/19 cfDNA^{high} cases without ctDNA^{pre+}, two had metastasis at diagnosis and another two had relapsed (Supplementary Table 9). Including cfDNA^{high} as a risk factor for adverse outcomes alongside ctDNA^{pre+} and metastases at diagnosis resulted in 41/72 (57%) patients classed as high risk at the time of diagnosis. These three risk factor approaches correlated with a poor prognosis for this group ($p = 0.01$, Supplementary Figure 6, Supplementary Table 9).

4. Discussion

The standard of care for patients with OS [2] results in an approximate 70% five-year overall survival, thus despite aggressive multi-drug treatment 30–40% die of their disease. The identification of metastases at diagnosis is the most reliable indication that a patient with OS has an unfavourable prognosis. However, other factors also contribute to outcome, including tumour size and site, and response to chemotherapy [47,48]. Nevertheless, even when combining all of these clinical variables, clinicians lack sufficient information to stratify patients adequately for treatment and to offer patients with OS a reliable prognosis.

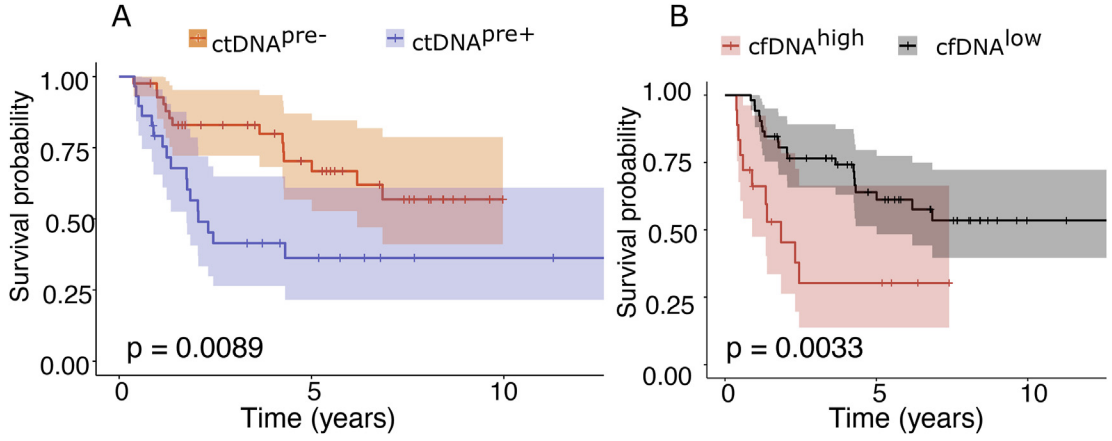

Fig. 2. **Detection of ctDNA or high levels of cfDNA pre-operatively correlates with survival** A) Detection of ctDNA and **B)** cfDNA pre-operatively correlate with overall survival (disease-related mortality or censorship). Survival analysis utilised a standard Cox proportional hazard model. The plots are censored after 12 years.

Here, we report a pipeline for the development and application of DNA methylation markers in OS and demonstrate their utility in detecting ctDNA in plasma. The *in silico* analysis of our markers predicted a high sensitivity and specificity for OS, which was subsequently confirmed experimentally by analysing both OS tumour and matching germline samples using the developed assays. These findings indicate that our methylation-based assays potentially can be applied to over 90% of patients with high-grade OS, as opposed to genomic alteration-based approaches which require the development of patient-specific assays due to the lack of recurrent alterations in OS. This high level of specificity for OS was retained when the performance of these markers was tested in plasma. We saw a decline in the sensitivity of the assay to 40% when performed on plasma using our two markers threshold. Of note, if only one marker were employed the sensitivity increased to 69%. Although lower than anticipated, our 40% detection rate was only slightly lower compared to two (46% [10], 57% [11]) of the three previous ctDNA OS studies which used different sequencing-based technologies. A third study [12] reported 90% sensitivity using ULP-WGS. However, only 10 patients were investigated, and therefore this approach warrants further investigation.

ctDNA assays have numerous potential applications in the management of OS for which knowledge of the sensitivity and specificity of the assay is important. The finding that ctDNA was detected preoperatively in 17 patients with clinically localised disease at presentation, 10 of whom subsequently developed a disease-related event, indicates that liquid biopsy has the potential for the early identification of such patients who are at high risk of adverse outcomes. While we were unable to show a statistically significant difference in survival in our localised disease cohort based on preoperative ctDNA detection, we anticipate that a larger validation cohort would demonstrate this. ctDNA assays will also be valuable for surveillance particularly in the absence of a tissue sample being available, and in the clinical trial setting by providing a baseline for monitoring disease response to treatments. This study, although the largest to date, is small in terms of translating the findings into clinical practice, therefore requires validation for use in the aforementioned scenarios.

The reason for failing to detect ctDNA in in 60% of patients based on the two marker threshold that was used to determine a "true positive" sample particularly in those presenting with metastases, is unclear. The volume of plasma analysed is an important factor in ctDNA detection [49], and we opted to use a low volume of plasma (1−2 ml) given the rarity of our samples. As a result, this initial study only utilised four of the 17 candidate markers identified in our *in silico* discovery testing; using more markers may increase the sensitivity of this approach. However, it is apparent from other studies that ctDNA detection rate varies across cancer types [50]. Given the similar sensitivities reported by different studies using different approaches, the low detection rate of ctDNA may be related to the biology of OS and local factors in the bone niche. False-positive ctDNA results in patients without OS using our methylation markers were rare (2.6%) and should not lead to misdiagnoses or inappropriate management provided results are interpreted in the context of the full clinical picture. This includes histological and radiological assessment which is currently standard practice as part of a multidisciplinary meeting.

The limitations of the study are largely attributable to the rarity of OS, the incidence of which is 0.27/100,000 per year in England [51]. The consequence of this includes the relatively small number of samples analysed within a heterogenous cohort of patients across all age groups, and a relatively short median follow-up of approximately 3.5 years. The small numbers of samples that were available for analysis post-treatment were disappointing but the finding that ctDNA is detectable prior to presentation of clinical relapse is encouraging and requires further investigation. The lack of access to an independent cohort prevented validation of our findings and highlights the challenges when pursuing translational research in rare cancers.

The study highlights the need for multi-centre collaboration, to enable the prospective systematic collection and sharing of patient samples with annotated clinical data, to recruit sufficient patient numbers for validation of these biomarkers and determination of their clinical utility in OSs, or any other rare disease. Multi-centre studies can be unattractive for a variety of reasons: they are expensive, time-consuming to establish and it can be difficult for different groups to agree and prioritise research aims and studies. Despite these challenges, there appears to be a growing appetite for the OS clinical and research communities to work collaboratively as highlighted in the UK ICONIC study (Improving Outcomes Through Collaboration in Osteosarcoma) [52] which provides the infrastructure to evaluate clinical and biological questions prospectively, and the Fight Osteosarcoma Through European Research (FOSTER) Consortium involving several countries in Europe with the aim of better stratification of patients for treatments and improving outcomes. It is our belief that such an approach is necessary to achieve clinical translation of plasma-based biomarkers for the management of OS.

Author contributions statement

Iben Lyskjær: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Validation, Visualization, Roles/Writing − original draft, Writing − review & editing.

Neesha Kara: Data curation, Formal analysis, Investigation, Visualization, Roles/Writing – original draft, Writing – review & editing.

Solange De Noon: Formal analysis, Validation, Visualization, Roles/Writing – original draft, Writing – review & editing.

Christopher Davies: Data curation, Formal analysis, Writing – review & editing.

Ana Maia Rocha: Data curation, Formal analysis, Writing – review & editing.

Anna-Christina Strobl: Data curation, Writing – review & editing.

Inga Usher: Data curation, Writing – review & editing.

Craig Gerrand: Data curation, Writing – review & editing.

Sandra J Strauss: Data curation, Writing – review & editing.

Daniel Schrimpf: Data curation, Writing – review & editing.

Andreas von Deimling: Data curation, Writing – review & editing.

Stephan Beck: Conceptualization, Funding acquisition, Roles/Writing – original draft, Writing – review & editing.

Adrienne M Flanagan: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Roles/Writing – original draft, Writing – review & editing.

Statement of author contribution

Conceptualization: IL, SB, AMF; Sample curation: AMF; Data curation: IL, NK, AMR, CD, ACS, IU, DS, AVD; Analysis: IL, NK, SDN; Clinical data: AMF, SDN, CG, SJS; Supervision: SB, AMF; Writing: IL, SB, AMF, SDN; Review and editing: All authors.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Funding for this project was received from The Tom Prince Cancer Trust, The Rosetrees and Stoneygate Trusts (M46-F1) and the Bone Cancer Research Trust (BRCT: 6519). IL is supported by the Lundbeck Foundation (R303-2018-3018) and previously by The Tom Prince Cancer Trust. NK was funded by Imperial College London. SB acknowledges support from the Wellcome Trust (WT-218274/Z/19/Z). The study was also supported by the National Institute for Health Research, UCL Genomics and Genome Engineering, UCL Great Ormond Street Institute of Child Health and Pathology Core facilities, the Cancer Research UK University College London Experimental Cancer Medicine Centre, and the RNOH Research and Development Department. AMR and CD were supported by The Tom Prince Cancer Trust. SDN is supported by The Jean Shanks Foundation and The Pathological Society of Great Britain and Ireland. SJS was supported by the National Institute for Health Research, the University College London Hospitals Biomedical Research Centre.

The authors thank Dr Sarah Ø. Jensen and Dr Jacob H. Fredsøe, Aarhus University in Denmark, for inputs to the biomarker discovery, Professor Gert Attard and Dr Anjui Wu for a valuable discussion of the experimental design as well as Dr Robert Lowe for sharing data from the Marmal-Aid database. The results generated in this article are partly based upon data generated by the TCGA research Network (http://cancergenome.nih.gov), and the Marmal-Aid database. The results published here are also in part based upon data generated by the Therapeutically Applicable Research to Generate Effective Treatments (https://ocg.cancer.gov/programs/target) initiative, phs000218. The data used for this analysis are available at https://portal.gdc.cancer.gov/projects. We acknowledge the RNOH Biobank Team and the RNOH Research and Development Office and all healthcare workers who cared for the patients. We give a special thanks to the patients for their donating invaluable samples for research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2022.03.002.

References

[1] Misaghi A, Goldin A, Awad M, Kulidjian AA. Osteosarcoma: a comprehensive review. SICOT-J 2018. https://doi.org/10.1051/sicotj/2017028.

[2] Gerrand C, et al. UK guidelines for the management of bone sarcomas. Clin Sarcoma Res 2016;6:7.

[3] Durfee RA, Mohammed M, Luu HH. Review of osteosarcoma and current management. Rheumatol. Ther. 2016. https://doi.org/10.1007/s40744-016-0046-y.

[4] Kager L, et al. Primary metastatic osteosarcoma: presentation and outcome of patients treated on neoadjuvant Cooperative Osteosarcoma Study Group protocols. J Clin Oncol 2003. https://doi.org/10.1200/JCO.2003.08.132.

[5] Kilgour E, Rothwell DG, Brady G, Dive C. Liquid biopsy-based biomarkers of treatment response and resistance. Canc Cell 2020. https://doi.org/10.1016/j.ccell.2020.03.012.
[6] Bronkhorst AJ, Ungerer V, Holdenrieder S. The emerging role of cell-free DNA as a molecular marker for cancer management. Biomolecular Detection and Quantification 2019. https://doi.org/10.1016/j.bdq.2019.100087.
[7] Morris VK, Strickler JH. Use of circulating cell-free DNA to guide precision medicine in patients with colorectal cancer. Annu Rev Med 2021. https://doi.org/10.1146/annurev-med-070119-120448.
[8] Cescon DW, Bratman SV, Chan SM, Siu LL. Circulating tumor DNA and liquid biopsy in oncology. Nat Can 2020. https://doi.org/10.1038/s43018-020-0043-5.
[9] McBride DJ, et al. Use of cancer-specific genomic rearrangements to quantify disease burden in plasma from patients with solid tumors. Gene Chromosome Canc 2010. https://doi.org/10.1002/gcc.20815.
[10] Barris DM, et al. Detection of circulating tumor DNA in patients with osteosarcoma. Oncotarget 2018. https://doi.org/10.18632/oncotarget.24268.
[11] Shulman DS, et al. Detection of circulating tumour DNA is associated with inferior outcomes in Ewing sarcoma and osteosarcoma: a report from the Children's Oncology Group. Br J Canc 2018. https://doi.org/10.1038/s41416-018-0212-9.
[12] Klega K, et al. Detection of somatic structural variants enables quantification and characterization of circulating tumor DNA in children with solid tumors. JCO Precis. Oncol. 2018. https://doi.org/10.1200/po.17.00285.
[13] Llobat L, Gourbault O. Role of micrornas in human osteosarcoma: future perspectives. Biomedicines 2021. https://doi.org/10.3390/biomedicines9050463.
[14] Suzuki MM, Bird A. DNA methylation landscapes: provocative insights from epigenomics. Nat Rev Genet 2008. https://doi.org/10.1038/nrg2341.
[15] Baylin SB, Jones PA. A decade of exploring the cancer epigenome-biological and translational implications. Nat Rev Cancer 2011. https://doi.org/10.1038/nrc3130.
[16] Koch A, et al. Analysis of DNA methylation in cancer: location revisited. Nat Rev Clin Oncol 2018. https://doi.org/10.1038/s41571-018-0004-4.
[17] Chen XG, Ma L, Xu JX. Abnormal DNA methylation may contribute to the progression of osteosarcoma. Mol Med Rep 2018. https://doi.org/10.3892/mmr.2017.7869.
[18] Tian W, Li Y, Zhang J, Li J, Gao J. Combined analysis of DNA methylation and gene expression profiles of osteosarcoma identified several prognosis signatures. Gene 2018. https://doi.org/10.1016/j.gene.2018.01.093.
[19] Rosenblum JM, et al. Predictive properties of DNA methylation patterns in primary tumor samples for osteosarcoma relapse status. Epigenetics 2015. https://doi.org/10.4161/15592294.2014.989084.
[20] Barenboim M, et al. DNA methylation-based classifier and gene expression signatures detect BRCAness in osteosarcoma. PLoS Comput Biol 2021. https://doi.org/10.1371/journal.pcbi.1009562.
[21] Behjati S, et al. Recurrent mutation of IGF signalling genes and distinct patterns of genomic rearrangement in osteosarcoma. Nat Commun 2017. https://doi.org/10.1038/ncomms15936.
[22] Wu CC, et al. Immuno-genomic landscape of osteosarcoma. Nat Commun 2020. https://doi.org/10.1038/s41467-020-14646-w.
[23] Bousquet M, et al. Whole-exome sequencing in osteosarcoma reveals important heterogeneity of genetic alterations. Ann Oncol 2016. https://doi.org/10.1093/annonc/mdw009.
[24] Klutstein M, Nejman D, Greenfield R, Cedar H. DNA methylation in cancer and aging. Canc Res 2016. https://doi.org/10.1158/0008-5472.CAN-15-3278.
[25] Gai W, Sun K. Epigenetic biomarkers in cell-free DNA and applications in liquid biopsy. Genes (Basel) 2019. https://doi.org/10.3390/genes10010032.
[26] Tissot C, et al. Circulating free DNA concentration is an independent prognostic biomarker in lung cancer. Eur Respir J 2015. https://doi.org/10.1183/13993003.00676-2015.
[27] Spindler KLG, Pallisgaard N, Vogelius I, Jakobsen A. Quantitative cell-free DNA, KRAS, and BRAF mutations in plasma from patients with metastatic colorectal cancer during treatment with cetuximab and irinotecan. Clin Canc Res 2012. https://doi.org/10.1158/1078-0432.CCR-11-0564.
[28] Lyskjær I, et al. Correlation between early dynamics in circulating tumour DNA and outcome from FOLFIRI treatment in metastatic colorectal cancer. Sci Rep 2019;9.
[29] Lyskjær I, De Noon S, Tirabosco R. DNA methylation-based profiling of bone and soft tissue tumours: a validation study of the 'DKFZ Sarcoma Classifier. J. Pathol. Clin. Res. 2021. https://doi.org/10.1002/cjp2.215.
[30] Colaprico A, et al. TCGAbiolinks: an R/Bioconductor package for integrative analysis of TCGA data. Nucleic Acids Res 2016. https://doi.org/10.1093/nar/gkv1507.
[31] Lowe R, Rakyan VK. Marmal-aid–a database for infinium HumanMethylation450. BMC Bioinf 2013. https://doi.org/10.1186/1471-2105-14-359.
[32] Koelsche C, et al. Sarcoma classification by DNA methylation profiling. Nat Commun 2021;12.
[33] Barrett T, et al. NCBI GEO: archive for functional genomics data sets - Update. Nucleic Acids Res 2013. https://doi.org/10.1093/nar/gks1193.
[34] Moriarity BS, et al. A Sleeping Beauty forward genetic screen identifies new genes and pathways driving osteosarcoma development and metastasis. Nat Genet 2015. https://doi.org/10.1038/ng.3293.
[35] Johansson A, Enroth S, Gyllensten U. Continuous aging of the human DNA methylome throughout the human lifespan. PLoS One 2013. https://doi.org/10.1371/journal.pone.0067378.
[36] Warden CD, et al. COHCAP: an integrative genomic pipeline for single-nucleotide resolution DNA methylation analysis. Nucleic Acids Res 2013. https://doi.org/10.1093/nar/gkt242.
[37] Untergasser A, et al. Primer3-new capabilities and interfaces. Nucleic Acids Res 2012. https://doi.org/10.1093/nar/gks596.
[38] Li LC, Dahiya R MethPrimer. Designing primers for methylation PCRs. Bioinformatics 2002. https://doi.org/10.1093/bioinformatics/18.11.1427.
[39] Arányi T, Tusnády GE. BiSearch: ePCR tool for native or bisulfite-treated genomic template. Methods Mol Biol 2007. https://doi.org/10.1385/1-59745-528-8:385.
[40] Robin X, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinf 2011. https://doi.org/10.1186/1471-2105-12-77.
[41] Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. Clin Biochem Rev 2008;29:49–52.
[42] R Core Team. R. A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2017. http://www.R-project.org/.
[43] Tomczak K, Czerwińska P, Wiznerowicz M. The Cancer Genome Atlas (TCGA): an immeasurable source of knowledge. Wspolczesna Onkologia; 2015. https://doi.org/10.5114/wo.2014.47136.
[44] Salah S, Ahmad R, Sultan I, Yaser S, Shehadeh A. Osteosarcoma with metastasis at initial diagnosis: current outcomes and

prognostic factors in the context of a comprehensive cancer center. Mol Clin Oncol 2014. https://doi.org/10.3892/mco.2014.325.

[45] Iacovelli R, Lanoy E, Albiges L, Escudier B. Tumour burden is an independent prognostic factor in metastatic renal cell carcinoma. BJU Int 2012. https://doi.org/10.1111/j.1464-410X.2012.11518.x.

[47] Bramer JAM, van Linge JH, Grimer RJ, Scholten RJPM. Prognostic factors in localized extremity osteosarcoma: a systematic review. Eur J Surg Oncol 2009. https://doi.org/10.1016/j.ejso.2009.01.011.

[48] Xin S, Wei G. Prognostic factors in osteosarcoma: a study level meta-analysis and systematic review of current practice. J. Bone Oncol. 2020. https://doi.org/10.1016/j.jbo.2020.100281.

[49] Keller L, Belloum Y, Wikman H, Pantel K. Clinical relevance of blood-based ctDNA analysis: mutation detection and beyond. Br J Canc 2021. https://doi.org/10.1038/s41416-020-01047-5.

[50] Zhang Y, et al. Pan-cancer circulating tumor DNA detection in over 10,000 Chinese patients. Nat Commun 2021. https://doi.org/10.1038/s41467-020-20162-8.

[51] G R, et al. UK guidelines for the management of bone sarcomas. Sarcoma; 2010. https://doi.org/10.1155/2010/317462.

[52] ICONIC. Improving outcomes through collaboration in OsteosarComa (ICONIC). Available at: https://clinicaltrials.gov/ct2/show/NCT04132895.