Enhanced detection of minimal residual disease by targeted sequencing of phased variants in circulating tumor DNA

David M. Kurtz, Joanne Soo, Lyron Co Ting Keh, Stefan Alig, Jacob J. Chabon, Brian J. Sworder, Andre Schultz, Michael C. Jin, Florian Scherer, Andrea Garofalo, Charles W. Macaulay, Emily G. Hamilton, Binbin Chen, Mari Olsen, Joseph G. Schroers-Martin, Alexander F. M. Craig, Everett J. Moding, Mohammad S. Esfahani, Chih Long Liu, Ulrich Dührsen, Andreas Hüttmann, René-Olivier Casasnovas, Jason R. Westin, Mark Roschewski, Wyndham H. Wilson, Gianluca Gaidano, Andrea Garofalo, Joanne Soo, Stefan Alig, and Ash A. Alizadeh

Circulating tumor-derived DNA (ctDNA) is an emerging biomarker for many cancers, but the limited sensitivity of current detection methods reduces its utility for diagnosing minimal residual disease. Here we describe phased variant enrichment and detection sequencing (PhasED-seq), a method that uses multiple somatic mutations in individual DNA fragments to improve the sensitivity of ctDNA detection. Leveraging whole-genome sequences from 2,538 tumors, we identify phased variants and their associations with mutational signatures. We show that even without molecular barcodes, the limits of detection of PhasED-seq outperform prior methods, including duplex barcoding, allowing ctDNA detection in the ppm range in participants. We profiled 678 specimens from 213 participants with B cell lymphomas, including serial cell-free DNA samples before and during therapy for diffuse large B cell lymphoma. In participants with undetectable ctDNA after two cycles of therapy using a next-generation sequencing-based approach termed cancer personalized profiling by deep sequencing, an additional 25% have ctDNA detectable by PhasED-seq and have worse outcomes. Finally, we demonstrate the application of PhasED-seq to solid tumors.

Analysis of somatic mutations from circulating cell-free DNA (ctDNA) has broad applications for detecting and characterizing cancer. Detection of ctDNA has been shown in principle in most cancer types. However, in practice, the current limits of detection (LOD) for ctDNA analysis are imperfect and are inherently restricted by two key factors: low-input DNA amounts from a typical blood collection and background error rates of current sequencing technologies.

Recent approaches have improved ctDNA minimal residual disease (MRD) performance by tracking multiple somatic mutations with error-suppressed sequencing. This approach has allowed detection limits as low as 2–10 parts in 100,000 from limited ctDNA using off-the-shelf panels or personalized assays. Detection of residual disease during or after treatment is a powerful prognostic tool, with detectable MRD representing an adverse sign even during radiographic remission. However, current detection limits are insufficient to universally detect residual disease in patients destined for disease relapse. This ‘loss of detection’ is exemplified in diffuse large B cell lymphoma (DLBCL). Here a threshold-response in ctDNA after only two cycles of curative-intent therapy is strongly prognostic for favorable clinical outcomes (major molecular response (MMR)). Despite this, nearly one-third of patients experiencing eventual disease progression do not have detectable ctDNA at this interim landmark when using either immunoglobulin or targeted sequencing, thus representing ‘false-negative’ measurements. Such high false-negative rates have also been observed using alternative methodologies to track disease in colon and breast cancers. More sensitive methods are therefore needed to improve residual disease detection across cancers.

Prior methods at lowering the LOD have focused on somatic variants detected on both of the complementary strands of parental DNA duplexes (Extended Data Fig. 1a). ‘Duplex
sequencing' reduces the background error rate due to the requirement of two concordant events for detection of a single-nucleotide variant (SNV). In previous studies using duplex sequencing for ctDNA analysis, an analytical LOD as low as 1 molecule in 400,000 was achievable. However, this approach is limited by inefficient recovery of DNA duplexes—recovery of both original strands occurs in a minority (typically 20–25%) of all recovered molecules. This inefficiency makes duplex sequencing suboptimal for real-world ctDNA detection, where input DNA from practical blood volumes is limited (~4,000–8,000 genomes per standard 10-ml blood collection tube) and maximal recovery of genomes is essential. To improve MRD detection, methods that simultaneously achieve low analytical detection limits and high molecular recovery for multiple mutations are still needed (Extended Data Fig. 1b).

Here we introduce an alternative to duplex sequencing for reducing the background error rate that involves detection of 'phased variants' (PVs), where two or more mutations occur in cis (that is, on the same strand of DNA; Fig. 1a and Extended Data Fig. 1a). Similarly
to duplex sequencing, this method provides lower error profiles due to the concordant detection of two separate non-reference events in individual molecules. However, unlike duplex sequencing, both events occur on the same sequencing read pair, thereby increasing the efficiency of genome recovery. We find that phased mutations are present in diverse cancer types, but occur in stereotyped portions of the genome in B cell malignancies, likely due to physiological and aberrant somatic hypermutation (aSHM)\(^2\). We therefore develop and apply PhasEd-seq, a new method to detect ctDNA through PVs to tumor fractions on the order of ppm. We demonstrate that PhasEd-seq can meaningfully improve detection of ctDNA in clinical samples both during therapy and before disease relapse. Finally, we apply PhasEd-seq to participants with solid tumors through personalized identification of PVs, demonstrating the broad applicability of PhasED-seq for improved MRD detection.

**Results**

**Genomic distribution of phased variants.** To identify malignancies where PVs could potentially improve disease detection, we assessed the frequency of PVs across cancer types. We analyzed publicly available whole-genome sequencing (WGS) data to identify sets of variants occurring at a distance of <170bp apart, which represents the typical length of a single cDNA fragment\(^1\) consisting of a single core nucleosome and associated linker. We identified and summarized the frequency of these putative phased variants, (Methods) controlling for the total number of SNVs, from 2,538 tumors across 24 cancer histologies including solid tumors and hematological malignancies (Fig. 1b, Extended Data Fig. 2 and Supplementary Table 1). Interestingly, PVs were most significantly enriched in two B cell lymphomas (DLBCL and follicular lymphoma (FL); \(P<0.05\) versus all other histologies), a group of diseases with known hypermutation driven by AID (AICDA).

**Mutational mechanisms underlying phased variants.** To investigate the origin of PVs, we compared the single-base substitution (SBS) mutational signatures\(^3\) contributing to SNVs occurring within 170bp of another SNV, and SNVs occurring in isolation (Methods). As expected, PVs were highly enriched in several mutational signatures associated with clustered mutations\(^4,5\). Signatures of clustered mutations associated with activity of AID (SBS84 and SBS85) were significantly enriched in PVs from B cell lymphomas and chronic lymphocytic leukemia (CLL), while signatures associated with activity of APOBEC3B (SBS2 and SBS13)—another known mechanism of kataegis hypermutation—were significantly enriched in PVs from multiple solid cancer histologies (Fig. 1c)\(^3,6\). Interestingly, PVs from multiple tumor types were also associated with SBS4, a signature associated with tobacco use. In contrast, aging-associated mutational signatures such as SBS1 and SBS3 were significantly enriched in isolated SNVs\(^7\).

**Phased variants occur in stereotyped genomic regions in lymphoid cancers.** To assess the genomic distribution of putative PVs, we binned these events into 1-kb regions across the genome. We observed a strikingly stereotyped distribution of PVs in individual lymphoid neoplasms (that is, DLBCL, FL, Burkitt lymphoma (BL) and CLL; Fig. 1d and Extended Data Fig. 3). In contrast, nonlymphoid cancers did not exhibit substantial recurrence of PVs in stereotyped regions. This lack of stereotype in PV-containing loci was true even when considering melanomas and lung cancers, diseases with frequent PVs.

Across lymphomas, the majority of hypermutated regions were shared between lymphoma subtypes, with the highest densities in known targets of SHM\(^8,9\), including BCL2, BCL6 and MYC, as well as the immunoglobulin loci IGH, IGK and IGL (Supplementary Table 2), with DLBCL harboring the most recurrently affected regions (Supplementary Fig. 1a). Among 1,639 1-kb regions recurrently containing PVs, nearly one-third fell into genomic areas previously associated with physiological or aberrant SHM in B cells. Specifically, 19% (315/1,639) were located in immunoglobulin regions, while 13% (218/1,639) were in portions of 68 previously identified targets of aSHM (Supplementary Table 2). While most PVs fell into noncoding regions of the genome, we also identified additional recurrently affected loci not previously described as targets of aSHM, including XBP1, LPP and AICDA, among others.

Interestingly, the distribution of PVs within each lymphoma correlated with known oncogenic features associated with the corresponding disease, including PVs in BCL2 in FL\(^9\) and PVs in MYC and ID3 in BL\(^9\) (Fig. 1d and Supplementary Fig. 1b–d). DLBCL molecular subtypes associated with distinct cell of origin\(^10\) also demonstrated distinct distributions of PVs (Supplementary Table 2), including significant enrichments in PVs in BCL2 in germinal center B cell-like (GCB) DLBCLs and IGH class-switch regions
(Sy1 and Sy3) in activated B cell-like (ABC) DLBCLs, consistent with previous reports\(^3\) (Supplementary Fig. 1e).

**Design and validation of PhasEd-seq panel for lymphoma.** To validate these PV-rich regions and assess their utility for disease detection from ctDNA, we designed a sequencing panel targeting putative PVs identified within WGS from three independent cohorts of individuals with DLBCL, as well as in individuals with CLL\(^{13-36}\) (Fig. 2a and Methods). This final PhasEd-seq panel targeted \~115 kb of genomic space focused on PVs, along with an additional \~200 kb targeting genes known to be recurrently mutated in B cell non-Hodgkin lymphomas (B-NHLs); this single panel was used for both identification of PVs from tumor and/or plasma samples and tracking residual disease (Fig. 2b). While the 115 kb of space dedicated to PV capture targets only 0.0035% of the human genome, it captures 26% of PVs observed by WGS (Extended Data Fig. 4a), yielding a \~7,500-fold enrichment over WGS.

**Recovery of predicted phased SNVs by PhasEd-seq** (Fig. 4a), yielding a \~7,500-fold enrichment over WGS. genome, it captures 26% of PVs observed by WGS (Extended Data space dedicated to PV capture targets only 0.0035% of the human samples and tracking residual disease (Fig. 2b). While the 115 kb of used for both identification of PVs from tumor and/or plasma samples and tracking residual disease (Fig. 2b). While the 115 kb of space dedicated to PV capture targets only 0.0035% of the human genome, it captures 26% of PVs observed by WGS (Extended Data Fig. 4a), yielding a \~7,500-fold enrichment over WGS.

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We next compared expected SNV and PV recovery to a prior panel, PhasEd-seq covered 3 times more SNVs and 2.9 times more PVs in available WGS data\(^{33-35}\). To validate these yield improvements, we profiled 16 pretreatment tumor or plasma DNA samples from participants with DLBCL (Supplementary Table 3). We applied both panels to each specimen and sequenced to high unique molecular depths (Fig. 3a and Extended Data Fig. 4d,e). Compared to the expected enrichment, we observed similar improvements in yield of SNVs by PhasEd-seq compared to the prior panel (2.7 times; median 304.5 versus 114). However, we found a larger improvement in the number of PVs recovered by PhasEd-seq (5.8 times; median 2,461 versus 423 PVs per participant; Fig. 3a). We also observed a robust correlation between the frequency of PVs in WGS and targeted data across 101 DLBCL samples (Fig. 3b).

**Validation and application of phased variant enrichment sequencing.** a. Comparison of SNV (left) and PV (right) yield for sequencing tumor and/or cfDNA and matched germ line by a previously established lymphoma CAPP-seq panel or PhasEd-seq (two-sided Wilcoxon signed-rank test). PVs included doublet, triplet and quadruplet phased events. b. Scatterplot showing the frequency and Pearson correlation of PVs in 1,000-bp bins for participants with DLBCL, identified by either WGS or PhasEd-seq. c. Scatterplots comparing the frequency of PVs by location (in 50-bp bins) for subtypes of lymphoma. Statistical analysis was performed by two-sided Wilcoxon rank-sum test of all 50-bp bins in a given gene against all other bins (Methods). See https://phasedseq.stanford.edu/ for additional details. d. Volcano plots summarizing the difference in relative frequency of PVs in specific loci between types of lymphoma, including ABC-DLBCL versus GCB-DLBCL (red, left); PMBCL versus DLBCL (blue, middle); and HL versus DLBCL (green, right; Methods). NS, not significant.

**Differences in phased variants between lymphoma subtypes.** Having validated the PhasEd-seq panel, we sought to assess the biological differences in PVs between various B cell malignancies, including DLBCL (\(n=101\)), primary mediastinal B cell lymphoma (PMBCL; \(n=16\)), FL (\(n=13\)), classical Hodgkin lymphoma (CLL; \(n=13\); Supplementary Table 4). We evaluated the quantity of SNVs and PVs in each of these lymphomas, as well as the number of ‘independent tumor reporters’ for possible disease monitoring from ctDNA, defined as PVs separated into 150-bp islands across our panel (Extended Data Fig. 5f–j and Methods). The number of SNVs identified per sample was not significantly different between lymphoma subtypes, with the exception of fewer SNVs in MCL. However, DLBCL, PMBCL and FL had a higher burden of PVs than classical Hodgkin lymphoma or...
DNA mismatches, and AID hotspots can contain a 5–10% or higher level of DNA damage leading to frequent PVs in lymphoma subtypes (Fig. 3c,d and Supplementary Fig. 2), including frequent PVs in BCL2 in GCB-DLBCL, PIM1 PVs in ABC-DLBCL and CIITA PVs in PMBCL. Additional loci enriched in specific lymphoma types are provided in Supplementary Table 5.

Recovery of phased variants through PhasED-seq. To facilitate detection of ctDNA using PVs, efficient recovery of DNA molecules is required. Hybrid-capture sequencing is potentially sensitive to DNA mismatches, and AID hotspots can contain a 5–10% or higher local mutation rate. Using in silico simulation of 150-nmers, we observed the expected decrease in predicted binding energy with an increasing number of mutations (Extended Data Fig. 5a). We assessed the effect on hybrid-capture sequencing efficiency using synthetic 150-bp DNA oligonucleotides with 0 to 10% difference from the reference sequence in MYC and BCL6, known targets of aSHM (Supplementary Table 6 and Methods). When subjecting an equimolar mixture of these oligonucleotides to capture and sequencing, molecules with as high as a 5% mutation rate were captured with nearly the same efficiency as their wild-type counterparts (85% versus 100%), while molecules with a 10% mutation rate were captured with only 27% relative efficiency (Fig. 4a). Notably, only 7% of cases had any region exceeding 10% mutation frequency across the panel (Methods and Extended Data Fig. 5b,c), and in all cases, the 90th percentile mutation rate was <5%, suggesting the majority of phased mutations are recoverable by hybrid capture.

Background profile and limit of detection of PhasED-seq. Previous methods for error-suppressed sequencing of ctDNA have used either a combination of molecular and in silico methods for error suppression, for example, integrated digital error suppression (iDES) or duplex sequencing to reduce background signal. However, each of these methods has limitations, either for detecting ultra-low tumor fractions or for efficient recovery of original DNA molecules. We compared the background profile and recovery of input genomes from plasma ctDNA samples from 12 healthy adults by PhasED-seq with both iDES-enhanced CAPP-seq and duplex sequencing. As expected, while iDES-enhanced CAPP-seq had a lower background profile than barcode deduplication alone, duplex sequencing offered even lower background signal for non-reference single-nucleotide substitutions (Fig. 4b; 3.3 × 10^-8 versus 1.2 × 10^-5, P < 0.0001). However, the rate of phased errors—that is, multiple non-reference bases occurring on the same sequencing fragment—was significantly lower than the rate of single errors in either iDES-enhanced CAPP-seq or duplex sequencing data. This was true for the incidence of both two (2x) or ‘doublet’ PVs and three (3x or ‘triplet’ PVs) substitutions on the same DNA molecule (Fig. 4b; 8.0 × 10^-12 and 3.4 × 10^-9, respectively, P < 0.0001), even without the use of barcode-mediated error suppression (Extended Data Fig. 5d). The background rate of PVs was also lower than that of small insertions and deletions (Extended Data Fig. 5d). Phased errors containing C-to-T or T-to-C transitions were more common than other types of PVs (Extended Data Fig. 5e). The rate of background PVs also decreased with increasing distance between positions (Extended Data Fig. 5f). When considering unique molecular depth, duplex sequencing recovered only 19% of all unique ctDNA fragments (Fig. 4c). In contrast, the unique depth of reads covering PVs within a genomic distance of <20 bp was nearly identical to the overall sample depth. Similarly, PVs of up to 80 bp in size had a depth greater than 50% of the median unique depth for a sample. Importantly, almost half (48%) of all PVs were less than 80 bp in length (Fig. 4d).

To compare PhasED-seq to alternative methods for ctDNA detection, we generated limiting dilutions of ctDNA from three participants with lymphoma into healthy control ctDNA, resulting in expected tumor fractions between 0.1% and 0.00005% (1:2,000,000; Methods and Supplementary Table 7). We compared the expected and observed tumor fractions in each of these dilutions using PhasED-seq and SNV-based methods (that is, CAPP-seq or duplex sequencing; Fig. 5a). All methods performed equally well down to tumor fractions of 0.01% (1 part in 10,000); however, below this level (for example, 0.001%, 0.0002%, 0.00001% and 0.000005%), both PhasED-seq and duplex sequencing significantly outperformed single-strand unique molecular identifier (UMI)-based SNV detection (P < 0.0001 for duplex, ‘2x’ PhasED-seq and ‘3x’ PhasED-seq; Fig. 5a). In addition, when compared to detection of SNVs from duplex molecules, tracking either two or three variants in-phase...
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CAPP-seq versus duplex, $P < 0.05$; total samples). In each sample, we assessed for tumor-specific SNVs or PVs from three participant samples used in the limiting dilution experiment, for a $n = 13$ samples. The difference between observed and expected tumor fractions for sample $< 0.0016$. B. Plot demonstrating the background signal of tumor-specific alleles in 12 unrelated control cfDNA samples, and the control cfDNA sample used for limiting dilution series ($n = 13$ total samples). In each sample, we assessed for tumor-specific SNVs or PVs from three participant samples used in the limiting dilution experiment, for a total of 39 assessments. Bars represent the mean across all 39 assessments; statistical comparison was performed by Wilcoxon rank-sum test. $P < 0.05$; CAPP-seq versus duplex, $P = 3.7 \times 10^{-5}$; CAPP-seq versus PhasED-seq (2×), $P = 4.4 \times 10^{-4}$; CAPP-seq versus PhasED-seq (3×), $P = 2.9 \times 10^{-4}$; duplex versus PhasED-seq (2×), $P = 9.0 \times 10^{-4}$; duplex versus PhasED-seq (3×), $P = 3.1 \times 10^{-4}$.

(that is, 2× or 3× PhasED-seq) more accurately identified expected tumor content, with superior linearity down to 1 part in 2,000,000 ($P = 0.005$ for duplex versus 2× PhasED-seq, $P = 0.002$ for 3× PhasED-seq; Methods). This was also true even without the use of molecular barcodes (Extended Data Fig. S5). We also assessed specificity of PVs by looking for evidence of tumor-derived SNVs or PVs in cfDNA samples from 12 unrelated healthy controls and the healthy control used for our limiting dilution. Here PhasED-seq showed the lowest background signal across methods (Fig. 5b). This lower error rate and background from PVs improves the detection limit for cfDNA disease detection. The detection limit could be accurately modeled as a binomial sampling process down to the ppm range, with increased detection from increasing number of independent reporters (Extended Data Fig. 5h–j and Methods).

Improvements in detection of low-burden minimal residual disease. To test the utility of the lower detection limits from PhasED-seq, we sequenced serial cfDNA from a participant undergoing front-line therapy for DLBCL (Fig. 6a). Using SNV-based methods (that is, CAPP-seq), ctDNA was undetectable after only one cycle of therapy. Subsequently, detectable ctDNA re-emerged $> 250$ d later, with eventual clinical and radiographic progression 5 months later, indicating falsely negative measurements with SNVs. Strikingly, all four of the plasma samples that were undetectable using SNVs during and after treatment had detectable ctDNA levels by PhasED-seq, with mean allelic fractions as low as 4 in 1,000,000. This increased sensitivity improved the lead time of disease detection by ctDNA compared to radiographic surveillance from 5 to 10 months with PhasED-seq.

We next assessed the performance of PhasED-seq in a cohort of 107 participants with large B cell lymphomas receiving standard immuno-chemotherapy (Supplementary Table 8). In total, we assessed 442 tumor, germline and cfDNA samples, including cfDNA before therapy ($n = 107$) and after one or two cycles of treatment ($n = 82$ or 88). Before therapy, participant-specific PVs were detectable by PhasED-seq in 98% of samples, with 97% specificity (Extended Data Fig. 6a). Importantly, ctDNA levels measured by PhasED-seq were highly correlated with those measured by prior SNV-based methods (Spearman rho $= 0.93$; Extended Data Fig. 6b). We next compared ctDNA levels measured by PhasED-seq and SNV-based methods from samples after initiation of therapy. In total, 79% (77/98) of samples with detectable ctDNA by PhasED-seq after one or two cycles were also detected by SNV-based methods (Fig. 6b). Among 98 samples detected by PhasED-seq, disease burden was significantly lower for those with undetectable (21%) versus detectable (79%) ctDNA levels using SNVs, with a $> 10$-fold difference in median ctDNA levels (tumor fraction $2.9 \times 10^{-4}$ versus $1.5 \times 10^{-4}$, $P < 0.001$; Fig. 6b). In total, an additional 10% (8/82) of samples after one cycle of therapy and 15% (13/88) of samples after two cycles of therapy had detectable ctDNA when comparing PhasED-seq with SNV-based CAPP-seq (Fig. 6c).

We previously described ctDNA molecular response criteria for DLBCL participants using SNV-based CAPP-seq, including MMR, defined as a 2.5-log reduction in ctDNA after two cycles of therapy. However, many participants had undetectable ctDNA when measured by SNVs at this landmark (Fig. 6d,e). Indeed, in the 88 participants with a sample available from this time point, 59% (52/88) had undetectable ctDNA by SNV-based CAPP-seq at their interim MMR assessment, after completing two of six planned cycles of therapy. Using PhasED-seq, 25% (13/52) of samples not detected by SNVs had evidence of ctDNA as confirmed by PVs, with levels as low as $\sim 3 \times 10^{-4}$ (Extended Data Fig. 7)—these 13 samples additionally detected by PhasED-seq represent potential false-negative tests by prior SNV-based methods. Similar results were seen at the early molecular response time point (that is, after one cycle of therapy; Extended Data Fig. 8).
While detection of ctDNA in DLBCL after one or two cycles of therapy is a known adverse prognostic marker, outcomes for participants with undetectable ctDNA at these time points are heterogeneous (Fig. 6e and Extended Data Fig. 8e). Importantly, even in participants with undetectable ctDNA by SNVs after one or two cycles of therapy, detection of ultra-low ctDNA levels by PhasED-seq was strongly prognostic for outcomes including event-free survival (EFS; Fig. 6f and Extended Data Fig. 8f). When combining detection by PhasED-seq with our previously described MMR threshold, participants could be stratified into three groups: (1) participants not achieving MMR, (2) participants achieving MMR but with persistent ctDNA and (3) participants with undetectable ctDNA (Fig. 6g). Interestingly, while participants not achieving MMR were at especially high risk for early events despite additional planned first-line therapy (that is, within the first year of treatment), participants with persistent low levels of ctDNA appeared to have a higher risk of later relapse or progression events. In contrast, participants with undetectable ctDNA after two cycles of therapy by PhasED-seq...
had overwhelmingly favorable outcomes. Similar results were seen at the early molecular response time point after one cycle of therapy (Extended Data Fig. 8g).

Given its enhanced sensitivity for detection of residual disease during therapy, we sought to explore disease detection via PhasED-seq at the time point of lowest disease burden, that is, at end of therapy (EOT). We assessed SNV-based CAPP-seq and PhasED-seq ctDNA detection in a cohort of 19 participants after therapy, of whom 5 had eventual disease progression. While only 2/5 participants who had eventual progression at this landmark had detectable disease using SNVs, PhasED-seq detected all 5/5 participants, resulting in improved stratification of outcomes (Fig. 6h). PhasED-seq also correctly identified all participants without clinical relapse as having no residual disease, including one participant with abbreviated therapy after only one cycle of treatment (Extended Data Fig. 9). Taken together, this suggests the performance of PV-based PhasED-seq outperformed SNV-based detection for residual disease, particularly during and after therapy (Fig. 6i).

Extension of PhasED-seq to solid tumors. While PVs are concentrated in stereotyped genomic loci B cell malignancies, this is not the case in most other cancer types (Extended Data Fig. 3). Despite this, most cancer types considered in the Pan-Cancer Analysis of Whole Genomes (PCAWG) dataset have a sufficient number of PVs in their genome to improve on the limit of detection for ctDNA from SNV-based or alternative approaches using a typical sequencing depth (Fig. 7a, Extended Data Fig. 10a–c and Methods). However, these PVs must first be identified and targeted on an individualized basis. To extend PhasED-seq beyond B cell cancers, a ‘personalized’ approach is therefore needed, including (1) up-front WGS from tumor–normal pairs to identify candidate PVs, (2) design of a personalized panel to select loci containing PVs and (3) targeted sequencing of ctDNA in these regions to monitor the previously defined PVs (Fig. 7b). To explore the potential utility of this approach, we performed personalized PhasED-seq for six participants with solid tumors (lung cancer, n = 5; breast cancer, n = 1). In all six cases, multiple PVs were identified from paired tumor–normal WGS data and subsequently validated through targeted resequencing (median 118 validated PVs, range 14–622). Similarly to our lymphoma PhasED-seq panel, the background rate of PVs was lower than the background rate for SNVs across all six personalized panels, even compared to duplex molecules (Fig. 7c).

We next assessed the performance of this approach for MRD detection in 24 plasma samples from these six patients. In nearly all samples, we recovered a sufficient number of cfDNA fragments evaluable for tumor content to detect tumor fractions at or below the limit of detection for SNV-based methods (Fig. 7c). Using SNV-based methods, ctDNA was detected in 9/24 of the plasma samples. As expected, PhasED-seq also detected ctDNA in these samples (Fig. 7d). Importantly, PhasED-seq also detected ctDNA in 6 additional samples that were negative when assessed by SNVs (Fig. 7d and Extended Data Fig. 10d). These additional samples had very low tumor fractions (median 0.0005%), below the background error rate of SNV-based methods. The lowest measurable tumor fraction was 0.000094%, or less than 1:1,000,000, from a pretreatment sample from a participant with stage 1 non-small-cell lung cancer (LUP649).

We analyzed serial samples from a participant with stage III lung adenocarcinoma treated with chemoradiotherapy (LUP814; Fig. 7e). CAPP-seq and PhasED-seq detected similar ctDNA levels before therapy; however, three samples after treatment initiation had undetectable ctDNA by CAPP-seq before ctDNA re-emerged at the time of biopsy-confirmed recurrent disease. Using PhasED-seq, we observed molecular residual disease in 3/3 (100%) of samples that were undetected by SNVs, with tumor fraction as low as 0.00016%. Furthermore, the trend in ctDNA quantification mirrored the participant’s disease, with an initial response to chemoradiotherapy but disease progression during immunotherapy. Importantly, this participant’s ctDNA remained detectable at all time points, including after completion of chemoradiotherapy 8 months before disease progression. Similar improvements for detection of low-burden disease after treatment were seen in LUP831 (Extended Data Fig. 10e), as well as BRCA001 for disease detection in anticipation of clinical diagnosis (Fig. 7f).

Discussion
The emergence of cfDNA in oncology has signaled a revolution in precision medicine. While ctDNA-based mutational genotyping and MRD detection are gaining adoption in the clinic, current approaches for disease surveillance have suboptimal sensitivity. We present a method to leverage PVs to improve on these limitations. Using WGS data, we identified putative PVs amenable to capture in a single cfDNA fragment (<170 bp) in multiple cancer subtypes. These PVs were associated with established mutational signatures associated with clustered mutations driven by mechanisms of hypermutation such as AID/AICDA and APOBEC3B.

We observed that cancers derived from B lymphocytes contain PVs in stereotyped genomic regions, while other tumor types contain such events throughout their genome. We therefore designed PhasED-seq, a hybrid-capture approach for disease characterization and MRD detection, and applied it to both lymphomas and solid tumors. We demonstrated in technical and biological experiments the superior error profile of PhasED-seq, establishing an improved sensitivity for PhasED-seq over alternative approaches for ctDNA detection, including error-suppressed SNV-based methods and duplex sequencing. This resulted in detection down to below 1 part per 1,000,000 in a limiting dilution series and clinical samples.
The lower background rate of PVs compared with SNVs for tracking known genotypes was expected, as PVs require multiple non-reference bases. We initially expected the background rate of SNVs from duplex sequencing and the rate of PVs to be similar, as both require two independent observations (in trans and cis, respectively). However, we found that the background rate of SNVs in duplex data was higher than that of PVs, even when considering all molecules (that is, single-stranded and double-stranded recovery) or without using molecular barcodes. We hypothesize that this is most likely due to a biological source of background SNVs. While ‘technical’ background from PCR error requires two events to create signal for both PhasED-seq and duplex sequencing, SNV ‘biological’ background—that is, true somatic mutations occurring in non-tumor cells—can be created from a single in vivo mutational event and would not be suppressed by duplex barcoding. In contrast, a PV of biological origin still requires two separate mutational events in the same cell, within <170 bp, to result in PV background signal. Consistent with this, while theoretical estimates of the error rate in duplex sequencing were <1 in 1 × 10^9, the observed rate of background signal from biological samples is ~1:1 × 10^5–1 × 10^6.
(ref. 13), with reported background SNV rates from duplex sequencing of cfDNA also in this range. Indeed, multiple recent reports of error-suppressed sequencing from cfDNA have demonstrated frequent low-level mutations that can be found in paired leukocyte samples, likely representing low-burden clonal hematopoiesis41,42, creating background for duplex sequencing.

To demonstrate the utility of the lower background from PhasEd-seq, we studied samples from participants with DLBCL with undetectable ctDNA assessed by prior methods. We demonstrated improved disease detection by PhasEd-seq, where participants with occult disease only detectable by PhasEd-seq had significantly inferior outcomes to participants without detectable disease. PhasEd-seq also improved on disease detection in DLBCL participants at the EOT landmark. Finally, we extended PhasEd-seq beyond B cell lymphomas. To apply PhasEd-seq to solid cancers that do not have stereotyped locations for PVs, a two-step approach is required, involving up-front tumor WGS to identify PVs, followed by development of a personalized panel to capture PVs. Notably, this type of personalized approach has been successfully applied to ctDNA for SNV-based detection in previous studies32,33,34,35. Applying this to participants with lung or breast cancer, we demonstrated improved disease detection as compared with SNV-based approaches (for example, CAPP-seq), including tumor fractions as low as ~1 in 1,000,000.

We note that this personalized approach could be used for any type of somatic alteration with intrinsically low background error rates, such as fusions or indels, similarly to the implementation here for PVs. Prior approaches using fusions for ctDNA36,37,38 have had limited sensitivity due to use of only a few fusions for each participant. However, recent improvements in genome-wide identification of structural variants39 makes these a potentially attractive alternative for PV-poor cancers. However, most cancer types have a sufficient number of PVs to enable PhasEd-seq.

This technical improvement in the performance of ctDNA detection at the time point of lowest disease burden potentially unlocks several new precision medicine approaches. For example, participants achieving molecular remissions without detectable ctDNA by PhasEd-seq after receiving chemotherapy might benefit from ‘dose de-escalation’ or an abbreviated course of therapy. Participants achieving molecular remissions without detectable ctDNA by prior methods (for example, CAPP-seq), including tumor fractions as low as ~1 in 1,000,000.

While better prognostication of outcomes for individual patients is useful by itself, for improved ctDNA methodologies such as PhasEd-seq to make a significant impact on patient outcomes, changes in treatment paradigms are required. Prospective clinical trials implementing PhasEd-seq to select patients for personalized therapies will therefore be necessary to establish its utility before wide clinical adoption.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgment, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41587-021-00981-w.

Received: 14 March 2020; Accepted: 11 June 2021; Published online: 22 July 2021

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Methods

Participant enrollment and clinical sample collection. All samples analyzed in this study were collected with informed consent from participants enrolled on studies approved by an Institutional Review Board that were in accordance with the Declaration of Helsinki and all relevant ethical regulations at their respective centers—Stanford University, MD Anderson Cancer Center, the National Cancer Institute, University of Eastern Piedmont, Essen University Hospital and CHU Dijon. Participants were not compensated for their contribution in this study.

Individuals with B cell lymphomas undergoing front-line therapy were enrolled on this study from six centers across North America and Europe, including Stanford University, MD Anderson Cancer Center, the National Cancer Institute, University of Eastern Piedmont, Essen University Hospital and CHU Dijon (France). An additional six participants with a diagnosis of solid tumors (lung cancer, n = 5, breast cancer, n = 1) were enrolled at Stanford University. In total, 144 tumor, 109 tumor and 219 germline samples from 219 participants and 76 cfDNA samples from controls were included in this study. Cell-free, tumor and germline DNA were isolated as previously described. All radiographic imaging was performed as part of standard clinical care.

Whole-genome sequencing analysis. Putative phased variant identification from whole-genome sequencing. WGS data were obtained from two sources. Data for lymphoid malignancies (DLBCL, FL, BL and CLL) were downloaded from the International Cancer Genome Consortium (ICGC) data portal on 7 May 2018. Data from all other histologies were part of the PCAWG dataset and were downloaded on 11 November 2019 (ref. 1). Only cancer histologies with at least 35 the International Cancer Genome Consortium (ICGC) data portal on 7 May 2018. Data from all other histologies were part of the PCAWG dataset and were downloaded on 11 November 2019 (ref. 1). Only cancer histologies with at least 35 available cases were considered; details of the dataset considered are provided in Supplementary Table 1. All samples had somatic mutations called from WGS using matched tumor and normal genotyping. Queries were limited to base substitutions obtained from WGS (single-, double-, triple- and oligo-nucleotide variants (SNVs, DNVs, TNVs and ONVs)). Having thus identified the cases and variants of interest, we next identified the number of putative PVs in each tumor. To function as a PV on a single cDNA molecule, two SNVs must occur within a genomic distance less than the length of a typical cDNA molecule (∼170 bp). We therefore defined putative PVs as two variants occurring on the same chromosome within a genomic distance of <170 bp. DNVs, TNVs and ONVs were considered as the set of their respective component SNVs. The number of SNVs and identity of putative PVs for each case are detailed in Supplementary Table 1. The raw number of SNVs and putative PVs, as well as the number of putative PVs controlling for the number of SNVs, is shown in Extended Data Fig. 2.

Mutational signatures of phased variants from WGS. To assess the mutational processes associated with phased and non-phased mutations across different cancer types/subtypes, we enumerated the mutational signatures of SNVs for each WGS case described above using the R package ‘deconstructSigs’. We began by dividing the list of SNVs for each participant into two groups: (1) SNVs contained within a possible PV; that is, with an adjacent or ‘nearest neighbor’ SNV <170 bp away, and (2) non-related SNVs (that is, non-phased), defined as those occurring ≥170 bp in distance from the closest adjacent SNV. We then applied ‘deconstructSigs’ using the 49 SBS signatures described in COSMIC* (excluding signatures linked to possible sequencing artifacts) to assess the contribution of each SBS signature to both candidate phased SNVs and unphased SNVs for each participant. To compare the contribution of each SBS signature to phased and isolated SNVs, we performed a Wilcoxon signed-rank test to compare the relative contribution of each SBS signature between these two categories for each cancer type. To account for multiple hypotheses, we applied Bonferroni’s correction, by considering any SBS signature that differed in contribution to phased versus unphased SNVs to be significant if the Wilcoxon signed-rank test resulted in a P value < 0.05/49 or 0.001. The distributions of these comparisons for each of the 49 SBS signatures, along with significance testing, are depicted in figures available at https://phasedseq.stanford.edu/. A summary of this analysis is also shown in Fig. 1c using a heat-map display, where the ‘heat’ represents the difference between the mean contribution of the SBS signature to PVs to the mean contribution to isolated/unphased variants.

Genomic distribution of phased variants from WGS. We assessed the recurrence frequency for PVs in each cancer type across the genome within each tumor type. Specifically, we first divided the human genome (build GRCh37/hg19) into 1-kb bins (3,095,689 total bins); then, for each sample, we counted the number of PVs (as defined above) contained in each 1-kb bin. For this analysis, we included any PV with at least one of its constituent SNVs falling within the 1-kb bin of interest. We then calculated the fraction of participants whose tumors harbored a PV for each cancer type within each genomic bin. To identify 1-kb bins harboring PVs across participants, we plotted the fraction of participants containing PVs in each 1-kb bin versus genomic coordinates (Fig. 1d and Extended Data Fig. 3); for bin 170 bp apart in each B

Identification of recurrent 1-kb bins with phased variants. To identify 1-kb bins that recurrently contain PVs in B lymphoid malignancies, we utilized WGS data from the following diseases: DLBCL, FL, BL and CLL. Any 1-kb bin in which >1 sample from these tumor types contained a PV was considered to recurrently contain PVs from B lymphoid malignancies. The genomic coordinates of 1-kb bins containing recurrent PVs in lymphoid malignancies are enumerated in Supplementary Table 2.

Design of Phased-seq panel for B lymphoid malignancies. Identification of recurrent PVs from whole-genome sequencing at high resolution. Given the prevalence of recurrent putative PVs from WGS data in ALL and CLL malignancies, we designed a targeted sequencing approach for their hybridization-mediated capture. Phased-seq, to enrich these specific PV events from tumor or cfDNA. In addition to the ICGC data described above, we also used WGS data from other sources in this design, including both B cell NHLs and CLL. We also considered our previous experience with targeted sequencing from cfDNA in NHLs. We began by designing a variable number of SNVs on each of the B cell tumor sample. We then identified genomic ‘windows’ that contained PVs as follows: for each chromosome, we sorted the PVs by genomic coordinates relative to the reference genome. We then identified the left-most position for any PV in any participant; this defined the left-hand (5′) coordinate seeding a desired window of interest, to be captured from the genome. We then extended this window by growing its 3′ end to capture successive PVs until a gap of >340 bp was reached, with 340 bp chosen as capturing two successive chromosomal-sized fragments of ∼170 bp. When such a gap was reached, a new window was started, and this iterative process of adding neighboring PVs was repeated again until the next gap of >340 bp was reached. This resulted in a BED file containing all possible PVs from all samples considered. Finally, each window was additionally padded by 50 bp on each side, to enable efficient capture from flanking sequences in rare scenarios when repetitive or poorly mapping intervening sequences might preclude their direct targeting for enrichment.

Having identified the regions of interest containing putative PVs, we then divided each window into 170-bp segments (that is, 170 bp apart). We then performed a Wilcoxon signed-rank test to assess the performance of Phased-seq for capturing both SNVs and PVs compared to our previously reported CAPP-seq selector for B cell lymphomas, we quantified the predicted number of both SNVs and PVs that would be recovered with each panel by limiting WGS in silico prediction to the capture targets of each approach (Extended Data Fig. 4a–c). The predicted number of variants was then compared using Wilcoxon signed-rank test. We also performed both CAPP-seq and Phased-seq on 16 samples from participants with DLBCL. In these samples, tumor or plasma DNA, along with matched germline DNA, was sequenced. The resulting number of variants were again compared by the Wilcoxon signed-rank test (Fig. 3a and Extended Data Fig. 4d,e). The sequencing depths for the samples included in this analysis are provided in Supplementary Table 3.

Identification of phased variants from targeted sequencing. Library preparation and sequencing. To generate sequencing libraries and targeted sequencing data, we applied CAPP-seq as previously described. Briefly, cell-free, tumor, and germline DNA were used to construct sequencing libraries through end repair, A-tailing and adaptor ligation using the KAPA Hyper Prep Kit according to the manufacturer’s instructions. Hybridization with ligation performed overnight at 4 °C. CAPP-seq adapters with UMIs were used for barcoding of unique DNA duplexes and subsequent duplication of sequencing read pairs. Hybrid captures were then performed for lymphoma samples using (SeqCap EZ Choice; NimbleGen) at 47°C for 48h and (xGen Lockdown Probe Pool; IDT) for solid tumors at 65°C for 16h. Lymphoma samples utilized an off-the-shelf panel, while solid tumor samples utilized a customized panel as described in the main text. Affinity capture was performed according to the manufacturer’s protocol, with the respective hybridization conditions conducted on an Eppendorf thermalycler. Following enrichment, libraries were sequenced using an Illumina HiSeq 4000 instrument with 2 × 150-bp paired-end reads.

Preprocessing and alignment. FASTQ files were demultiplexed and UMIs were extracted using a custom pipeline as previously described. FASTQ files were then processed using fastp without global trimming, with an unqualified percentage limit of 50% and a required length of 35. All other parameters were kept as default. For more efficient parallelization and to minimize barcode collisions, sample
FASTQ files were then split into multiple files based on fragment UMIs. Each FASTQ file was then aligned to the human genome (build GRCh37/hg19) using the BWA ALN algorithm without fragment size estimation, and deduplicated using both samtools ‘markdup’ and our previously described in-house algorithm. UMI-specific files were then merged to generate final sample alignment files. Molecular barcode-mediated error suppression and background polishing (that is, idES) were then performed as previously described.

Identification of phased variants. For detailed methods of identification of PVs and MRD detection, see the Supplementary Methods.

Assessment of hybridization bias. To assess the effect of mutations on hybridization efficiency, we first estimated the affinity of mutated molecules to wild-type capture baits in silico by considering DNA fragments harboring 0–30% mutations across the entire fragment. For each mutation condition across this range, we first randomly sampled 10,000 regions, each 150 bp in length, from across the whole genome. We then mutated these 150-mers in silico to simulate the desired mutation rate in three different ways: (1) mutating ‘clustered’ or contiguous bases starting from the ends of a sequence, (2) mutating clustered bases started from the middle of the sequence, or (3) mutating bases selected at random positions throughout the sequence. We then used the ‘energy.c’ package to calculate the theoretical binding energy (kcal mol$^{-1}$) between the mutated and wild-type sequences, in relying on a nearest-neighbor model using established thermodynamic parameters.

We then replicated this in silico experiment by testing the effects of some mutated sites in vivo. Specifically, we synthesized wild-type and annealed oligonucleotides (IDI) to form DNA duplexes harboring 0–10% mutations at defined positions relative to the human reference genome sequence. We then captured these synthetic DNA molecules together at equimolar concentrations and quantified the relative capture efficiency of mutated duplexes compared to the wild-type, unmutated species (Fig. 4a). Two sets of oligonucleotide sequences were selected from the coding regions of BCL6 (ref. 35) and MYC (ref. 36) to capture AID-mediated aSHMs associated with each gene; we ensured the preserved mappability of the mutated species by BWA ALN (Supplementary Table 6). These synthetic oligonucleotide duplexes were then subjected to library preparation and then captured and sequenced using PhasED-seq, performed in triplicate using distinct samples. This allowed assessment of the relative efficiency of hybrid capture and molecular recovery as directly compared to wild-type molecules identical to the reference genome.

Assessment of limit of detection with dilution series. To empirically define the analytical sensitivity of PhasED-seq, we utilized a limited dilution series of ctDNA from three participants that were spiked into healthy control ctDNA at defined concentrations. Our dilution series contained samples with an expected mean tumor fraction of 0.1%, 0.01%, 0.001%, 0.0002%, 0.0001% and 0.00005% or ranging from 1 part in 1,000 to 1 part in 2,000,000. The sequencing characteristics and ctDNA quantification via CAPP-seq, duplex sequencing and PhasED-seq are provided in Supplementary Table 7. Tumor fractions were normalized to the observed and expected tumor fraction for each participant at each dilution (δ

where \( \delta_{i,j} \) = \( \text{tumorfrac}_{i,j} - \text{tumorfrac}_{i,0} \) (1)

This value was calculated for participants \( j = 1, 2, 3 \) and concentrations \( j = (0.001\%, 0.0002\%, 0.0001\%, 0.00005\%) \) for each ctDNA detection method (CAPP-seq, duplex, doublet PhasED-seq and triplet PhasED-seq). The performance of each method was then compared to each other by paired \( t \)-test across the set of participants and concentrations. Results are shown in Fig. 5a, as well as in Extended Data Fig. 5g without the use of barcode deduplication (that is, samtools markdup without UMI-mediated error suppression).

Extension of PhasED-seq to solid tumors. Whole-genome sequencing to identify phased variants. To demonstrate the utility of PhasED-seq for solid tumors, we first performed WGS on tumor and matched normal DNA from six tumors (lung cancer, \( n = 5 \); breast cancer, \( n = 1 \)) to ~30x read depth (Supplementary Table 9). Sequencing reads were aligned to hg19 and deduplicated with samtools markdup, as described above. Per GATK best practices, tumor and normal deduplicated BAM files were processed with GATK IndelRealigner and BaseRecalibrator before variant calling, using recommended parameters (GATK v3.8–1–g-f5c13e2). Variant calling was performed using three methods: VarScan2 (v2.3.9)\(^49\), Mutect (v1.1.7)\(^50\) and Strelka2 (v2.9.1)\(^51\). Mutect and VarScan2 VCF files were annotated by annovar (v2018April), and Strelka VCF files were annotated by Oncotator (v1.9.8.0). Variants called by each method were combined and filtered according to the following criteria: (1) pass caller-intrinsic quality filters (for example, base quality, orient bias and depth bias); (2) depth \( \geq 10 \times \), AF \( \geq 5\% \); (3) variant identified by \( \geq 2 \) caller filters. SNVs passing all filters were then assessed for possible phased relationships—any pair of SNVs \( \leq 170 \) bp from their nearest neighbor was considered a possible PV. We also genotyped PVs directly from WGS reads, considering any candidate PV’s with at least two supporting reads, 10x depth and 5% tumor fraction. Candidate PVs were then assessed and prioritized for tumor specificity, considering the (1) presence in individual tumor reads as phased relationships, (2) absence of read support in matched normal, (3) presence of other non-reference bases on the supporting reads, (4) base quality, (5) mapping quality and (6) uniqueness of genomic positions. Candidate PVs were then selected for targeted resequencing below.

Targeted resequencing to validated phased variants. After identifying candidate PVs from each of these six tumors, we designed 120-bp biotinylated hybrid-capture oligonucleotides targeting the regions of interest (Integrated DNA Technologies). We then performed hybrid capture resequencing of the tumor–normal pairs to high unique molecular depth to create a validated list of PVs for tumor monitoring. A PV was considered to be validated if it was present in the tumor at higher than 5% AF and had no read support in the matched germline DNA.

Measurement of ctDNA in participants with solid tumors. We applied the above personalized hybrid-capture panels targeting PVs’ to plasma samples from each of these six participants, sequencing to high unique molecular depth. We also sequenced 24 control healthy ctDNA samples with each panel to assess specificity. Tumor fraction was defined as the number of reads containing an a priori defined PV over the total number of reads covering a PV position (that is, PV tumor fraction, defined in ‘Determination of tumor fraction in a sample from phased variants’ in the Supplementary Methods). Most samples had been assessed for ctDNA content using SNV-based CAPP-seq approaches previously, providing covariates specific to PhasED-seq. The results of these experiments are shown in Fig. 7c–f and Extended Data Fig. 10e.

Statistical analyses. All P values reported in this paper are two-sided unless otherwise noted. Comparisons of matched samples and populations were performed using the Wilcoxon signed-rank test, whereas comparisons of samples drawn from unrelated populations were performed using the Wilcoxon rank-sum test. Comparisons of paired samples were performed by paired \( t \)-test. Survival probabilities were estimated using the Kaplan–Meier method; survival of groups of participants based on ctDNA levels were compared using the log-rank test. Other statistical tests are noted in the main text where used. All analyses were performed using MATLAB (v2018b), R (v3.4.1), Perl (v5.10.1) and GraphPad Prism (v8.0.2).

The contribution of known mutational processes to phased and isolated SNVs from WGS was assessed with the ‘deconstructSigs’ R package using the COSMIC signature set (v2) as described. Calculation of AUC accounting for survival and censorship was performed using the R ‘survivalROC’ package (v1.0.3) with default settings. The PhasED-seq software (v1.0) was developed using C++17.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
WGS data were obtained from the PCARWG dataset available at https://dcc.icgc.org/ pcawg. Additional lymphoma WGS data were obtained from the ICGC website (malignant lymphoma project, MALY-DE), as well as from work by Morin et al.\(^34\) and Qian et al. Raw data for samples used in the dilution series are available at http://phasedseq.stanford.edu/. This website provides users with software for phased variant monitoring (PhasED-seq v1.0). Included here are the list of SNVs and PVs to detected tumor DNA from each of the three samples used in this dilution series, as well as the sequencing files for each concentration condition. Owing to restrictions related to dissemination of germline sequence information included in the informed consent forms used to enroll study participants, we are unable to provide access to other raw sequencing data. Reasonable requests for additional data will be reviewed by the senior authors to determine whether they can be fulfilled in accordance with these privacy restrictions. Requests for additional materials related to this work should be directed to A.A.A.

Code availability
PhasED-seq (v1.0) was used to generate the results in this work and is freely available for academic research use at http://phasedseq.stanford.edu/, including a downloadable compiled version of the software. For academic users, the source code is available upon request.

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Acknowledgements
This work was supported by the National Cancer Institute (R01CA233975 and R01CA188298 to A.A.A. and M.D.; R01CA241076 to D.M.K.), the Virginia and D. K. Ludwig Fund for Cancer Research (A.A.A. and M.D.), the Damon Runyon Cancer Research Foundation (PST no. 09-16 to D.M.K. and DR-CI no. 71-14 to A.A.A.), the American Society of Hematology Scholar Award (A.A.A. and M.D.), the CRK Faculty Scholar Fund (M.D.), the Stinehart/Reed Award (A.A.A.), the CRK Faculty Scholar Fund (M.D.) and the SDW/DT and Shanahan Family Foundations (A.A.A.). A.A.A. is a Scholar of The Leukemia & Lymphoma Society.

Author contributions
D.M.K., J.S., M.D. and A.A.A. developed the concept, designed the experiments and analyzed the data. D.M.K., J.S. and M.D. A.A.A. wrote the manuscript. D.M.K., J.S. and L.C.T.K. developed the phased variant identification and monitoring workflows with input from M.D. and A.A.A. D.M.K., J.S., S.A., J.J.C., B.J.S, M.C.J., F.S., C.W.M., M.O., A.F.M.C. and E.J.M. performed molecular biology experiments related to the technical performance of PhasED-seq and detection of ctDNA in clinical samples. D.M.K., J.S., L.C.T.K., A.S., A.G., E.G.H., B.C., J.G.S.M., A.F.M.C., M.S.E. and C.L.L. performed bioinformatic analyses. Participant specimens were provided by D.M.K., E.J.M., U.D., A.H., R.-O.C., J.R.W., M.R., W.H.W., G.G., D.R., M.D. and A.A.A. Participant enrollment, sample collection and data curation were organized and performed by D.M.K., U.D., A.H., R.-O.C., J.R.W., M.R., W.H.W., G.G., D.R., M.D. and A.A.A. All authors reviewed the manuscript.

Competing interests
D.M.K. reports paid consultancy from Roche and Genentech. A.A.A. reports research funding from Celgene and Pfizer, ownership interests in FortySeven and CiberMed, and paid consultancy from Roche, Genentech, Janssen, Pharmacyclics, Gilead, Celgene and Chugai. M.D. reports research funding from Varian Medical Systems, AstraZeneca and Illumina, ownership interest in CiberMed, and paid consultancy from Roche, AstraZeneca, Novartis, Genentech, Illumina, RefleXion, Gristone Oncology, Boehhringer Ingelheim and BioNTech. M.D., A.A.A., D.M.K., J.C. and M.S.E. report patent filings related to cancer biomarkers. M.D., A.A.A., D.M.K. and J.C. report ownership interest in Foresight Diagnostics. R.O.C. is a member of the Roche advisory board and received research support from Roche. D.R. received research support from Gilead, Janssen, Roche and AbbVie, outside the submitted work. E.J.M. has served as a paid consultant for DeciBio. The remaining authors declare no competing interests.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41587-021-00981-w.
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41587-021-00981-w.
Correspondence and requests for materials should be addressed to M.D. or A.A.A.
Peer review information Nature Biotechnology thanks Paul Spellman, Christian Steidl and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.
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Extended Data Fig. 1 | Comparison of duplex sequencing to phased variant sequencing. a, A schema comparing error-suppressed sequencing by duplex sequencing vs. recovery of phased variants. In duplex sequencing, recovery of a single SNV observed on both strands of an original DNA double-helix (that is, in trans) is required. This requires independent recovery of two molecules by sequencing as the plus and minus strands of the original DNA molecule go through library preparation and PCR independently. In contrast, recovery of PVs requires multiple SNVs observed on the same single strand of DNA (that is, in cis). Thus, recovery of only the plus or the minus strand (rather than both) is sufficient for identification of PVs. b, A model showing the two possible reasons for limited sensitivity for ctDNA MRD assays. An assay can be limited by either having i) an insufficient number of cfDNA fragments evaluable for tumor content, or ii) an inadequate error-profile. This plot demonstrates the analytical sensitivity as the number of evaluable cfDNA fragments increase with either the amount of plasma input or the number of mutations tracked, until eventually becoming limited by the background signal (grey). Separate plots shown for single-stranded and double-stranded SNV based methods, assuming 8.92 ng cfDNA/mL plasma; 50% efficiency of library preparation, and 20% efficiency of duplex sequencing.
Extended Data Fig. 2 | Enumeration of SNVs and PVs in diverse cancers from WGS. a–d. Univariate scatter plots showing the number of a) SNVs, b) 2x-PVs (2 SNVs in phase), c) 3x-PVs, and d) total 2x-PVs, controlling for total number of SNVs, from WGS data for 24 different histologies of cancer. Data are presented as median and interquartile range. (FL-NHL, follicular lymphoma; DLBCL-NHL, diffuse large B cell lymphoma; Burkitt-NHL, Burkitt lymphoma; Lung-SCC, squamous cell lung cancer; Lung-Adeno, lung adenocarcinoma; Kidney-RCC, renal cell carcinoma; Bone-Osteosarc, osteosarcoma; Liver-HCC, hepatocellular carcinoma; Breast-Adeno, breast adenocarcinoma; Panc-Adeno, pancreatic adenocarcinoma; Head-SCC, head and neck squamous cell carcinoma; Ovary-Adeno, ovarian adenocarcinoma; Eso-Adeno, esophageal adenocarcinoma; Uterus-Adeno, uterine adenocarcinoma; Stomach-Adeno, stomach adenocarcinoma; CLL, chronic lymphocytic leukemia; ColoRect-Adeno, colorectal adenocarcinoma; Prost-Adeno, prostate adenocarcinoma; CNS-GBM, glioblastoma multiforme; Panc-Endocrine, pancreatic neuroendocrine tumor; Thy-Adeno, thyroid adenocarcinoma; CNS-PiloAstro, piloastrocytoma; CNS-Medullo, medulloblastoma.).
Extended Data Fig. 3 | Distribution of PVs in stereotyped regions across the genome. Distribution of PVs occurring in stereotyped regions across the genome of multiple cancer types. In this plot, the genome was divided into 1000 bp bins, and the fraction of samples of a given histology with a PV in each 1000 bp bin was calculated. Only bins that have at least a 2 percent recurrence frequency in any cancer subtype are shown.
Extended Data Fig. 4 | Performance of PhasED-Seq for recovery of PVs across lymphomas. **a**, Univariate scatter plot comparing the fraction of all PVs across the genome identified by WGS (n = 79) that were recovered by our previously reported lymphoma CAPP-Seq panel (left) compared to PhasED-Seq (right). **b**, Univariate scatter plot comparing the expected yield of SNVs per case identified from WGS using a previously established lymphoma CAPP-Seq panel or the PhasED-Seq panel. **c**, Univariate scatter comparing the expected yield of PVs per case identified from WGS using a previously established lymphoma CAPP-Seq panel or the PhasED-Seq panel. Data from three independent publicly available cohorts are shown in **a-c**. **d,e**, Plots showing the improvement in recovery of PVs by PhasED-Seq compared to CAPP-Seq in 16 patients sequenced by both assays. This includes improvement in **d**) two SNVs in phase (that is, 2x or ‘doublet PVs’) and **e**) three SNVs in phase (3x or ‘triplet PVs’). Statistical testing in panels **a-e**) performed by 2-sided Wilcoxon signed-rank test. **f**, A cartoon describing the terminology for phased variants in this manuscript. The figure shows one region of an individual’s cancer genome (300 bp). Phased variants on a single strand of DNA of DNA can occur with different numbers of SNVs, including 2 variants in phase (doublets) and 3 in phase (triplets). For the purpose of detecting ctDNA, ‘independent reporters’ are defined as PVs that will typically co-segregate on separate cfDNA molecules, resulting in independent evaluable fragments. Given the size of cfDNA molecules, these are separated in 150 bp regions. **g-j**, These panels show the number of SNVs and PVs identified for patients with different types of lymphomas. These panels show the number of **g**) SNVs, **h**) doublet PVs, **i**) triplet PVs, and **j**) independent PV reporters; bars represent median and interquartile range. *P < 0.05 by two-sided Wilcoxon rank sum test; comparisons only shown for all histologies vs DLBCL. (DLBCL, diffuse large B-cell lymphoma; GCB, germinal center B-cell like DLBCL; ABC, activated B-cell like DLBCL; PMBCL, primary mediastinal B-cell lymphoma; FL, follicular lymphoma; HL, Hodgkin lymphoma; MCL, mantle cell lymphoma).
Extended Data Fig. 5 | Technical aspects of PhasED-Seq by hybrid-capture sequencing. a, Theoretical binding energy for 150-mers across the genome. Mutations were either clustered to one end (green), clustered in the middle (blue), or randomly throughout the sequence (red). Data represent the median and IQR from 10,000 in silico simulations. b, Histograms of summary metrics of the mutation rate of 151-bp windows from all patients in this study. c, The percentile of mutation rate across all mutated 151-bp windows across all patients in this study. d, Rate of background-signal in the PhasED-Seq panel for multiple variants, including SNVs (red), PVs (blue), and indels (green). Different methods of error-suppression for each variant type are shown. Bars represent median and IQR. UMI, unique molecular identifiers; PhasED-Seq 2x, doublet PVs; PhasED-Seq 3x, triplet PVs. e) Error-rate for SNVs (left), doublet PVs (middle), and triplet PVs (right) by type of mutation. For triplet PVs, the x and y-axis represent the first and second type of base alteration in the PV. f, Error rate for doublet PVs across n = 12 healthy cfDNA samples as a function of inter-SNV distance. Data show mean and standard deviation. g, Limiting dilution series simulating cfDNA similar to Fig. 5a; cfDNA from 3 independent patient samples were used in each dilution. In this plot, PhasED-Seq is assessed without the use of UMIs. Data are presented as mean and range. *, P < 0.05; CAPP-Seq vs duplex, P = 3.2e-5; CAPP-Seq vs PhasED-Seq (2x), P = 1.6e-4; CAPP-Seq vs PhasED-Seq (3x), P = 1.9e-5; duplex vs PhasED-Seq (2x), 0.017; duplex vs PhasED-Seq (3x), 0.0046. h, Theoretical rate of detection for a sample with a given number of PV-containing regions, according to binomial sampling, assuming unique sequencing depth of 4000–6000x (shaded area; 5000x shown as line). i, Observed rate of detection given a true tumor fraction, with varying numbers of PV-containing regions. Filled-in points represent ‘wet’ experiments; open points represent in silico dilution experiments. Data represent mean and range. j, Predicted vs observed rate of detection for samples from the dilution series shown in panels h and i. Error-bars are as described in h) and i) above (see the Supplementary Methods).
Extended Data Fig. 6 | Comparison of ctDNA quantitation by PhasED-Seq to CAPP-Seq and clinical applications. a, ROC curve of the performance for detection of ctDNA from SNVs (that is, CAPP-Seq) and PVs using PhasED-Seq. Positive samples are 107 pretreatment plasmas, negative samples are 40 control plasmas assessed for evidence of ctDNA using 107 personalized mutation lists for 4,280 total samples. Sensitivity and specificity at optimum point and AUC are shown. b, Quantity of ctDNA (measured as log10(haploid genome equivalents/mL)) as measured by CAPP-Seq vs. PhasED-Seq in individual samples. Samples taken prior to cycle 1 of RCHOP therapy (that is, pretreatment), prior to cycle 2, and prior to cycle 3, are shown in independent colors (blue, green, and red respectively; 277 total samples). Undetectable levels fall on the axes. Spearman correlation and P-value are shown.
Extended Data Fig. 7 | Detection of ctDNA after two cycles of systemic therapy. a, Scatterplot shows the log-fold change in ctDNA after 2 cycles of therapy measured by CAPP-Seq or PhasED-Seq for patients receiving RCHOP therapy. Dotted lines show the previously established threshold of a 2.5-log reduction in ctDNA for molecular response. Undetectable samples fall on the axes; the correlation coefficient represents a Spearman rho for the samples detected by both CAPP-Seq and PhasED-Seq. b, Detection rate of ctDNA samples after 2 cycles of therapy by PhasED-Seq vs CAPP-Seq. Patients with eventual disease progression are shown in red, while patients without eventual disease progression are shown in blue. c, ROC curve for detection of ctDNA after 2 cycles of treatment. Positive samples include 24 samples from patients with eventual disease progression, and therefore are known to have residual disease. Negative samples are from 4,280 tests on healthy controls as described in Extended Data Fig. 6a. d, Kaplan-Meier plots and two-sided log-rank test showing the event-free survival of 69 patients achieving an MMR stratified by ctDNA detection with CAPP-Seq (top) or PhasED-Seq (bottom).
Extended Data Fig. 8 | Detection of ctDNA after one cycle of systemic therapy. 

a, Scatterplot showing the log-fold change in ctDNA after 1 cycle of therapy measured by CAPP-Seq or PhasED-Seq for patients receiving RCHOP therapy. Dotted lines show the previously established threshold of a 2-log reduction in ctDNA for molecular response. Undetectable samples fall on the axes; the correlation coefficient represents a Spearman rho for the samples detected by both CAPP-Seq and PhasED-Seq. 

b, Detection rate of ctDNA samples after 1 cycle of therapy by PhasED-Seq vs CAPP-Seq. Patients with eventual disease progression are shown in red, while patients without eventual disease progression are shown in blue. 

c, ROC curve for detection of ctDNA after 2 cycles of treatment. Positive samples include 22 samples from patients with eventual disease progression, and therefore are known to have residual disease. Negative samples are from 4,280 tests on healthy controls as described in Extended Data Fig. 6a. 

d, Waterfall plot showing the change in ctDNA levels measured by CAPP-Seq after 1 cycle of first-line therapy in patients with DLBCL. Patients with undetectable ctDNA by CAPP-Seq are shown as ‘ND’ (‘not detected’), in darker colors. The colors of the bars also indicate the eventual clinical outcomes for these patients. 

e, A Kaplan–Meier plot showing the event-free survival for 33 DLBCL patients with undetectable ctDNA measured by CAPP-Seq after 1 cycle of therapy. 

f, A Kaplan–Meier plot and two-sided log-rank test showing the event-free survival of 33 patients shown in e (undetectable ctDNA by CAPP-Seq at this same time-point (cycle 2, day 1)). 

g, A Kaplan–Meier plot and two-sided log-rank test showing the event-free survival for 82 patients with DLBCL stratified by ctDNA at cycle 2, day 1 separated into 3 strata – patients failing to achieve an early molecular response (red), patients with an early molecular response who still have detectable ctDNA by PhasED-Seq and/or CAPP-Seq (grey), and patients who have a stringent molecular remission (undetectable ctDNA by PhasED-Seq and CAPP-Seq; blue).
Extended Data Fig. 9 | Performance of ctDNA detection at the end of systemic therapy. a, ROC curve for detection of ctDNA after the completion of planned systemic therapy. Positive samples include 5 samples from patients with eventual disease progression, and therefore are known to have residual disease. Negative samples are from 4,280 tests on healthy controls as described in Extended Data Fig. 6a. b, The ctDNA profile of a patient with stage 4 DLBCL undergoing systemic chemotherapy, with pretreatment PET scan shown on the left. This patient only received one cycle of EPOCH-R chemotherapy from 6 planned treatments (dashed arrows – planned therapy that was not given). Following this, the patient self-discontinued treatment. This patient was found to have cleared their ctDNA by PhasED-Seq and continues in clinical remission after > 4 years.
Extended Data Fig. 10 | Extension of PhasED-Seq to solid tumors. a, A mathematical model showing the expected total unique molecular depth (blue) and duplex molecular depth (green) from an optimized hybrid-capture workflow (Chabon et al.41; Methods). b, A comparison in projected sensitivity for ctDNA detection using PVs versus structural variants (SVs) for various histologies from the PCAWG dataset. Comparison assumes a personalized sequencing panel targeting only patient-specific variants, 64 ng of DNA input and 20 million sequencing reads, using the model of molecular recovery from a. c, A comparison in expected sensitivity for ctDNA detection using PVs versus duplex sequencing and SNVs for various histologies from the PCAWG dataset. Comparison assumes a personalized sequencing panel targeting only patient-specific variants, 64 ng of DNA input and 20 million sequencing reads, using the model of molecular recovery from a. d, Detection of ctDNA for the 6 cases of patients with solid tumors, including lung cancer (n = 5) and breast cancer (n = 1) using SNV-based detection (that is, CAPP-Seq) or PhasED-Seq with a personalized panel. Detection of ctDNA in patient plasma samples are shown in blue; samples detectable with PhasED-Seq but not SNV based approaches are in light blue. Specificity of the assay was assessed using 24 healthy control samples; detection of evidence of ctDNA by PhasED-Seq in these are shown on the right in pink across all 6 personalized panels, indicating 97% (139/144) specificity; CAPP-Seq on the same samples showed 95% (137/144) specificity. e, The ctDNA profile of a patient with stage 3 lung adenocarcinoma (LUP831) undergoing combined chemo-radiotherapy (CRT) and immunotherapy, measured by both CAPP-Seq and PhasED-Seq. The left panel shows the measured tumor fraction in the tumor biopsy sample using both methods. The right panel shows the tumor fraction from plasma DNA, including a sample detected by PhasED-Seq that is undetected by CAPP-Seq. ND: not detected.
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Software and code

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Data collection

Sequencing libraries were prepared as described in the methods and sequenced on the Illumina HiSeq4000 platform using 2x150bp paired end reads. Primary processing of sequencing data was performed with BWA [0.5.9-r16], samtools (versions 0.1.18 and 1.3.1), and GATK [v3.8-1.0-g15c1c3ef]. Variant calling was performed by VarScan2 [v2.3.9], Mutect [v1.1.7], and Strelka2 [v2.9.1] with variant annotation by annovar (version 2018April16) and Oncotator [v1.9.8.0].

Data analysis

Statistical analyses were performed using MATLAB (R2018a), R [version 3.4.1], and perl [v5.10.1] in a CentOS 6.7 Linux environment or with GraphPad Prism (version 8.0.2). The contribution of known mutational processes to phased and isolated SNVs from WGS data was assessed with the deconstructSigs R package using the COSMIC signature set [v2] as described in the methods. WGS of primary Calculation of AUC for survival and censorship was performed using the R ‘survivalROC’ package version 1.0.3 with default settings. The Phased-Seq software was developed using C+17. Phased-Seq v.1.0 was used to generate the results in this work and is freely available for academic research use at http://phasedseq.stanford.edu.

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The count of SNVs and phased variants from WGS data for all cancer types analyzed, along with the distribution of phased variants from WGS in lymphoid malignancies, is provided in the Supplementary Tables. The coordinates of regions of the genome with enriched phased variants from WGS are also provided in the Supplementary Tables. Anonymized and summarized clinical and demographic data from patients where PhasEd-Seq was used for disease detection are also provided. Whole genome sequencing data was obtained from the Pan-Cancer Analysis of Whole Genomes (PCAWG) from https://dcc.icgc.org/pcawg. Additional lymphoma WGS was obtained from the International Cancer Genome Consortium website (malignant lymphoma project, MALY-DE), as well as from DOI: 10.1182/blood-2013-02-483723/j and 10.1016/j.cell.2014.11.01334. Raw data for samples used in the dilution series is available at http://phasedseq.stanford.edu. This website provides users with software for phased-variant monitoring (PhasEd-Seq v1.0). Included here are the list of SNVs and PVS used to detected tumor DNA from each of the three samples used in this dilution series, as well as the sequencing files for each concentration condition. Owing to restrictions related to dissemination of germ-line sequence information included in the informed consent forms used to enroll study subjects, we are unable to provide access to other raw sequencing data. Reasonable requests for additional data will be reviewed by the senior authors to determine whether they can be fulfilled in accordance with these privacy restrictions. Requests for additional materials related to this work should be directed to A.A.A.

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Sample size
The goal of our study was to determine if a more sensitive cDNA assay has additional prognostic value in large B-cell lymphoma patients with undetectable cDNA by current methodologies (i.e., CAPP-Seq) after 1 or 2 cycles of standard immuno-chemotherapy. We powered our study to detect a meaningful difference (35% difference in event-free survival at 24 months) between PhasEd-Seq detectable and undetectable patients. A sample size of 34 patients is needed to detect this difference with 80% power and alpha=0.05 (assuming a 1:1 ratio between detectable and undetectable patients by PhasEd-Seq). However, these 34 patients must have undetectable cDNA by CAPP-Seq after 1 or 2 cycles of therapy. Previous literature suggests that ~50% of patients will have undetectable cDNA by CAPP-Seq at these milestones, therefore ~78 total subjects are required to achieve the necessary sample size. This justifies our sample size of 82 subjects with cDNA available after 1 cycle of therapy and 88 with cDNA after 2 cycles of therapy.

Data exclusions
Samples deemed to be technical failures during DNA isolation or library preparation were excluded. Phased variants were filtered using the PhasEd-Seq algorithm as described in the Methods sections “Identification of phased variants and allelic quantitation” and “Genotyping phased variants from pretreatment samples”.

Replication
Demonstration of the lower error profile from phased variants as compared to SNVs was performed in 12 independent samples with consistent results. Hybridization experiments were performed in three replicates. Disease detection in the limiting dilution series was performed using three independent patient-derived cell-free DNA samples. Results describing the clinical significance of cDNA detection using PhasEd-Seq in the setting of undetectable cDNA by alternative methods (such as CAPP-Seq) were replicated with samples after both one cycle or two cycles of therapy, as well as at the end of therapy (3 sample-sets at different time-points n=82, 88, and 19 samples). The performance of PhasEd-Seq in cancers outside of B-cell lymphomas was shown from 6 patients with solid tumors. All attempts at replication were successful.

Randomization
Patients and controls were enrolled in the study based on a confirmed diagnosis of B-cell lymphoma receiving standard first-line immuno-chemotherapy. Additional subjects were enrolled based on a confirmed diagnosis of lung cancer or breast cancer. Therefore, randomization of individuals to different groups is not applicable as there was no intervention.

Blinding
As there was no therapeutic intervention in this observational biomarker study, no blinding was performed.

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**Population characteristics**

Study participants include patients with B-cell lymphomas, including diffuse large B-cell lymphoma, primary mediastinal B-cell lymphoma, Hodgkin lymphoma, follicular lymphoma, and mantle cell lymphoma. Blood samples were collected at Stanford University (USA), MD Anderson Cancer Center (USA), the National Cancer Institute (USA), University of Eastern Piedmont (Italy), Essen University Hospital (Germany), CHU Dijon (France). To extend PhasED-Seq to solid tumors, additional subjects with a diagnosis of lung cancer or breast cancer were collected at Stanford University (USA). The median age was 57 (range 20-84), and 47% of participants were female. Additional clinical and demographic details are provided with the paper.

**Recruitment**

All subjects were enrolled with Institutional Review Board-approved protocols and all biospecimens analyzed in this study were collected with informed consent. Patients receiving first-line therapy for large B cell lymphomas at these centers were enrolled separately at each institution for observational study of blood-based biomarkers with serial blood samples collected and stored locally. Patients were treated with first-line therapy according to local standards. To extend PhasED-Seq to solid tumors, subjects with a diagnosis of limited stage (stage I-III) lung cancer or breast cancer undergoing treatment at Stanford University were enrolled for observational study of blood-based biomarkers with serial blood samples collected and stored locally.

These patients represent a typical population receiving cancer care at tertiary academic medical centers, which could differ from other patient populations.

**Ethics oversight**

All samples analyzed in this study were collected with informed consent from subjects enrolled on Institutional Review Board approved protocols that complied with all relevant ethical regulations at their respective centers, including Stanford University, MD Anderson Cancer Center, the National Cancer Institute, University of Eastern Piedmont, Essen University Hospital, and CHU Dijon.

Note that full information on the approval of the study protocol must also be provided in the manuscript.