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Longitudinal Plasma cfDNA and Genomic Profiling During Chemotherapy in Canine Multicentric Lymphoma: A Pilot Study

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Simple Summary

Canine multicentric lymphoma often responds to chemotherapy, but recurrence remains common. In this pilot study, we investigated whether repeated blood sampling could capture changes in plasma cell-free DNA (cfDNA) and genomic variants alongside clinical assessments in seven dogs with multicentric B-cell lymphoma. Five dogs were sampled through approximately 30 days of treatment, and one dog had an additional sample at documented relapse. Plasma cfDNA decreased from baseline to approximately day 30 in all five dogs with paired samples, while longitudinal variant patterns also changed during treatment. In the dog sampled at relapse, cfDNA and several tracked variant signals increased concurrently with clinical progression. Although larger studies with additional molecular confirmation are needed, these findings support the feasibility of patient-specific serial plasma profiling for investigating molecular changes during treatment and follow-up in canine lymphoma.

Abstract

Serial plasma DNA profiling offers a minimally invasive approach for investigating molecular changes during treatment of canine multicentric lymphoma. In this exploratory pilot study, longitudinal plasma cell-free DNA (cfDNA) concentrations and genomic variants were evaluated in seven dogs with B-cell multicentric lymphoma receiving a vincristine–L-asparaginase–doxorubicin-based chemotherapy protocol. Samples were collected before treatment (T0), approximately 7 days after treatment initiation (T1), and, when available, approximately 30 days after initiation (T2); one dog was additionally sampled at documented relapse (Tx). Targeted sequencing of 140 cancer-related genes was performed with matched buffy coat filtering and a 0.5% variant allele frequency (VAF) inclusion threshold. Longitudinal analysis tracked all eligible T0-defined plasma variants for each dog across subsequent samples. Five dogs contributed T0–T2 comparisons, and plasma cfDNA decreased in all five (10.4–99.9%; median decrease, 64.1%). Longitudinal variant trajectories also changed during treatment, with decreases in mean VAF of the baseline-defined variant set in several responding dogs. In the single relapse-associated sample, cfDNA increased by 755.6% relative to T2, accompanied by increases in several tracked variant signals and clinical progression. These observations support the feasibility of serial plasma genomic profiling and provide preliminary evidence that within-dog molecular changes can accompany clinical disease dynamics. Larger prospective cohorts with additional relapse

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sampling and tumor-matched or orthogonal molecular confirmation will be needed to determine the clinical monitoring value of these longitudinal molecular changes.

Keywords: canine lymphoma; cell-free DNA; liquid biopsy; targeted sequencing; longitudinal monitoring

1. Introduction

Canine lymphoma is one of the most common malignant hematopoietic neoplasms in dogs, with reported annual incidence rates ranging from 24 to 114 cases per 100,000 dogs [1,2]. Lymphoma accounts for approximately 7–24% of all canine tumors and 83% of canine hematopoietic malignancies [3], with multicentric lymphoma being the most prevalent form, characterized by generalized peripheral lymphadenopathy [4].

Multi-agent chemotherapy remains the mainstay of treatment for canine multicentric lymphoma. CHOP-based protocols, consisting of cyclophosphamide, doxorubicin, vincristine, and prednisolone, are widely used for high-grade canine lymphoma and have achieved remission rates greater than 85%, with reported survival times ranging from approximately 8 to 12 months [5,6]. Despite favorable initial response rates, canine lymphoma is rarely curable, and disease relapse remains a major clinical challenge. Although numerous studies have evaluated modifications of CHOP-based and other multi-agent chemotherapy protocols to improve treatment efficacy, reduce toxicity, and optimize long-term outcomes, relapse remains common even among dogs that initially achieve clinical remission [7–10]. These limitations highlight the need for biomarkers capable of monitoring treatment response, detecting minimal residual disease (MRD), and identifying relapse-associated disease progression at an earlier stage.

Current response assessment in clinical practice primarily relies on physical examination and serial measurements of peripheral lymph nodes according to Veterinary Cooperative Oncology Group (VCOG) criteria [11]. Although these approaches remain indispensable in routine clinical practice, they may not adequately capture MRD, emerging treatment resistance, or molecular relapse preceding clinically detectable progression. Furthermore, conventional tissue biopsies are limited by the inability to perform repeated sampling and the difficulty of accessing deep-seated tumors.

To overcome these limitations, several circulating blood-based biomarkers have been investigated in canine multicentric lymphoma, including YKL-40, thymidine kinase 1 (TK1), C-reactive protein (CRP), monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), interleukin-10 (IL-10), and haptoglobin [12–16]. Although some of these biomarkers are associated with disease status, clinical stage, or treatment response, their prognostic and longitudinal monitoring utility remains incompletely established, highlighting the need for more tumor-specific biomarkers capable of directly reflecting disease dynamics.

Liquid biopsy enables minimally invasive analysis of plasma cell-free DNA (cfDNA) and its tumor-derived component, circulating tumor DNA (ctDNA). Previous canine studies have demonstrated the feasibility of plasma genomic profiling and longitudinal blood-based cancer monitoring, while quantitative cfDNA assays have examined associations with cancer status and outcome [17–20]. Total cfDNA includes DNA from both tumor and non-tumor sources. Tumor-derived DNA may contain single-nucleotide variants (SNVs), insertions/deletions (indels), and copy number alterations (CNAs). For SNVs and indels, variant allele frequency (VAF) describes the proportion of sequencing observations supporting the alternate allele at a locus; CNAs require separate analytical approaches. Distinguishing tumor-associated plasma variants from germline, hematopoietic, and technical background is therefore a central consideration in cfDNA sequencing. Parallel sequencing

of leukocyte DNA can reduce germline and hematopoietic confounding, although it does not by itself prove tumor origin. Accordingly, matched buffy coat genomic DNA was analyzed alongside plasma cfDNA in the present study to exclude variants detected in the cellular fraction.

In human diffuse large B-cell lymphoma (DLBCL), longitudinal ctDNA measurements have been associated with treatment response and outcome, and molecular recurrence has been detected in some settings before clinical progression [21,22]. Although assay performance and clinical interpretation are context dependent, these studies provide a strong biological rationale for investigating analogous longitudinal plasma biomarkers in canine lymphoma. The biological and genomic similarities between canine lymphoma and human non-Hodgkin lymphoma further support this comparative approach [23,24].

Targeted next-generation sequencing (NGS) permits simultaneous evaluation of multiple genomic loci from a single plasma sample [25] and canine studies have demonstrated the feasibility of detecting cancer-associated genomic alterations in blood [26]. Recent studies have further expanded the veterinary evidence base. Nascimento et al. reported longitudinal concordance between a blood-based lymphoma assay and VCOG-defined clinical status during chemotherapy, while also concluding that the assay was not sufficient for stand-alone monitoring [27]. Megquier et al. systematically evaluated preanalytical factors and reported longitudinal liquid-biopsy changes with disease status; importantly, their paired analyses also demonstrated substantial, but not complete, concordance between tumor and cfDNA mutations, supporting the biological relevance of plasma genomic signals while emphasizing the value of tissue confirmation when assigning tumor origin [28]. Prospective NGS-based screening studies and recent reviews likewise support the growing clinical scope of canine liquid biopsy while emphasizing validation, standardization, and interpretation alongside conventional diagnostics [29,30].

We hypothesized that serial plasma cfDNA concentrations and the VAFs of baseline-defined plasma genomic variants would change during chemotherapy in dogs with multicentric B-cell lymphoma, and that these within-dog molecular changes would, in some cases, accompany changes in clinically measured lymph node burden. The primary objective of this pilot study was to evaluate the feasibility of serial plasma cfDNA quantification and targeted genomic profiling during chemotherapy. Secondary exploratory objectives were to characterize longitudinal plasma variant trajectories, describe their relationship with clinical treatment response, and examine molecular changes at documented relapse when available.

2. Materials and Methods

2.1. Study Design and Cohort

This prospective longitudinal observational pilot study consecutively enrolled seven client-owned dogs with cytologically and/or histopathologically confirmed multicentric lymphoma between September 2024 and December 2025. Dogs with high-grade lymphoma, including treatment-naïve and relapsed cases, were eligible for enrollment. B-cell immunophenotype was confirmed by flow cytometry in all seven dogs. Exclusion criteria included severe cardiovascular disease, clinically significant anemia, and coagulopathy. Previous administration of glucocorticoids or chemotherapeutic agents was also an exclusion criterion, except for dogs enrolled with relapsed lymphoma. Clinical stage, substage, chemotherapy protocol, and available outcome data were recorded for all dogs. The study protocol was approved by the Institutional Animal Care and Use Committee of National Taiwan University (Approval No. NTU113-EL-00097), and informed owner consent was obtained before enrollment.

2.2. Clinical Evaluation and Response Assessment

Clinical tumor burden was assessed by serial caliper measurements of target peripheral lymph nodes, selected from enlarged mandibular, prescapular, axillary, inguinal, and popliteal nodes when available, and summarized as the sum of the longest diameters (sumLD). Clinical response categories were recorded according to VCOG criteria as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD) [11]. For descriptive comparisons, percent change was calculated as 100 × (follow-up value—reference value)/reference value. T0 was the reference for treatment-associated comparisons; T2 was the reference for the relapse-associated comparison in Dog 2. Clinical response assessments were performed independently of plasma variant selection. Because dogs were enrolled either at initial diagnosis or after relapse following a previous treatment course, the documented interval from prior treatment to relapse was recorded for relapsed dogs, along with the subsequent observation interval after enrollment for all dogs. These descriptive intervals are not presented as uniformly defined progression-free survival (PFS) or overall survival (OS) endpoints.

2.3. Longitudinal Sampling Schedule

All dogs were assigned to a planned 16-week vincristine–L-asparaginase–doxorubicin (VLA)-based chemotherapy protocol consisting of four four-week cycles. During each cycle, vincristine (0.7 mg/m^2, intravenously) was scheduled in Week 1, L-asparaginase (400 IU/kg, intramuscularly) in Week 2, and doxorubicin (30 mg/m^2 for dogs weighing $\geq$ 15 kg or 1 mg/kg for dogs weighing $<$ 15 kg, intravenously) in Week 3, followed by a week without VLA chemotherapy in Week 4. Prednisolone was administered once daily only during the first cycle, with doses tapered from 2 mg/kg in Week 1 to 1.5 mg/kg in Week 2, 1 mg/kg in Week 3, and 0.5 mg/kg in Week 4; it was not administered in Cycles 2–4. The sampling schedule comprised T0 before the first vincristine administration, T1 approximately 7 days after treatment initiation and before L-asparaginase administration, T2 approximately 30 days after treatment initiation at the beginning of the second cycle and before the second vincristine administration, and an additional relapse-associated time point (Tx) when available. All seven dogs contributed T0 and T1 samples; five (Dogs 1–5) contributed T2 samples, and Dog 2 contributed the only Tx sample, resulting in a total of 20 longitudinal plasma samples. T2 represents an on-treatment assessment and does not imply completion of chemotherapy. Molecular sampling availability was distinct from the duration of clinical follow-up. The Tx observation was obtained at documented relapse, rather than through a series of pre-relapse surveillance samples.

2.4. Plasma Processing and cfDNA Extraction

Peripheral blood was collected in Streck Cell-Free DNA BCT tubes (Streck, La Vista, NE, USA). Plasma was separated by centrifugation and stored at −80 °C until extraction. Plasma DNA was extracted using the iCatcher12 Automated Nucleic Acid Purification System (CatchGene, New Taipei City, Taiwan). Plasma DNA concentrations were measured using a Qubit 4 Fluorometer and the Qubit dsDNA High Sensitivity (HS) Assay (Thermo Fisher Scientific, Waltham, MA, USA). Samples exceeding the quantification range of the HS assay were remeasured using the Qubit dsDNA Broad Range (BR) Assay (Thermo Fisher Scientific, Waltham, MA, USA). The total recovered DNA amount was calculated by multiplying the measured DNA concentration (ng/μL) by the final elution volume (μL). Plasma DNA concentration (ng/mL) was then determined by dividing the total recovered DNA amount by the input plasma volume (mL), allowing normalization across samples with different plasma input volumes.

To preserve DNA for downstream library preparation and sequencing, direct fragment-size analysis of extracted plasma DNA was not routinely performed. Instead, library fragment-size distributions were assessed using the Qsep system (BiOptic Inc., New Taipei City, Taiwan) after library preparation and before sequencing. This quality-control procedure evaluated the prepared sequencing libraries but was not intended to characterize the fragment-size distribution of the original plasma DNA or exclude potential genomic DNA contamination.

2.5. Targeted Next-Generation Sequencing

Plasma-derived DNA was analyzed using a custom hybrid-capture panel targeting 140 cancer-related genes over 734,511 bp. Libraries were prepared using Twist Bioscience target enrichment technology (Twist Bioscience, South San Francisco, CA, USA) with unique molecular identifiers (UMIs) and unique dual indexes (UDIs) and sequenced as paired-end 150 bp reads on an Illumina NovaSeq X Plus platform (Illumina, San Diego, CA, USA). The average target-region sequencing depth was approximately 2000× at the read level, before UMI-based consensus generation. Although UMIs were incorporated during library preparation, UMI-based read-family collapsing, consensus generation, and molecular error suppression were not performed in the bioinformatic analysis. Therefore, the reported sequencing depth represents read-level coverage rather than unique molecular depth.

2.6. Bioinformatic Analysis

Sequencing reads were aligned to CanFam3.1 using BWA-MEM (v0.7.17). Candidate variants were called using Mutect2 in GATK (v4.4) and annotated using SnpEff (v5.1). Two levels of variant analysis were employed for distinct analytical purposes. A broader candidate-variant dataset was used for exploratory gene-level characterization, whereas a more stringently filtered dataset was used for longitudinal variant tracking and cross-dog variant-sharing analysis.

The final analytical dataset was generated using filtering thresholds established before analysis and applied consistently across all dogs. These criteria included Mutect2 PASS status, variant-calling confidence (tumor log odds (TLOD)), sequencing depth (DP), alternate allele depth (AD_alt), and mapping-related quality-control metrics. Variants detected in matched buffy coat genomic DNA were excluded. Additional background-variant filtering was performed using an existing canine genomic DNA reference dataset, excluding variants exceeding the established background-frequency criterion, including those not detected in the corresponding matched buffy coat sample. A VAF threshold of ≥0.5% was applied for inclusion in the final analytical dataset.

Variant retention was determined independently of clinical response and subsequent longitudinal VAF trajectories. Although matched buffy coat filtering reduces germline and hematopoietic-background contributions, it cannot establish tumor origin. In canine lymphoma, circulating malignant lymphocytes may also be present in the cellular fraction, potentially resulting in the exclusion of true tumor-associated variants. Because matched tumor tissue sequencing was unavailable, retained plasma variants were not considered tumor-confirmed somatic alterations and are referred to as plasma genomic variants throughout this study.

2.7. Analytical Performance Assessment

Analytical performance across low variant allele frequencies was characterized using two complementary dilution-based approaches because standardized canine reference materials containing validated low-frequency somatic variants are not readily available. First, synthetic reference sequences containing predefined cancer-associated variants were

introduced into a canine genomic DNA background. Second, an independent genomic DNA dilution model was established using two previously characterized canine DNA sources with known sequence differences. Both models were evaluated across serial dilution levels spanning high to low variant fractions, including levels below the predefined 0.5% VAF analysis threshold, with two independent replicates at each level. Samples were processed using the same targeted sequencing and bioinformatic workflow applied to the study samples. Analytical performance was assessed based on recovery of predefined positive variants across dilution levels. Background false-positive calls were additionally evaluated using approximately 20,020 predefined negative-control genomic positions in the canine genomic DNA model. These experiments were used to characterize low-VAF detection performance and to empirically support the analysis threshold used in this study. Both dilution-based analytical validation models used 10 ng of DNA input for library preparation. The longitudinal clinical plasma cfDNA libraries were prepared using approximately 10 ng of DNA input, with 19 libraries using 10 ng and one library (Dog 2 T2) using the entire available amount of 9.7 ng. To assess sequencing conditions across clinical samples, read-level coverage at plasma-positive variant positions was summarized for each longitudinal cfDNA library. Although the analytical validation and clinical samples used comparable amounts of input DNA, independent molecular depth was not determined because UMI-based consensus generation was not performed. Therefore, the predefined 0.5% VAF threshold was considered an operational analysis cutoff supported by the analytical validation results under the tested conditions, rather than a universally established detection limit.

2.8. Variant Annotation and Baseline-Defined Selection

Variants were annotated by genomic position, reference and alternate alleles, gene, protein consequence, and variant class. For longitudinal analysis, each dog's tracking set consisted of all plasma variants detected at T0 that satisfied the predefined final analytical criteria described in Section 2.6, including VAF $\geq$ 0.5% and the specified quality-control and background-filtering requirements. The same baseline-defined set was evaluated across all subsequent available samples from that dog, regardless of whether individual variants persisted, increased, decreased, or fell below the baseline inclusion threshold during follow-up.

Variants first detected after T0 were not incorporated into the fixed baseline tracking set and therefore did not contribute to its longitudinal mean VAF. The broader exploratory candidate-variant landscape was analyzed separately and did not determine the composition of the baseline tracking set.

2.9. Gene-Level Landscape Analysis

A broader set of candidate plasma genomic variant calls was used for exploratory gene-level characterization. Candidate variant records were summarized by individual sample, gene annotation, and variant class, and visualized using heatmaps and stacked bar plots to illustrate inter-individual heterogeneity and temporal variation during treatment and follow-up.

This exploratory dataset was generated using less stringent candidate-level criteria than those applied to the final analytical dataset described in Section 2.6. Consequently, the counts in the exploratory gene-level analysis represent sample-level candidate variant records rather than final retained variants. These exploratory counts were not interpreted as confirmed somatic alterations or quantitative measures of tumor burden and were not used to select variants for longitudinal tracking or cross-dog variant-sharing analyses.

2.10. Longitudinal Plasma Variant Dynamics

Longitudinal molecular dynamics were assessed by tracking the VAFs of the complete baseline-defined plasma variant set for each dog, as specified in Section 2.8. Mean VAF of that fixed set was calculated at each available time point as a descriptive summary of the aggregate longitudinal signal. Individual-variant trajectories were retained in the longitudinal analysis so that the mean did not obscure heterogeneous behavior among variants. Molecular trajectories were compared descriptively with independently assigned VCOG response categories and longitudinal lymph-node measurements; no inferential statistical test of molecular–clinical association was performed because of the small cohort and unequal sampling.

2.11. Shared and Dog-Specific Variant Analysis

Shared and dog-specific plasma genomic variants were evaluated across the seven dogs using the final stringently filtered dataset described in Section 2.6. For each dog, retained variants from all available longitudinal sampling time points were combined and deduplicated using genomic identifiers defined by chromosome, genomic position, reference allele, and alternate allele. Each unique variant was counted once per dog, regardless of its detection at multiple time points.

Variant intersections among dogs were summarized using an UpSet plot. Variants detected in at least three dogs were tabulated for exploratory assessment. Because matched tumor tissue sequencing was unavailable and recurrent technical or genomic-background effects could not be completely excluded, these recurrent plasma variants were not interpreted as established lymphoma driver mutations.

3. Results

3.1. Cohort Characteristics

A total of seven dogs with multicentric lymphoma were included in this longitudinal study (Table 1). The cohort consisted of six males and one female, with ages ranging from 8 to 16 years (median, 11 years). Multiple breeds were represented, including Welsh Corgi ($n = 2$), Maltese ($n = 1$), Cavalier King Charles Spaniel ($n = 1$), Shiba Inu ($n = 1$), Afghan Hound ($n = 1$), and mixed breed ($n = 1$). Dogs 2, 3, 4, and 6 were enrolled at relapse, whereas Dogs 1, 5, and 7 were enrolled at their initial diagnosis. Detailed clinical and demographic characteristics are summarized in Table 1.

Table 1. Clinical characteristics, treatment, and follow-up outcomes of dogs with multicentric lymphoma. All seven dogs had B-cell lymphoma and were assigned to a planned VLA chemotherapy protocol. Dogs 2, 3, 4, and 6 entered the study after relapse following prior treatment; Dogs 1, 5, and 7 were enrolled at initial diagnosis. The prior-treatment-to-relapse interval is reported only for dogs enrolled with relapsed disease (Dogs 2, 3, 4, and 6). Follow-up after enrollment is the interval from study entry to death, loss to follow-up, or the last documented clinical assessment, as indicated in the status column. These intervals have different starting points and should not be interpreted as a common PFS or OS measure. Longitudinal clinical responses during study treatment and follow-up are presented separately in subsequent sections. Substage a, absence of systemic clinical signs; substage b, presence of systemic clinical signs.

Dog	Immunophenotype	Stage	Substage	Chemotherapy Protocol	Prior Relapse Interval (Days)	Follow-up After Enrollment (Days)	Status at Last Follow-up
1	B-cell	IV	b	VLA	—	38	Lost to follow-up
2	B-cell	IV	a	VLA	1025	242	Deceased
3	B-cell	IV	b	VLA	120	93	Deceased

Table 1. *Cont.*

Dog	Immunophenotype	Stage	Substage	Chemotherapy Protocol	Prior Relapse Interval (Days)	Follow-up After Enrollment (Days)	Status at Last Follow-up
4	B-cell	IV	a	VLA	718	29	Lost to follow-up
5	B-cell	III	b	VLA	—	358	Last clinical assessment
6	B-cell	IV	a	VLA	320	22	Deceased
7	B-cell	IV	b	VLA	—	18	Deceased

VLA, vincristine–L-asparaginase–doxorubicin-based chemotherapy protocol; —, not applicable (treatment-naïve at enrollment). The prior relapse interval refers to the documented period after previous treatment and before relapse leading to study enrollment; it is not a post-enrollment PFS estimate. Follow-up after enrollment refers to the period from enrollment to death, loss to follow-up, or last clinical assessment. Dogs 2, 3, 4, and 6 were enrolled at relapse; Dogs 1, 5, and 7 were enrolled at initial diagnosis. The reasons for absence of T2 samples in Dogs 6 and 7 were deaths before their scheduled T2 collections. Dog 2 entered the study after relapse 1025 days following prior treatment and died 242 days after enrollment; its additional Tx plasma sample was collected during the study at documented subsequent progression/relapse.

3.2. Longitudinal Liquid Biopsy Workflow and Sampling Design

The overall study workflow and longitudinal sampling schedule are illustrated in Figures 1 and 2. Serial plasma samples were collected at predefined time points during treatment and follow-up, enabling an integrated assessment of clinical response, plasma cfDNA dynamics, and plasma genomic-variant dynamics over time. All seven dogs contributed T0 and T1 samples, whereas T2 samples were available from five dogs (Dogs 1–5). Dog 4 completed T2 sampling on day 29 after enrollment and was subsequently lost to follow-up. Dogs 6 and 7 died on days 22 and 18 after enrollment, respectively, before the scheduled T2 sampling. Their causes of death could not be determined from the available clinical information. Therefore, the absence of T2 molecular samples in these two dogs was attributable to death rather than loss to follow-up.

3.3. Analytical Performance Characterization

Both dilution-based models showed progressively reduced variant recovery at decreasing nominal variant fractions. At the predefined 0.5% VAF analysis threshold, the observed site-level detection rates were 90.6% and 90.2% in the synthetic-reference and canine genomic DNA models, respectively (Supplementary Table S1). Detection performance declined at nominal variant fractions below 0.5% in both models. Among 20,020 predefined negative-control genomic positions, five background variant calls were observed at or above 0.5% VAF. Together, these findings provided empirical support for the predefined 0.5% VAF operational analysis threshold used in this study.

Matched buffy coat genomic DNA was sequenced for all 20 plasma sampling time points using 30 ng of DNA input per library. At genomic positions corresponding to plasma-detected variants, mean read-level coverage ranged from 753× to 1792× across samples, with minimum locus-specific depths ranging from 176× to 395×. The proportions of positions covered at >300× and >500× ranged from 96.8% to 100% and from 87.1% to 99.4%, respectively (Supplementary Table S2). These results indicate generally high coverage at plasma-positive positions, although some loci had lower sequencing depths that may limit detection of low-frequency variants in matched buffy coat DNA.

Both dilution-based analytical models were evaluated using 10 ng of DNA input per library. Among the 20 longitudinal clinical plasma cfDNA libraries, 19 were prepared using 10 ng of DNA, whereas the Dog 2 T2 library used the entire available amount of 9.7 ng. Across the clinical samples, mean read-level sequencing depth at plasma-positive variant

positions ranged from 1590× to 3049×, while median depth ranged from 1520× to 2713× (Supplementary Table S3). These findings demonstrate closely comparable DNA input conditions between analytical characterization and clinical testing, together with generally high read-level sequencing coverage across longitudinal samples.

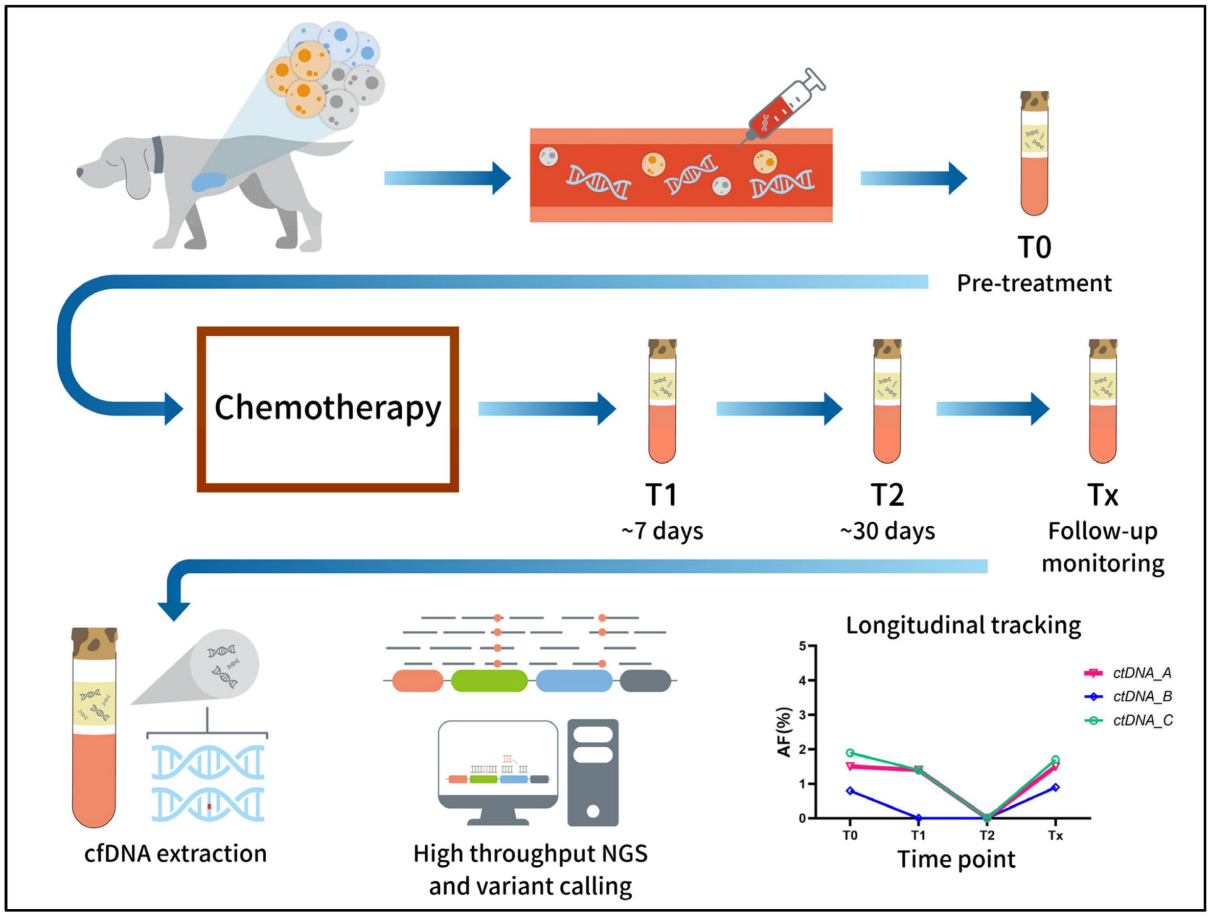


Figure 1. Overview of the study design and analytical workflow. Schematic representation of the longitudinal liquid biopsy workflow. Dogs diagnosed with multicentric lymphoma were enrolled for serial plasma sampling during treatment and follow-up. Peripheral blood samples were collected at predefined time points, including T0 (pre-treatment baseline), T1 (approximately 7 days after treatment initiation), T2 (approximately 30 days after treatment initiation), and Tx (relapse-associated sampling when available). T0 occurred before administration of chemotherapy. Plasma-derived cfDNA was analyzed using targeted next-generation sequencing (NGS), followed by plasma variant identification and longitudinal tracking of the complete baseline-defined variant set. Molecular findings were compared descriptively with independently assessed clinical response to characterize within-dog disease dynamics over time.

3.4. Gene-Level Plasma Genomic-Variant Landscape

Exploratory analysis of the broader candidate-variant dataset revealed substantial molecular heterogeneity among dogs and across longitudinal sampling time points (Figure 3). The number and composition of candidate variant records varied among samples, with multiple candidate calls observed in several genes within individual samples. The identified candidate calls included missense, synonymous, frameshift, in-frame insertion/deletion, splice-associated, and stop-gain/stop-loss variants.

These counts represent exploratory candidate-level observations rather than the final retained variant burden. Candidate variant counts did not show a uniform relationship with clinical response status and were therefore not used as a quantitative surrogate for

tumor burden. Instead, longitudinal molecular dynamics were assessed using the fixed baseline-defined variant sets described in Section 2.8.

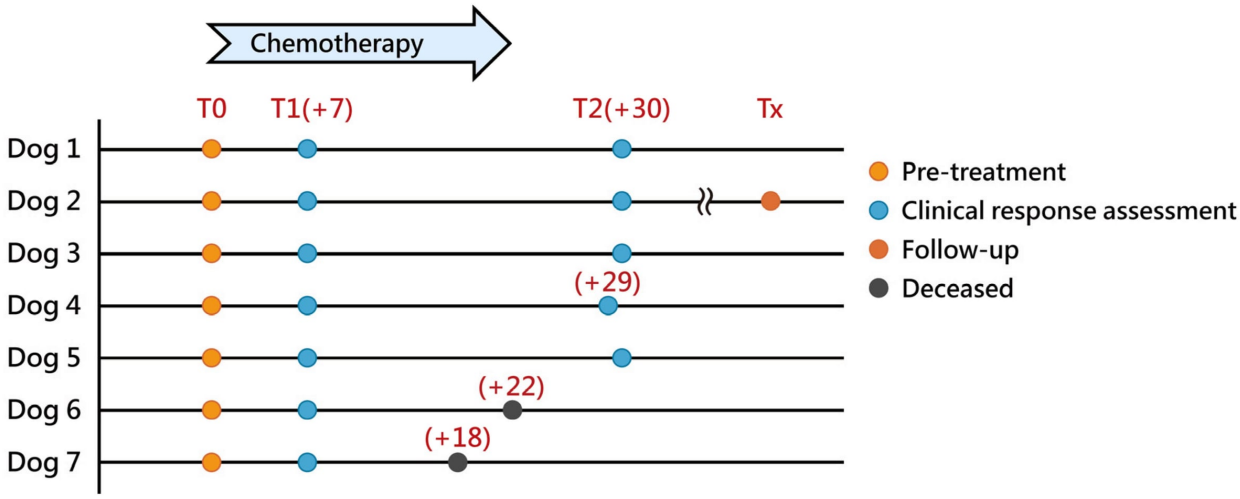


Figure 2. Longitudinal sampling timeline and clinical follow-up of seven dogs with multicentric lymphoma undergoing chemotherapy. Each horizontal line represents an individual dog (Dogs 1–7). Plasma samples were collected at baseline before chemotherapy initiation (T0), approximately 7 days after treatment initiation (T1), and approximately 30 days after treatment initiation (T2), when available. Orange circles indicate pre-treatment sampling, blue circles indicate longitudinal sampling associated with clinical response assessment, orange-red circles indicate additional follow-up sampling, and dark gray circles indicate death. Dog 4 underwent T2 sampling on Day 29 rather than Day 30. Dog 2 underwent an additional follow-up sampling (Tx) after the initial treatment assessment period. Dogs 6 and 7 died on Days 22 and 18, respectively, before the planned T2 assessment. Consequently, paired T0–T2 samples were available for five dogs. The chemotherapy arrow represents the treatment period schematically and is not drawn to scale. A break in the Dog 2 timeline indicates that the interval between T2 and Tx is not shown to scale.

3.5. Longitudinal Trajectories of Baseline-Defined Plasma Genomic Variants

All plasma variants meeting the predefined criteria at T0 were tracked longitudinally in each dog to characterize within-dog molecular dynamics during treatment and follow-up (Figure 4). Individual variants showed heterogeneous trajectories, while the mean VAF of the fixed baseline-defined set decreased after treatment initiation in several dogs with CR or PR. Dog 2 showed an increase in several baseline-defined variant signals at the relapse-associated Tx sample after lower values during treatment, concurrent with clinically documented PD. Together, these data illustrate longitudinal changes in patient-specific plasma genomic signals alongside clinical disease dynamics.

3.6. Descriptive Comparison of Plasma cfDNA Changes and Tumor Burden Dynamics

Tumor burden was assessed by serial measurements of target peripheral lymph nodes and summarized as the sumLD. Percent changes in tumor burden (ΔsumLD) were used to evaluate longitudinal changes in clinical disease burden. Plasma cfDNA concentrations were measured at corresponding time points, and changes in plasma cfDNA (ΔcfDNA) were compared with changes in tumor burden (Table 2, Figure 5). Detailed longitudinal measurements for all dogs and sampling intervals are provided in Supplementary Table S4.

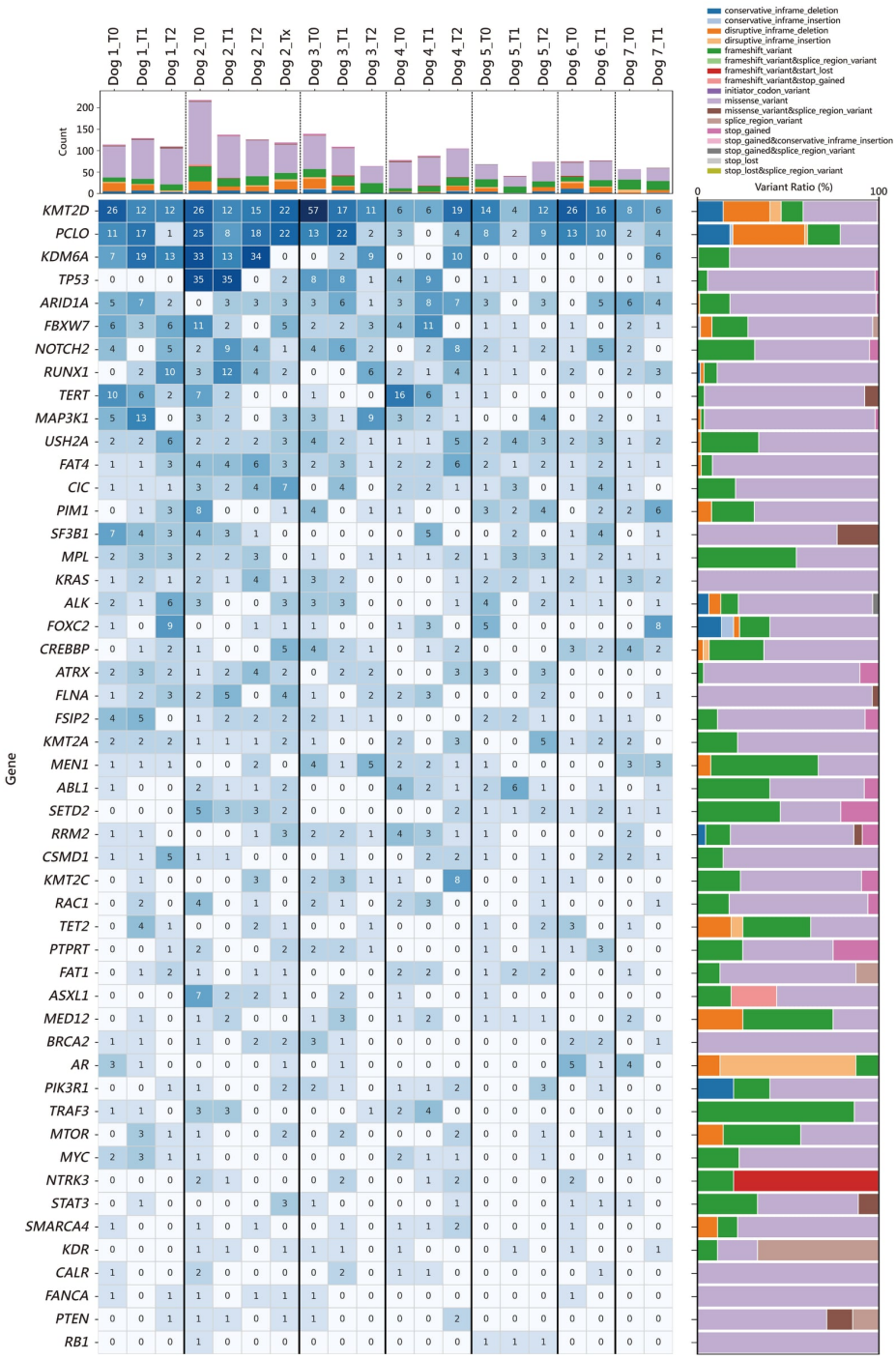


Figure 3. Gene-level landscape and variant composition of plasma genomic alterations detected by longitudinal liquid biopsy. Candidate variant records identified using broader exploratory filtering criteria were summarized at the gene level across serial samples. The upper bar plot shows the number of candidate variant records per sample, ordered by dog and longitudinal sampling time point. Dashed vertical lines indicate boundaries between individual dogs. The central heatmap illustrates the distribution of gene-level candidate alterations across serial plasma samples, highlighting inter-individual heterogeneity and temporal variation. The right stacked bar plot summarizes the relative proportions of functional variant classes associated with each gene, including missense, synonymous, frameshift, in-frame insertion/deletion, splice-associated, and stop-gain/stop-loss variants. The blue color gradient in the heatmap represents the number of candidate variant records, with darker shades indicating higher counts. Colors in the stacked bar plots represent different functional variant classes, as indicated in the legend. These exploratory candidate counts do not represent the final stringently filtered variant dataset used for longitudinal tracking or cross-dog comparisons.

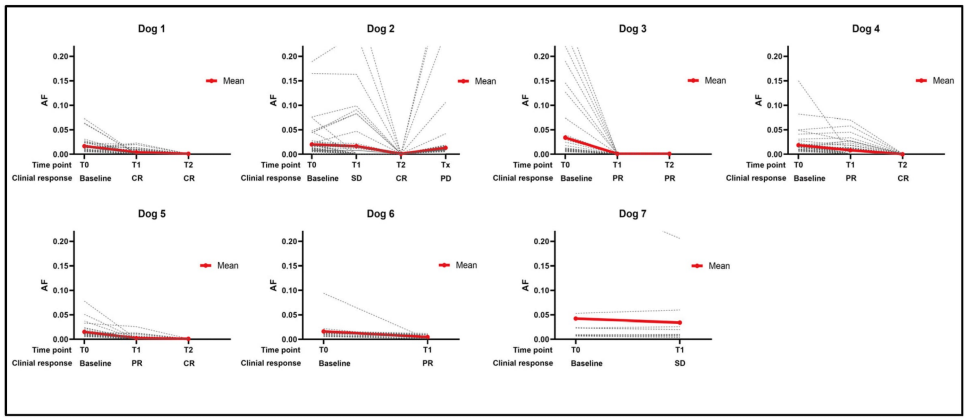


Figure 4. Longitudinal trajectories of plasma-derived genomic variants during chemotherapy and follow-up in individual dogs with multicentric lymphoma. Each panel represents an individual dog. Thin gray dashed lines represent the longitudinal VAF trajectories of individual plasma genomic variants that met the predefined analytical criteria at the pre-treatment baseline (T0), including VAF ≥ 0.5%, sufficient sequencing quality and coverage, and absence from matched buffy coat genomic DNA according to the filtering criteria. The same complete T0-defined variant set was tracked across all subsequent available sampling time points. Thick red lines represent the mean VAF of this baseline-defined variant set at each time point. T0 represents the pre-treatment baseline; T1 and T2 represent approximately 7 and 30 days after treatment initiation, respectively; and Tx represents the relapse-associated sample available for Dog 2. Clinical response was assessed independently according to VCOG criteria. The baseline tracking set was defined using the final analytical criteria described in Section 2.6 and was independent of the broader exploratory candidate-variant dataset used for Figure 3. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; VAF, variant allele frequency.

Table 2. Longitudinal changes in tumor burden and plasma cfDNA concentration in dogs with multicentric lymphoma. Changes in clinically measured tumor burden and plasma cfDNA concentration at treatment-associated and recurrence-associated time points are shown for evaluable dogs. Tumor burden was assessed by serial caliper-based measurements of peripheral lymph nodes and summarized as the sum of the longest diameters (sumLD). Percent changes in tumor burden (ΔsumLD) and plasma cfDNA concentration (ΔcfDNA) were calculated relative to the reference time point specified below. Clinical responses were assigned according to Veterinary Cooperative Oncology Group (VCOG) response criteria.

Dog [1]	Clinical Interval [2]	sumLD (cm)	ΔsumLD (%)	cfDNA (ng/mL)	ΔcfDNA (%)	Clinical Response
Dog 1	Pre-treatment	10.50		2640.00		
	T2 (~30 days; on-treatment)	0.00	−100%	3.89	−100%	CR
Dog 2	Pre-treatment	12.40		4.74		
	T2 (~30 days; on-treatment)	3.10	−75%	2.43	−49%	CR
	Recurrence	9.00	190%	20.79	756%	PD
Dog 3	Pre-treatment	14.50		25.26		
	T2 (~30 days; on-treatment)	5.00	−66%	4.43	−82%	PR
Dog 4	Pre-treatment	10.50		7.04		
	T2 (~30 days; on-treatment)	0.00	−100%	2.53	−64%	CR
Dog 5	Pre-treatment	18.70		5.96		
	T2 (~30 days; on-treatment)	0.00	−100%	5.34	−10%	CR

[1] Dogs 6 and 7 did not contribute T2 samples because they died before their scheduled T2 collections. Consequently, the T0–T2 treatment-response comparison includes only Dogs 1–5. [2] For treatment-response comparisons, ΔsumLD and ΔcfDNA were calculated between the pre-treatment baseline (T0) and the approximately day-30 on-treatment assessment (T2). For the recurrence-associated comparison in Dog 2, changes were calculated between T2 and the relapse-associated time point (Tx), with T2 used as the reference.

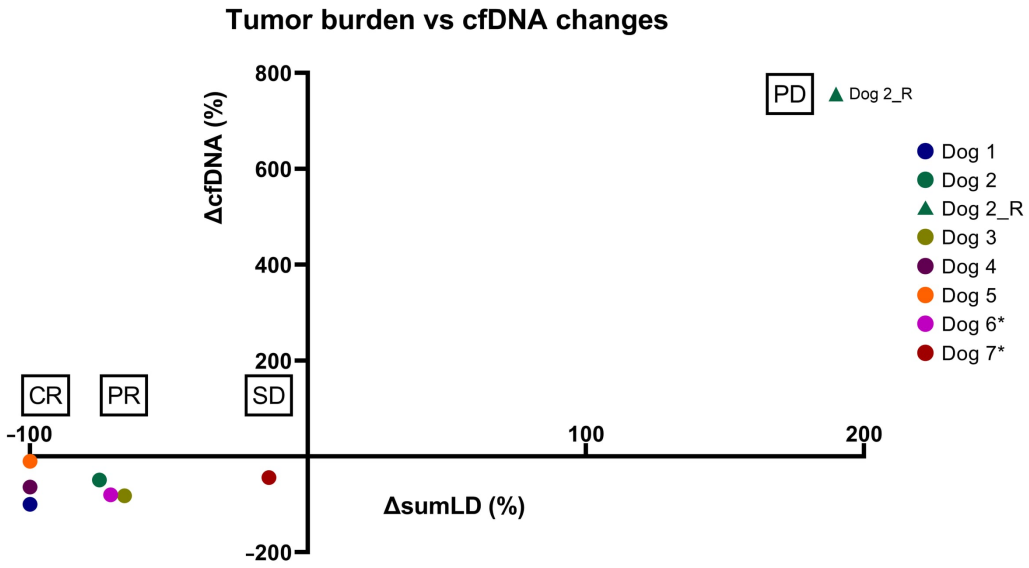


Figure 5. Descriptive comparison of changes in tumor burden and plasma cfDNA across longitudinal samples. ΔcfDNA was plotted against percent change in tumor burden (ΔsumLD), calculated from serial measurements of target peripheral lymph nodes. Each point represents an evaluable longitudinal sampling interval. For Dogs 1–5, changes were calculated between T0 and T2. For the recurrence-associated interval in Dog 2, changes were calculated between T2 and the recurrence-associated Tx sample. Dog 2_R denotes the recurrence-associated interval between T2 and Tx in Dog 2. Asterisks (*) indicate Dogs 6 and 7, for which only T0–T1 intervals were available because both dogs died before the scheduled T2 sampling. Points are color-coded by dog and annotated according to clinical response category (CR, PR, SD, and PD). The plot illustrates the relationship between molecular and clinical changes observed during treatment and disease progression. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

Across the five evaluable T0–T2 treatment-response comparisons, plasma cfDNA concentration decreased in all dogs that had CR or PR at T2, although the magnitude of decrease varied substantially among dogs. In Dog 2, the relapse-associated interval showed concurrent increases in sumLD and plasma cfDNA. Dogs 6 and 7 contributed only early-treatment T0–T1 intervals and were therefore shown in Figure 5 for descriptive context but were not included in the T0–T2 treatment-response comparison in Table 2. These data indicate that total plasma cfDNA can change alongside clinically measured disease burden in individual dogs, but cfDNA is not tumor-specific and the small number of evaluable intervals precludes inference regarding monitoring performance.

Dog 1 exhibited an exceptionally high baseline plasma DNA concentration of 2640 ng/mL at T0, which decreased to 33.58 ng/mL at T1 and 3.89 ng/mL at T2 (Supplementary Table S5). The baseline sample was quantified using the Qubit dsDNA BR Assay because its concentration exceeded the quantification range of the HS assay. However, direct fragment-size analysis of extracted plasma DNA was not performed, and potential genomic DNA contamination could not be definitively excluded. To assess the influence of this extreme baseline value, an additional descriptive sensitivity analysis was performed excluding Dog 1. Plasma DNA concentrations decreased in all four remaining dogs with paired T0–T2 samples, with reductions ranging from 10.4% to 82.5% (median, 56.4%). These findings indicate that the observed direction of longitudinal plasma DNA changes was not solely driven by Dog 1, although the small sample size and potential preanalytical variability warrant cautious interpretation.

3.7. Shared and Dog-Specific Plasma Genomic Variants Across the Cohort

To characterize inter-individual patterns of plasma genomic variation, final retained variants were compared across the seven dogs after aggregation of all available longitudinal samples and deduplication by genomic identifier (Figure 6). Most variants were unique to individual dogs, whereas a smaller subset was shared among multiple dogs.

For Dog 7, the final filtered dataset contained 11 unique variants across T0 and T1, including one *KMT2D* and two *ARID1A* variants. In contrast, the broader exploratory dataset used for Figure 3 contained eight *KMT2D* and six *ARID1A* candidate variant records at T0. This difference reflects the use of broader sample-level candidate variant criteria for Figure 3 and more stringent filtering followed by dog-level aggregation and deduplication for Figure 6.

Seventeen recurrent variants were identified in at least three dogs and are summarized in Table 3. Of these, 16 were detected in three dogs and one in four dogs. These recurrent variants represent exploratory findings that warrant further investigation, particularly through tumor-matched sequencing to clarify their biological origins and potential relevance to canine lymphoma.

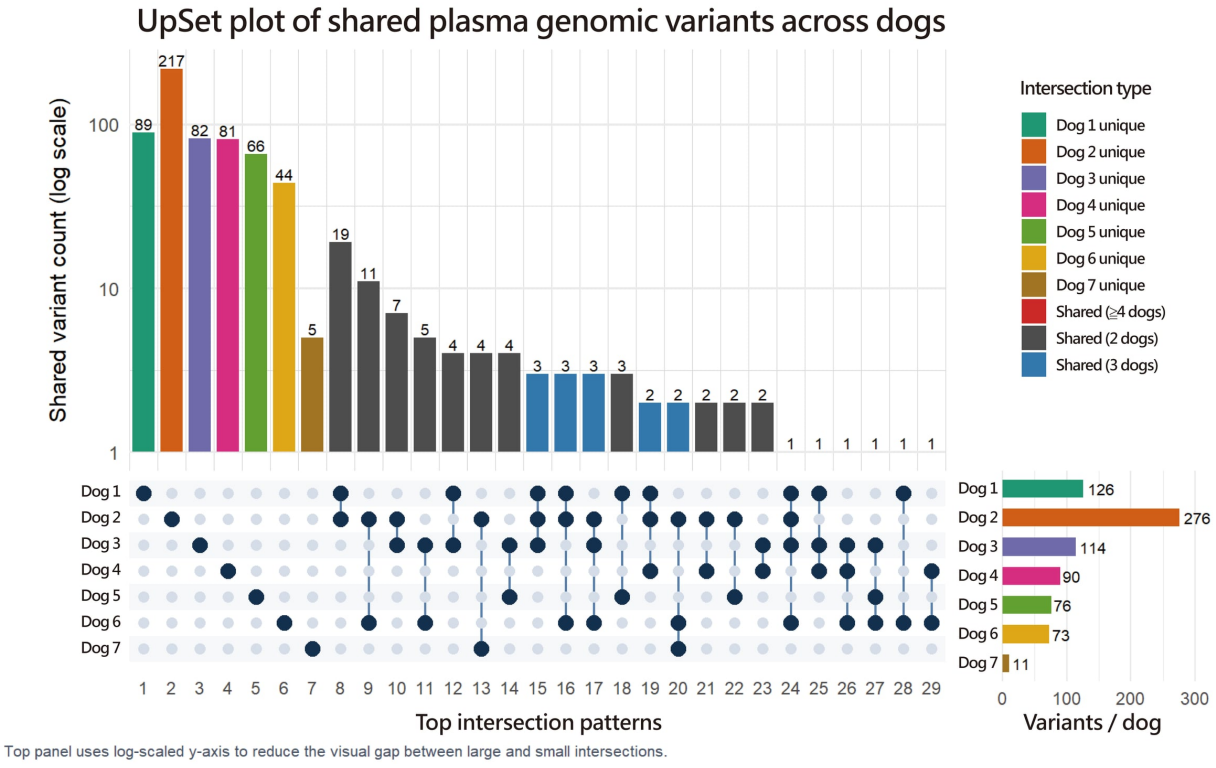


Figure 6. UpSet analysis of shared and dog-specific final filtered plasma genomic variants across seven dogs. Variants retained after the predefined stringent filtering procedures were combined across all available longitudinal samples for each dog and deduplicated using genomic identifiers defined by chromosome, position, reference allele, and alternate allele. Each unique variant was counted once per dog. The set sizes therefore represent dog-level unique retained variants rather than sample-level candidate variant records. For Dog 7, the set size of 11 represents the combined and deduplicated final variant set across T0 and T1. The upper intersection bars are displayed on a logarithmic scale to improve visualization of both large and small intersections. Different colors in the intersection bars distinguish dog-specific variants and variants shared among two, three, or four dogs, as indicated in the legend. The horizontal bars represent the total number of unique retained variants for each dog.

Table 3. Recurrently detected plasma genomic variants across multiple dogs. Seventeen recurrent plasma genomic variants detected in at least three dogs are summarized. Variants were derived from the final filtered dataset used for the cross-dog variant-sharing analysis in Figure 6, following the predefined quality-control and background-filtering procedures described in Section 2.6. Each row represents a distinct genomic variant assigned to a study-specific Variant ID. Binary indicators denote variant presence (1) or absence (0) in individual dogs, and the Occurrence column indicates the number of dogs in which each variant was detected. Variant classes are provided without displaying complete genomic allele sequences. Given the absence of matched tumor tissue sequencing, these recurrent plasma variants should not be interpreted as confirmed lymphoma driver mutations.

Chromosome	Gene	Variant ID	Variant type	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6	Dog 7	Occurrence
Chr18	*PCLO*	PCLO-1	conservative_in-frame_deletion	1	1	1	0	0	1	0	4
Chr9	*ABL1*	ABL1-1	missense_variant	1	1	0	1	0	0	0	3
ChrX	*AR*	AR-1	disruptive_in-frame_insertion	1	1	0	0	0	1	0	3
Chr2	*ARID1A*	ARID1A-1	missense_variant	0	1	0	0	0	1	1	3
Chr25	*BRCA2*	BRCA2-1	missense_variant	1	1	0	0	0	1	0	3
Chr6	*CREBBP*	CREBBP-1	frameshift_variant	0	1	0	0	0	1	1	3
Chr15	*FBXW7*	FBXW7-1	missense_variant	1	1	1	0	0	0	0	3
Chr27	*KMT2D*	KMT2D-1	missense_variant	1	0	1	1	0	0	0	3
Chr27	*KMT2D*	KMT2D-2	disruptive_in-frame_deletion	0	1	1	0	0	1	0	3
Chr27	*KMT2D*	KMT2D-3	disruptive_in-frame_deletion	1	1	0	0	0	1	0	3
Chr27	*KMT2D*	KMT2D-4	missense_variant	0	1	1	0	0	1	0	3
Chr27	*KMT2D*	KMT2D-5	disruptive_in-frame_deletion	1	1	1	0	0	0	0	3
Chr27	*KMT2D*	KMT2D-6	disruptive_in-frame_deletion	0	0	1	0	1	1	0	3
Chr27	*KMT2D*	KMT2D-7	frameshift_variant	0	0	1	1	0	1	0	3
Chr9	*STAT3*	STAT3-1	missense_variant	0	1	1	0	0	1	0	3
Chr34	*TERT*	TERT-1	missense_variant	1	1	0	1	0	0	0	3
Chr38	*USH2A*	USH2A-1	missense_variant	1	1	1	0	0	0	0	3

4. Discussion

This pilot study shows the feasibility of integrating serial plasma cfDNA quantification and targeted genomic profiling with conventional clinical assessment in canine multicentric B-cell lymphoma. The principal contribution is longitudinal: a fixed, baseline-defined set of plasma variants could be followed repeatedly during chemotherapy, while total cfDNA and variant-level signals showed measurable within-dog changes over time. Together with the sample-level genomic landscape, this approach provides a framework for patient-specific serial plasma profiling in a setting where repeated tissue sampling is impractical.

The longitudinal observations are consistent with emerging evidence supporting the potential value of liquid biopsy in canine cancer monitoring. Nascimento et al. reported that serial blood-based lymphoma test results were concordant with VCOG-defined disease status in many observations, including changes associated with remission and relapse [27]. Similarly, Megquier et al. reported longitudinal changes in canine liquid-biopsy metrics associated with disease status and observed increased tumor fraction preceding clinical relapse in some dogs with lymphoma or osteosarcoma [28]. In the present cohort, Dog 2 exhibited increased plasma cfDNA concentration and several baseline-defined plasma variant signals at the relapse-associated sampling time point, accompanied by clinical progression.

Although this represents a single relapse-associated observation rather than evidence of predictive lead time, the concordant molecular and clinical changes support further investigation of serial plasma profiling for disease monitoring. These findings are also biologically consistent with established ctDNA dynamics in human B-cell lymphoma [21,22,31].

Total plasma cfDNA provided complementary quantitative information. All five dogs with paired T0–T2 measurements showed decreased plasma cfDNA concentrations during chemotherapy, despite substantial differences in their baseline values. Notably, this directional pattern remained consistent in all four evaluable dogs after excluding Dog 1, which had an exceptionally high baseline plasma DNA concentration (2640 ng/mL). This sensitivity analysis supports the observation that the overall downward trend was not solely driven by a single extreme measurement. However, total plasma cfDNA includes DNA released from both tumor and non-tumor cells, and its concentration may also be influenced by inflammation, tissue injury, treatment-related cellular turnover, and preanalytical factors [17,20,28]. Accordingly, longitudinal cfDNA concentration is best interpreted alongside clinical findings and genomic profiling rather than as an isolated tumor-specific measurement.

The genomic findings further highlight the relevance of patient-specific monitoring strategies. The plasma variant landscape showed substantial inter-individual heterogeneity, with most retained variants detected in individual dogs and a smaller subset shared across the cohort. Rather than relying exclusively on recurrent variants, our longitudinal analysis used a fixed set of eligible variants identified at baseline to provide a consistent reference for subsequent measurements. This predefined approach reduces the risk of retrospectively selecting variants based on treatment outcomes and facilitates reproducible within-dog comparisons. The broader exploratory candidate-variant analysis and the final filtered longitudinal dataset provide complementary perspectives on genomic heterogeneity, although their different filtering levels and counting units should be considered when comparing the corresponding figures.

Interpretation of plasma genomic variants requires consideration of their biological origin. Matched buffy coat sequencing was incorporated to reduce germline and hematopoietic-background variant calls, providing an important additional filtering step. Previous canine studies have demonstrated concordance between tumor and plasma genomic alterations, supporting the biological rationale for plasma-based genomic profiling [17,28]. However, matched tumor sequencing was not available for the present cohort, and the hematopoietic nature of lymphoma introduces additional complexity when interpreting cellular-fraction controls. Therefore, retained calls are described as plasma genomic variants rather than individually confirmed tumor-derived mutations. This distinction allows the longitudinal molecular observations to be reported without assuming tumor origin for every detected variant.

Analytical characterization provided empirical support for the predefined 0.5% VAF operational analysis threshold. Two complementary dilution-based models, evaluated using 10 ng of DNA input and two independent library preparation and sequencing replicates per dilution level, yielded observed site-level detection rates of approximately 90% at a nominal VAF of 0.5%. Clinical plasma libraries were prepared under closely comparable input conditions, with 10 ng used for 19 samples and 9.7 ng for Dog 2 at T2. These findings support the feasibility of detecting low-frequency plasma variants under the tested conditions. Nevertheless, the limited number of independent replicates did not establish a formal limit of detection at 95% detection probability (LOD95), and read-level sequencing coverage should not be equated with UMI-consensus molecular depth. Further analytical validation will be needed to characterize performance across variant classes, DNA inputs, and genomic contexts.

Several limitations should be considered when interpreting these findings. The cohort comprised seven dogs, with only five providing paired T0–T2 samples and one providing an additional relapse-associated sample. The absence of scheduled T2 samples from dogs that died before collection introduces potential survivor bias, while differences in prior treatment exposure, baseline disease status, and follow-up duration limit direct comparisons across dogs. The study was not designed or powered to establish prognostic performance, relapse-detection sensitivity, or predictive lead time. In addition, direct fragment-size assessment of extracted plasma DNA was unavailable, preventing definitive exclusion of high-molecular-weight genomic DNA contamination, particularly for the unusually high baseline measurement from Dog 1. Matched tumor sequencing and orthogonal confirmation were also unavailable, limiting assignment of individual plasma variants to lymphoma. These considerations define the scope of the findings as exploratory longitudinal observations rather than validated clinical monitoring endpoints.

Future studies should evaluate this approach in larger cohorts with standardized sampling schedules, systematic relapse surveillance, and prespecified molecular endpoints. Where feasible, tumor-matched sequencing, orthogonal variant confirmation, and expanded analytical validation would strengthen the interpretation of plasma genomic signals. Integration of additional molecular features, including copy-number alterations, tumor-fraction estimates, and fragmentomic characteristics, may further improve longitudinal assessment. Recent canine studies and reviews also support continued investigation of liquid biopsy as an adjunct to conventional clinical assessment rather than a replacement for established diagnostic methods [29,30]. Overall, the present findings provide preliminary longitudinal evidence and a methodological framework for further evaluating plasma cfDNA quantification and genomic profiling as complementary approaches to clinical monitoring in canine lymphoma.

5. Conclusions

This prospective pilot study demonstrates the feasibility of integrating serial plasma cfDNA quantification and targeted genomic profiling with conventional clinical assessment in canine multicentric B-cell lymphoma. The observed within-dog molecular changes during chemotherapy, together with the relapse-associated increase in cfDNA and selected variant signals in one dog, provide preliminary support for the potential value of patient-specific longitudinal plasma monitoring.

Although the small cohort, incomplete longitudinal sampling, and lack of tumor-matched confirmation limit clinical generalizability, these findings establish a practical framework for future prospective studies. Serial plasma profiling may serve as a complementary approach to clinical examination, imaging, and tissue-based diagnosis, with further validation needed to define its role in treatment-response assessment and relapse monitoring.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/ani16233794/s1. Table S1, Detection performance of two dilution-based analytical models across nominal variant fractions; Table S2, Sequencing quality metrics and locus-specific coverage of matched buffy coat genomic DNA at plasma-positive variant positions; Table S3, DNA input and read-level sequencing coverage at plasma-positive variant positions across longitudinal plasma cfDNA samples; Table S4, Longitudinal measurements of tumor burden (sumLD) and plasma cfDNA concentration for all dogs at each sampling time point (T0, T1, T2, and follow-up where available); Table S5, Plasma DNA quantification and recovery characteristics across longitudinal sampling time points in seven dogs with multicentric B-cell lymphoma.

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draft preparation, F.-M.L.; writing—review and editing, F.-M.L. and S.-L.W.; visualization, F.-M.L. and S.-H.L.; supervision, F.-M.L., Y.-C.K. and S.-L.W.; project administration, F.-M.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with institutional animal care and use guidelines and approved by the Institutional Animal Care and Use Committee of the National Taiwan University (Approval number: NTU113-EL-00097).

Informed Consent Statement: Written informed consent was obtained from the owners of all client-owned dogs included in this study prior to enrollment and sample collection.

Data Availability Statement: The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request. Due to privacy considerations and proprietary aspects of the assay, certain raw sequencing data may not be publicly available.

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Conflicts of Interest: F.-M.L., S.-H.L., F.-C.H., K.C. and Y.-C.K. are employees of PETcision, which developed the assay evaluated in this study. F.-M.L., S.-H.L., F.-C.H., K.C. and Y.-C.K. are inventors on patents related to the technology described in this work. F.-M.L., K.C. and Y.-C.K. are shareholders of PETcision. F.-M.L. serves as Chief Operating Officer (COO) and Y.-C.K. serves as Chief Executive Officer (CEO) of PETcision. The remaining authors declare no competing interests. The study design, data collection, data analysis, interpretation of the results, and preparation of the manuscript were performed by the authors independently.

Abbreviations

The following abbreviations are used in this manuscript:

MRD	minimal residual disease
VCOG	Veterinary Cooperative Oncology Group
cfDNA	cell-free DNA
ctDNA	circulating tumor DNA
SNV	single-nucleotide variant
Indel	insertion/deletion
CNA	copy number alteration
VAF	variant allele frequency
NGS	next-generation sequencing
sumLD	sum of the longest diameters
ΔsumLD	percent change in tumor burden
CR	complete response
PR	partial response
SD	stable disease
PD	progressive disease
ΔcfDNA	percent change in plasma cfDNA concentration

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