Comparing *Bam*HI-W and CE-marked assays to detect circulating Epstein-Barr Virus (EBV) DNA of nasopharyngeal cancer patients in a non-endemic area

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ABSTRACT

Objectives: Plasma Epstein-Barr Virus (EBV)-DNA is a well-established prognostic biomarker in nasopharyngeal carcinoma (NPC). Different methods for assessment include single-copy gene targeted, European Conformity (CE)-marked assays, which are mostly employed in non-endemic settings, vs multiple-copy gene targeted, in-house *Bam*HI-W based assays, which currently represent the most widely used method for EBV-DNA quantification. To date, evidence concerning the commutability of these different assays is still limited.

Materials and methods: From August 2016 to March 2018, 124 plasma and 124 whole blood (WB) samples from 93 NPC patients were collected at different time-points for each patient. EBV-DNA viral load was quantified in pre- (n = 12) and post-treatment (n = 9), follow-up (n = 53), and recurrent/metastatic (R/M) (n = 50) phase. For each sample, one in-house *Bam*HI-W vs three different CE-marked plasma assays were compared; the performance of plasma vs WB matrix was also assessed. Quantitative agreement of EBV-DNA values was evaluated by linear correlation and Bland-Altman analysis.

Results: A statistically significant (p = 0.0001) agreement between all CE-marked and the *Bam*HI-W assays was found using plasma matrix, regardless of clinical phase. The results obtained in copies/ml were comparable to those expressed in IU/ml. When using WB matrix, the number of positive detections increased in the post-treatment phase.

Conclusions: Our retrospective comparison supported an agreement between Plasma *Bam*HI-W and CE-marked assays in measuring EBV-DNA for non-endemic NPC patients. There were no significant interferences from different measurement units (IU/ml vs copies/ml). Further evaluations are needed to better clarify the role of WB.

Abbreviations: EBV, Epstein-Barr Virus; NPC, nasopharyngeal carcinoma; CE, European Conformity; WB, whole blood; R/M, recurrent/metastatic; RT-PCR, real-time polymerase chain reaction; PCR, polymerase chain reaction; IVD, In Vitro Diagnostic; WHO, World Health Organization; IU, International Unit; INT, Istituto Nazionale dei Tumori; EBER, EBV-encoded RNAs; AJCC, American Joint Committee for Cancer; RT, radiotherapy; CTRT, concomitant chemo-radiotherapy; ICT, induction chemotherapy; CT, chemotherapy; LLoQ, lower limit of quantification; mOS, median Overall Survival.

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Introduction

Nasopharyngeal carcinoma (NPC) is endemic in Asian countries (20–50/100.000 new cases per year), whereas incidence is low (0.5/100.000/year) in Europe and the Western world [1], defined as 'non-endemic'. Epstein-Barr Virus (EBV) reversion from latent to lytic state is one of the main drivers in NPC pathogenesis [2].

Plasma EBV-DNA detection was identified as prognostic biomarker in endemic and non-endemic NPC patients, [3–7] offering a helpful tool in treatment planning and patients' monitoring. In endemic areas, USA and clinical trials, the most used EBV-DNA quantification method is in-house *Bam*HI-W assay, [8–10] targeting multiple-repeat fragments. European regulatory agencies recommend "European Conformity" (CE)-labelled diagnostic tests, intended for In Vitro Diagnostic use (IVD), according to the Guidance on IVD Medical Devices Directive 98/79/EC. Most of these CE-assays target single-copy genes. NPC guidelines [11–12] suggest to include circulating EBV-DNA measurement in pre- and post-treatment workup due to its prognostic value [13]. However, there is limited evidence [14] on a formal comparison among these methods. Further technical discrepancies (e.g. assay design, extraction protocol, results reporting, etc.) [9] lead to uncertainties on the interpretation and commutability of EBV-DNA quantification assays [8,15]. *Bam*HI-W assay measures a variable repeat EBV-DNA genomic region, potentially leading to higher sensitivity, but also to possible inter-subject quantification bias. Therefore, we compared the performance of four real-time PCR-based assays measuring plasma EBV-DNA: three commercial (CE-IVD marked) single-copy gene vs one in-house *Bam*HI-W targeting assays.

In addition, given the role of EBV-DNA testing in NPC, it would be useful to increase its sensitivity to refine treatment tailoring and monitoring strategies, potentially improving patients' survival outcomes [4,5]. Thus, we collected whole blood (WB) in addition to plasma samples, and we assessed the performance of CE- and *Bam*HI-W assays using WB. WB can identify EBV-DNA both in cells (not only tumor cells) and circulating cell-free DNA, bearing an advantage over plasma for EBV-DNA assessment [16].

Materials and methods

Study population

From August 2016 to March 2018, peripheral blood samples (12 ml) were collected in EBV-related NPC patients managed at the Fondazione IRCCS Istituto Nazionale dei Tumori (INT) of Milano and Fondazione IRCCS Policlinico San Matteo of Pavia. EBV infection was detected in primary tissue samples by EBV-encoded RNAs (EBER) in situ hybridization. Baseline patients' clinical characteristics are summarized in Table 1. Samples were collected from 93 NPC patients (86 at INT and 7 at Pavia).

Sample collection

Peripheral blood samples were collected in K_2EDTA tubes. Plasma was separated from peripheral blood by centrifugation. WB and plasma samples were de-identified and stored in aliquots at −80 °C until use.

Throughout NPC patients' management, samples collection was not systematic, and it was performed at different time-points for each patient. Overall, four phases of sample collection were retrospectively identified: 1) within 2 weeks before curative treatment start; 2) early post-curative treatment (within 4 weeks since RT end); 3) post-treatment follow-up (Supplementary Table 1); 4) R/M disease monitoring (before treatment start and then every 2–3 months).

EBV-DNA detection

EBV-DNA levels were measured on plasma and WB samples by quantitative real-time polymerase chain reaction (RT-PCR). For each sample, we compared four different assays: three are commercial, CE-IVD marked and target a single-copy gene. These were performed according to manufacturer's instructions and results were calculated in International Unit per milliliter (IU/ml) and copies/ml. The fourth is an in-house assay amplifying the repetitive *Bam*HI-W sequence; results were calculated in copies/ml.

Analyses were performed by 3 different automated systems in two laboratories: m2000 (Abbott Molecular, Des Plaines, IL) and ELITeInGenius (ELITechGroup Spa, Torino, Italy) systems were used at INT, while QIAsymphony RGQ (QIAGEN, Hamburg, Germany) was used at Pavia for two different assays (Artus and *Bam*HI-W). For assays' characteristics see Supplementary Table 2.

Data analysis

Analyses were conducted considering the viral load obtained

Table 1
Baseline clinical characteristics of study population.

Characteristics	N (% or range)
Gender	
M	68 (73.1)
F	25 (26.9)
Median Age	48 (19–75)
Treatment at diagnosis	
ICT followed by CTRT	40 (43)
CTRT^{+}	47 (50.5)
RT*	3 (3.2)
CT**	3 (3.2)
Neck surgery	
No	75 (80.6)
Yes	17 (18.3)
Unknown	1 (1.1)
Stage (AJCC Cancer Staging Manual, VIII Edition) at diagnosis	
I	1 (1.1)
II	14 (15.1)
III	31 (33.3)
IVa	32 (34.4)
IVb	11 (11.8)
Unknown	4 (4.3)
T stage at diagnosis	
T_{1-2}	48 (51.6)
T_{3-4}	33 (35.5)
T_x***	8 (8.6)
Unknown	4 (4.3)
N stage at diagnosis	
N_{0-1}	26 (28.0)
N_2	37 (39.8)
N_3	23 (24.7)
N_x***	3 (3.2)
Unknown	4 (4.3)
Relapse	
No	49 (52.7)
Yes	32 (34.4)
Locoregional	*21 (65.6)*
Distant	*9 (28.1)*
Locoregional + distant	*2 (6.3)*
N/A (ab initio metastatic disease)	11 (11.8)
Unknown	1 (1.1)
Death	
No	78 (83.9)
Yes	14 (15.0)
Unknown	1 (1.1)
Time of sampling	
Pre-treatment	12
Post-treatment	9
Follow-up	53
Recurrent/Metastatic	50
Median follow-up, months (range)	40 (2–307)

*Exclusive radiotherapy for early-stage disease; **palliative chemotherapy; ***patients at relapse, no data about disease at diagnosis; $^{+}$for stage III-IV disease except for one case classified as cT2 cN0 (II stage).

through *Bam*HI-W plasma assay as a reference [10]. We compared the obtained log10-transformed viral load using each CE-method (Artus, Abbott, ELITech) with this reference. Considering the current use of World Health Organization (WHO) standard IU/ml as measurement unit for EBV-DNA, [17] we compared CE-methods in IU/ml vs *Bam*HI-W in copies/ml. We repeated the analyses considering all methods in copies/ml.

Quantitative agreement was assessed through linear regression analysis which allowed the estimation of the regression line (estimated slope and intercept) [14]. Bland-Altman plots allowed the estimation of the bias between each pair of methods, its variance and 95 % confidence intervals. Samples lying outside the 95 % confidence interval were considered as outliers.

We verified EBV-DNA prognostic value by comparing viral load in pre- vs post-treatment samples +/- recurrence. Early post-curative treatment (phase 2) and follow-up (phase 3) samples were all considered as "post-treatment" samples. We compared the ability of each method to detect EBV-DNA (including "detected and quantified" values and "detected below lower limit of quantification (LLoQ)" values), as a preliminary indicator of its prognostic capability. We compared log10 viral load obtained from plasma vs WB assays.

Results

We evaluated EBV-DNA viral load in 248 samples (124 plasma and 124 WB) by considering four assays. Each sample was labeled according to the sampling setting (12 pre-treatment, 9 early post-curative treatment, 53 post-treatment follow-up and 50 R/M disease).

Agreement between BamHI-W and CE-assays in plasma

We found agreement between all CE- and *Bam*HI-W assays, with high correlation (Fig. 1**A-C**). All estimated slopes were < 1, suggesting a positive proportional bias for *Bam*HI-W. All estimated intercepts were < 0, implying that *Bam*HI-W can provide larger values than CE-methods. Correlation was always statistically significant ($p < 0.0001$).

At Bland-Altman analysis, all CE-assays had a mean difference in the 0.5–1 log interval, with a variance in the order of 1 log, and < 8 % outliers out of total samples (Table 2). Comparing all CE-methods, Artus agreed with *Bam*HI-W with the lowest bias, the smallest amplitude of agreement interval, and the highest percentage of values within 1 log difference. Bland-Altman plot showed a narrow distribution within the mean of Artus (Fig. 1**D**). Abbott had larger agreement intervals and a more scattered distribution of differences compared to Artus. The difference had a slight tendency to increase with the magnitude of the average value detected (Fig. 1**E**). ELITech had a similar distribution to Artus, with larger intervals and bias (Fig. 1**F**).

We repeated the same analysis transforming CE-methods values from IU/ml to copies/ml, to match *Bam*HI-W measurement unit. The

Table 2
Results of Bland Altman analysis for the plasma matrix.

	Artus (IU/ml)	Abbott (IU/ml)	ELITech (IU/ml)
Bias	−0.75	−1.00	−0.77
Variance	0.92	1.14	0.97
Confidence Interval	1.06 −2.55	1.23 −3.23	1.14 −2.68
% in 1 log	55.56	53.17	55.56
Outliers (%)	10 (8 %)	2 (2 %)	8 (6 %)

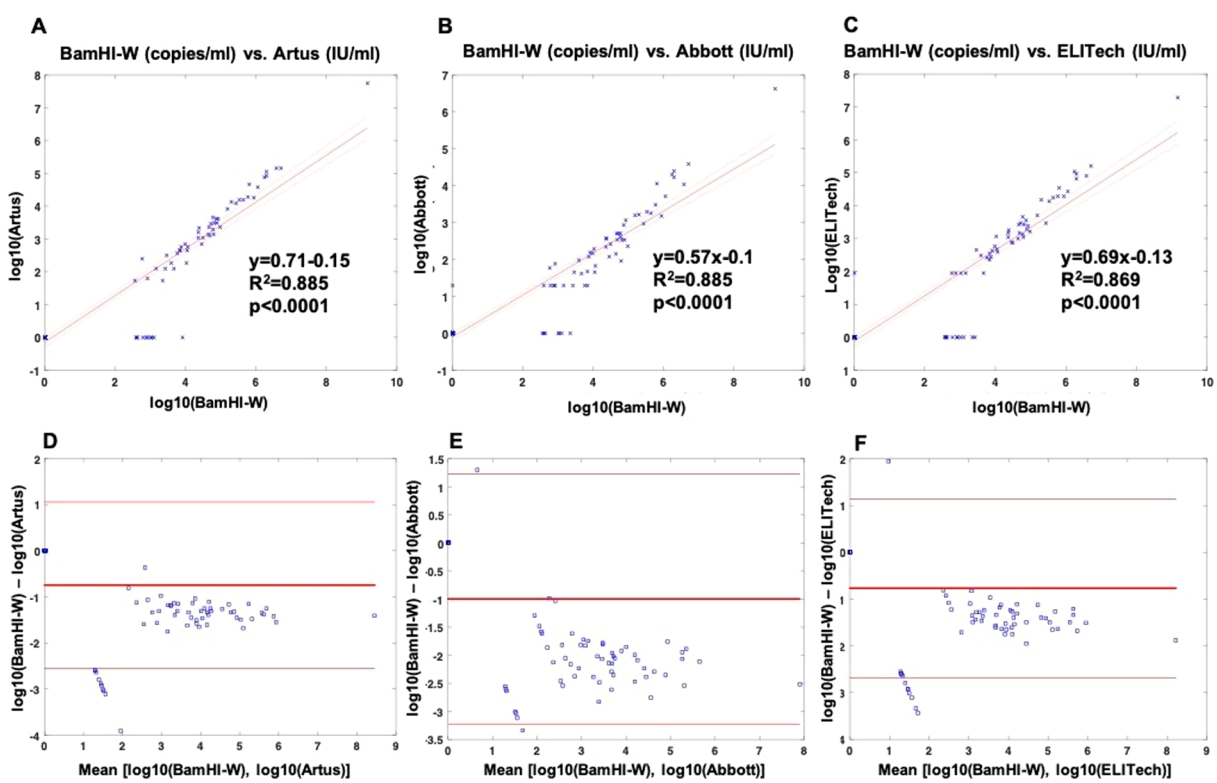

Fig. 1. Concordance analysis between *Bam*HI-W (copies/ml) and CE-assays (IU/ml). A, B, C: Scatter plots representing the correlation between *Bam*HI-W expressed in copies/ml and CE-assays expressed in IU/ml in plasma samples. The full line represents the estimated linear fit. Dashed lines represent confidence intervals. **1A:** *Bam*HI-W vs Artus, **1B:** *Bam*HI-W vs Abbott, **1C:** *Bam*HI-W vs ELITech. **D, E, F:** Bland-Altman plots representing the agreement between *Bam*HI-W expressed in copies/ml and CE-assays expressed in IU/ml in plasma samples. The full line represents the bias. Dashed lines represent confidence intervals. **1D:** *Bam*HI-W vs Artus, **1E:** *Bam*HI-W vs Abbott, **1F:** *Bam*HI-W vs ELITech.

transformation was obtained using a conversion factor, according to different manufacturers: the results obtained in copies/ml (Fig. 2) were comparable to those expressed in IU/ml.

At Bland-Altman analysis, the comparison among three CE-assays showed a mean difference in the −0.23/+0.22 log interval, with a variance < 1log (0.55–0.61) (Supplementary Fig. 1).

Comparison between plasma and whole blood

We first compared EBV-DNA viral loads obtained in plasma and WB samples by *Bam*HI-W assay. At regression analysis, the results coming from the two matrices were correlated (Fig. 3**A**), with WB providing slightly larger values. Bland-Altman analysis (Fig. 3**B**) showed a small bias (0.15 log) with a very scattered distribution of the differences, as demonstrated by the large variance (1.21 log) and confidence interval (-2.21, 2.52 log). This is mostly due to the number of zeros (no EBV-DNA detected), which was larger with plasma than with WB matrix in post-treatment samples.

Then, we compared plasma EBV-DNA levels obtained by *Bam*HI-W assay to the results by CE-assays on WB matrix. Correlation and Bland-Altman analyses were summarized in Supplementary Table 3.

EBV-DNA detection capability

A summary of the test performance based on sensitivity, specificity, false positive and false negative rates is reported in Table 3. Using all methods, post-treatment (phase 2 + 3) plasma samples (i.e., without apparent macroscopic disease) had significantly lower EBV-DNA concentrations than R/M and pre-treatment samples (Supplementary Fig. 2). With WB, the number of positive detections increased in post-treatment samples (Fig. 4).

Pre-treatment:

Plasma samples

In pre-treatment plasma samples (Fig. 4), *Bam*HI-W and Abbott methods always detected positive EBV-DNA, whereas Artus and ELITech did not detect EBV-DNA in 1/12 samples. This sample corresponded to a patient staged as cT2 cN0 cM0 (stage II AJCC VIII TNM) with a baseline *Bam*HI-W value ≤ 1 log.

WB samples

In pre-treatment WB samples (Fig. 4), the number of not-detected EBV-DNA increased compared to baseline plasma values. In detail, EBV-DNA level was not-detected by *Bam*HI-W and Artus in 1 and 3/12 cases (8 % and 25 %, respectively). Abbott and ELITech provided non-zero values for all samples.

Post-treatment:

Plasma samples

In post-treatment (Fig. 4), all methods provided 59/62 (95.2 %) not-detected EBV-DNA plasma samples. Of the three non-zero samples, one was detected by all methods and corresponded to a case, sampled 26 months after curative treatment completion, and relapsed (loco-regional recurrence) 5 months later. The two other samples without agreement among all methods corresponded to one patient sampled at 12 and 15 months after curative treatment completion, without recurrence after 41 months since EBV-DNA detection.

Among all not-detected post-treatment EBV-DNA samples (N = 59), corresponding to 55 patients, 5/55 patients recurred (3 loco-regional recurrences, 1 distant, 1 both) after a median of 14 months (range

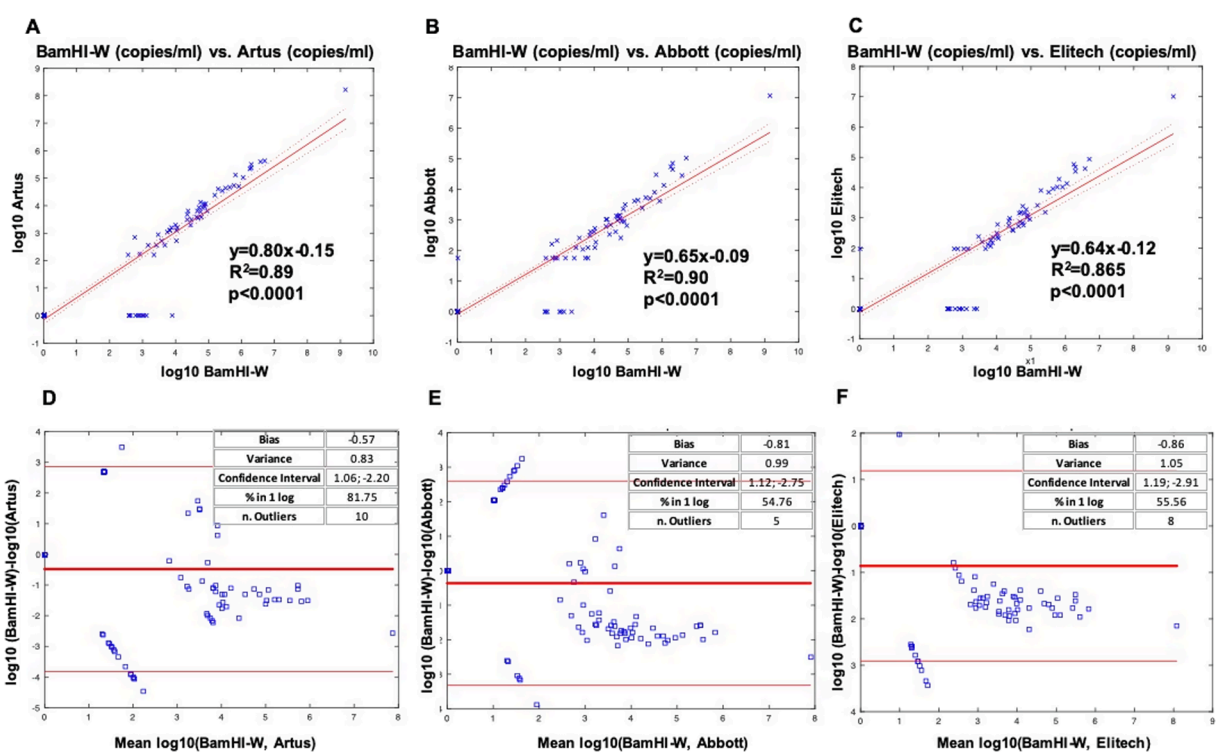

Fig. 2. Concordance analysis between *Bam*HI-W (copies/ml) and CE-assays (copies/ml). A, B, C: Scatter plots representing the correlation between *Bam*HI-W and CE-assays in plasma samples, both expressed in copies/ml. The full line represents the estimated linear fit. Dashed lines represent confidence intervals. **2A:** *Bam*HI-W vs Artus, **2B:** *Bam*HI-W vs Abbott, **2C:** *Bam*HI-W vs ELITech. **D, E, F:** Bland-Altman plots representing the agreement between *Bam*HI-W and CE-assays in plasma samples. The full line represents the bias. Dashed lines represent confidence intervals. **2D:** *Bam*HI-W vs Artus, **2E:** *Bam*HI-W vs Abbott, **2F:** *Bam*HI-W vs ELITech.

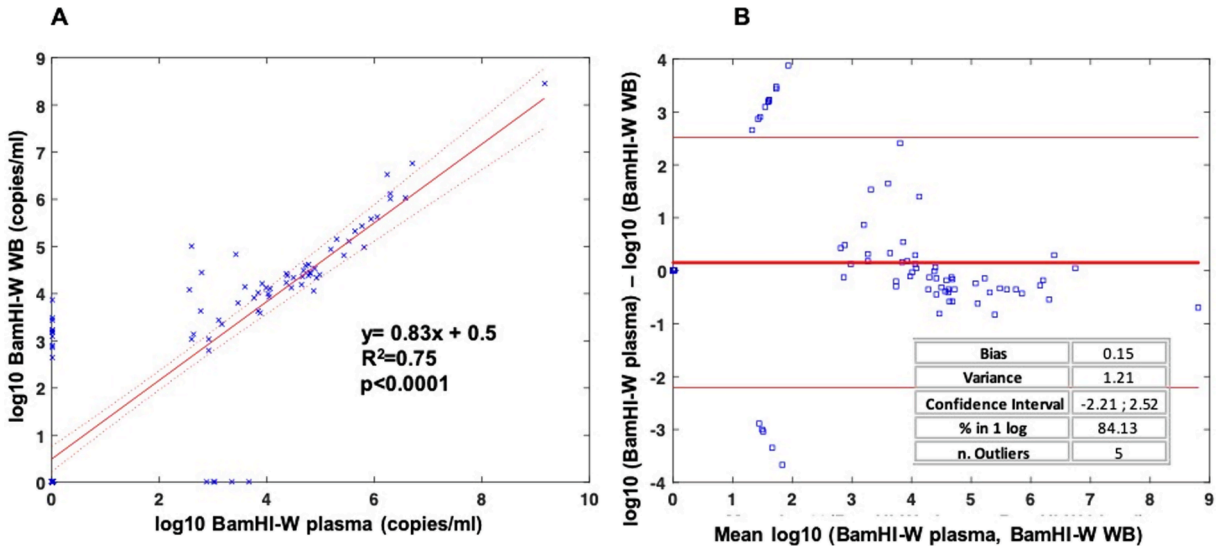

Fig. 3. ***Bam*HI-W: comparison of EBV-DNA levels between plasma and WB samples. 3A:** Correlation analysis. The full line represents the estimated linear fit. Dashed lines represent confidence intervals. **3B:** Bland-Altman plot. The full line represents the bias. Dashed lines represent confidence intervals.

Table 3
Different assays' performance based on sensitivity, specificity, false positive, and false negative rates.

	Pre-treatment			Post-treatment			R/M disease		
	Artus	Abbott	ELITech	Artus	Abbott	ELITech	Artus	Abbott	ELITech
Agreement (%)	92 %	100 %	92 %	100 %	97 %	97 %	84 %	88 %	82 %
Not detected by CE-assays (false negative, %)	8 %	0 %	8 %	0 %	2 %	2 %	16 %	12 %	18 %
Detected by CE-assays (false positive, %)	0 %	0 %	0 %	0 %	2 %	2 %	0 %	0 %	0 %
Sensitivity	0.92	1.00	0.92	1.00	0.50	0.50	0.81	0.86	0.79
Specificity	N/A	N/A	N/A	1.00	0.98	0.98	1.00	1.00	1.00

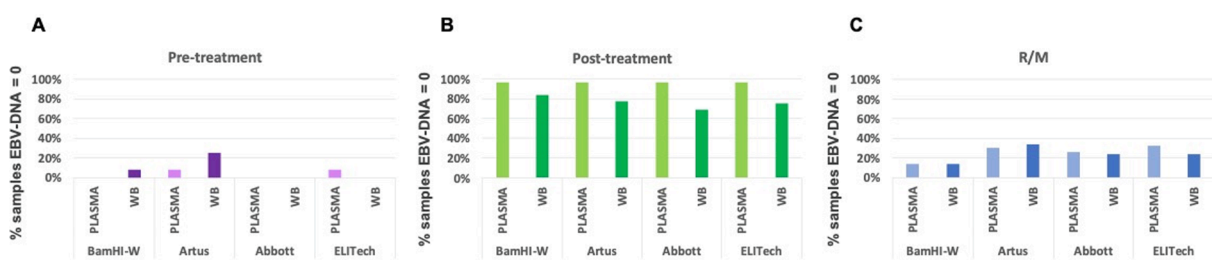

Fig. 4. Not-detected EBV-DNA samples using plasma and WB matrix at different clinical phases. Data are represented as percentage of the population (pre-treatment N = 12; post-treatment N = 62; R/M N = 50).

2–16) since EBV-DNA analysis. In detail, 3/5 had positive plasma EBV-DNA at the time of clinical/radiological recurrence.

WB samples

In post-treatment WB samples, *Bam*HI-W did not detect EBV-DNA in 52/62 samples (84 %), Artus in 48 (77 %), Abbott in 43 (69 %), and ELITech in 47 (76 %) (Fig. 4). Not-detected samples were less than those obtained with plasma. Of 5 patients with not-detected post-treatment plasma EBV-DNA experiencing disease recurrence, only 1 was detectable by WB without a full agreement among methods (detected by Abbott and Artus vs not-detected by *Bam*HI-W and ELITech). This recurrent patient, considered as false negative by post-treatment plasma EBV-DNA, had low tumor stage at baseline and disease recurrence (cT1cN0cM0, stage I AJCC VIII TNM) and was treated by RT (at diagnosis) and surgery (at recurrence). Time elapsed between WB EBV-DNA test and recurrence detection was 14 months.

R/M disease:

Plasma samples

In R/M plasma samples (Fig. 4), *Bam*HI-W did not detect EBV-DNA in 7/50 samples (14 %), Artus in 15 (30 %), Abbott in 13 (26 %), and ELITech in 16 (32 %).

Regarding the 7 not-detected EBV-DNA samples (corresponding to 6 patients) by *Bam*HI-W, also all CE-methods agreed in the lack of detection [median Overall Survival (mOS) 38 months, range 4–212]. The 11 not-detected samples by CE-methods (corresponding to 9 patients) had viral load $\leq$ 1 log in *Bam*HI-W and low total tumor burden and/or only loco-regional disease recurrence (mOS 49 months, range 18–212). The mOS of the 32 concordant positive samples (corresponding to 20 patients) was 37 months (range 2–171).

WB samples

In R/M WB samples (Fig. 4), *Bam*HI-W did not detect EBV-DNA in 7/50 samples (14 %), Artus in 17 (34 %), Abbott and ELITech in 12 (24 %). These not-detected WB samples are not fully overlapping with not-detected plasma samples: 5 were not-detected both with plasma and WB, whereas the majority (15 samples) were detected by *Bam*HI-W with plasma but were not-detected by at least one WB method. Overall, among 7 not-detected (by *Bam*HI-W and CE-assays) plasma R/M samples, none was detected by all methodologies on WB.

Discussion

Our analysis showed a statistically significant ($p = 0.0001$) agreement between *Bam*HI-W and all CE-methods considered, in terms of plasma EBV-DNA viral load quantification. Plasma EBV-DNA values concordance among *Bam*HI-W and CE-assays was confirmed in both correlation and Bland-Altman analyses without significant variation (i.e., in the order of 1log).

This study first compared three single-copy gene CE-methods (e.g. Artus, Abbott, ELITech) vs one *Bam*HI-W-based assay in a non-endemic NPC patients' cohort [14].

Our results support comparability of plasma EBV-DNA values measured by *Bam*HI-W and CE-assays. This is advocated for better interpretation and commutability of EBV-DNA assays in real-world settings. No significant interferences from different measurement units were found and CE-methods results were confirmed when transformed from 'IU/ml' into 'copies/ml'.

CE-assays could overcome the main *Bam*HI-W potential drawback, that is circulating EBV-DNA inter-subject variability; this could lead to better defined EBV-DNA cut-off values, expressed in international units, which to date are lacking in non-endemic areas [4,6,18,19]. Indeed, pre- and post-treatment EBV-DNA prognostic significance in NPC is well established, and can be crucial to determine different treatment and/or monitoring strategies [11]. In this respect, a recent multicenter study has shown how patients with EBV-DNA–negative NPC had better survival outcomes than their EBV-DNA–positive counterpart; moreover, authors validated a new staging system by incorporating pre-treatment plasma EBV-DNA to TNM stage, which outperformed AJCC VIII TNM alone prognostic performance [7]. All these aspects should encourage a more widespread plasma EBV-DNA quantification in NPC patients' routine clinical management, throughout standardized cutoffs and a reproducible method in each clinical setting [13].

Moreover, concordance among CE-assays (Artus vs Abbott, Artus vs ELITech, Abbott vs ELITech) was better than their comparison with *Bam*HI-W. We underline that discrepancies among different assays may be not only due to inter-laboratory variability, but also assay design, PCR efficiency, amplicon size, extraction volume [20,21] other than the different gene target (multiple vs single-copy gene).

Dissecting *Bam*HI-W vs CE-assays performance in plasma EBV-DNA quantification in pre- and post-treatment and R/M disease, we demonstrated the concordance of all methods in identifying positive EBV-DNA cases.

In detail, in pre-treatment phase, we observed correlation between all methodologies except for one case with low tumor burden (cT2N0, stage II AJCC VIII TNM), where *Bam*HI-W method only resulted positive.

In R/M phase, patients ($n = 9$) with discordant samples, i.e., samples not-detected by CE-methods and with ≤ 1 log viral load by *Bam*HI-W, had low tumor burden and/or only loco-regional disease recurrence. The mOS of this subgroup was longer than mOS of patients ($n = 20$) with concordant samples, i.e., samples detected both at *Bam*HI-W and CE-assays ($n = 32$) (49 months, range 18–212 vs 37 months, range 2–171, respectively). Indeed, low tumor burden, both at disease presentation and recurrence, is known to influence the accuracy of EBV-DNA detection, [6,22–25] and could play a role in this minor discordance between single vs multiple-copy gene target assays.

In post-treatment phase, all the methods agreed in finding patients at high risk of recurrence. Among the not-detected (by all methods) post-treatment plasma EBV-DNA values (59 samples, 55 patients), five cases experienced disease recurrence after a median of 14 months (range 2–16) since EBV-DNA analysis and could be considered as "false negative". Only 1/5 false negative plasma post-treatment EBV-DNA values was detectable on WB without agreement among all methods. Nonetheless, the small sample size and the retrospective nature of the study, in the lack of a longitudinal circulating EBV-DNA assessment, prevent from drawing any conclusions on plasma and WB sensitivity rate.

Persistent positive circulating EBV-DNA after curative treatment has a well-known negative prognostic value [4] and these patients may represent the best enriched population for adjuvant trials [26–29]. Moreover, identification of circulating EBV-DNA during follow-up has shown to correlate with an increased risk of recurrence and to anticipate its radiological or clinical appearance [5].

Given the limitations of our analysis (small sample size, retrospective design with lack of prospective sample collection and evaluation, different treatment modalities), further prospective and larger studies are awaited to confirm the comparability of different plasma EBV-DNA assays (e.g., single-copy gene vs *Bam*HI-W). WB failure to show a clear advantage over plasma may be attributable to the fact that circulating B-cells, the main reservoir of EBV, represent a small subset of circulating leukocytes and are found in variable numbers per ml of blood; hence, WB sensitivity for EBV-DNA detection may be affected [30]. In conclusion, the role of WB deserves more extensive evaluation, as its better performance compared to plasma in post-treatment setting should be considered as purely hypothesis-generating.

Conflicts of interest: Taverna Francesca, Alfieri Salvatore, Campanini Giulia, Marceglia Sara, Mazzocchi Arabella, Giardina Federica, Comoli Patrizia, Gloghini Annunziata, Quattrone Pasquale, Romanò Rebecca, Bergamini Cristiana, Apollonio Giulia, Filippini Daria Maria, Orlandi Ester, Baldanti Fausto declare no conflict of interest concerning the topic of this publication.

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Data accessibility: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics statement: The present study was approved by the Ethical Committee of Fondazione IRCCS Istituto Nazionale Tumori of Milan (Trial code: 131/20). Written informed consent was obtained from all participants before they entered the study.

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collected the patients' data. TF, CG, MS and CP analyzed the data. MS performed the statistical analyses. GA and QP contributed to the analysis and interpretation of the results. MA, OE, LDL, LL, BF and BP critically reviewed and approved the final version of the manuscript.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.oraloncology.2022.106229.

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