Epstein–Barr virus biomarkers for nasopharyngeal carcinoma in non-endemic regions


Abstract
The Epstein–Barr virus (EBV) plays a key role in the development of undifferentiated nasopharyngeal carcinoma (uNPC). In uNPC endemic regions EBV-specific antibodies and plasma EBV DNA load are used as markers for the early detection of uNPC and monitoring of the disease. In non-endemic regions, such studies were practically not conducted. The aim of this study was to compare the clinical significance of EBV serological markers and plasma EBV DNA levels for uNPC patients in a non-endemic region, Russia. The results obtained indicate that both viral capsid antigen/immunoglobulin A (VCA/IgA) antibodies and plasma EBV DNA copies can effectively be used for nasopharyngeal carcinoma (NPC) diagnosis. Besides, plasma EBV DNA load was found to be a more sensitive marker of uNPC than VCA/IgA antibody titres, as it reflected the effect of the therapy in stages of remission and relapse of the disease more precisely. Our study, for the first time, demonstrates that the simultaneous use of plasma EBV DNA loads and VCA/IgA antibody levels are indispensable markers for uNPC in non-endemic regions: a serological marker can be more effectively used for NPC screening, but EBV DNA copies are better for monitoring the disease. However, both markers turned out to be practically unsuitable for assessing the clinical status of patients. Serological markers did not correlate with any signs of the tumour process estimated by tumour, node and metastasis (TNM) classification and the plasma EBV DNA loads correlated only with the size of the pathologically altered lymph nodes (N). Additional study is required to confirm these findings.

INTRODUCTION
Epstein–Barr virus (EBV), a member of the human herpes-viruses family, has unique biological properties. Over 90% of the world’s population is infected with EBV, usually without clinical manifestations in carriers of the virus. Furthermore, the virus is recognized as an etiologic agent for a number of benign and malignant diseases [1], one of which is nasopharyngeal carcinoma (NPC). NPC is a malignant tumour, and EBV has been found to play a key role in its development. In the nasopharynx, the virus stimulates the pathological process in which pre-cancerous lesions progress to the development of cancer [2].

The global incidence of NPC is characterized by geographic and ethnic variability [3]. Its prevalence is highest in the southern provinces of China and South-East Asia (25–30 cases per 100 000 people per year), and is less prevalent in the North African Arabs and the indigenous people of Greenland and Alaska [4]. In Western countries, NPC is observed at a rate of less than 0.5 per 100 000 people [5]. This rate is similar in the territory of the former Soviet Union, including Russia, where in 2013 the prevalence of NPC was recorded to affect 0.14% of men and 0.06% of women [6].

According to the World Health Organization (WHO) classification, NPC can be morphologically divided into two types: keratinizing squamous cell carcinoma and non-keratinizing squamous cell carcinoma, of which undifferentiated nasopharyngeal carcinoma (uNPC) is a subtype [7]. In general, uNPC is characterized by abundant lymphoid infiltration, which is composed of lymphocytes, histiocytes, eosinophils and other reactive cells [8, 9].

Molecular epidemiological studies have shown that uNPC is associated with EBV, independent of the geographical and ethnic origins of the patient [10]. However, in order for the virus to become oncogenic, a carrier of the virus needs to be exposed to harmful external environmental factors in combination with internal factors such as immunodeficiency, a human leukocyte antigen (HLA)-specific host genotype or...
genetic predisposition to the development of NPC etc. [11–14]. All uNPC cases are EBV-positive, and unlike other EBV-associated pathologies, the viral DNA is present in all tumour cells at the early stages of tumour development [15]. Almost all uNPC cases are characterized by high levels of EBV-specific antibodies [16, 17], which appear long before tumour development [18]. These findings suggest that EBV is involved in the pathogenesis of uNPC while it is still in the preclinical stage of the disease. Therefore, the detection of immunoglobulin A (IgA) antibodies against viral capsid antigen (VCA/IgA) have become widely used for uNPC screening in endemic regions [19], however, high immunoglobulin G (IgG) antibody titres to VCA (VCA/IgG) also accompany this disease.

In 1999, Lo and colleagues [20] demonstrated for the first time that the plasma EBV DNA load is an important marker of NPC. Their findings were later confirmed by a number of studies which demonstrated that the number of EBV DNA copies in plasma is especially useful for the diagnosis, identification of residual (clinically latent) tumours after chemotherapy [22]. However, while elevated levels of plasma EBV DNA are closely related with tumour size, tumour growth and response to chemotherapy [23–25], the IgA antibody titres to VCA are usually associated with an increased risk of developing NPC and therefore, are mainly useful for diagnosis [26, 27]. From this it follows that the diagnostic and prognostic values of both markers can differ [28–31]. Therefore, it is important to find the optimal use of VCA/IgA antibodies and plasma EBV DNA markers or a combination of each, for diagnosis and monitoring of the disease. This was done in NPC patients from endemic regions [29, 31, 32]; however, the significance of these markers in NPC patients from non-endemic regions has not yet been properly investigated. As mentioned before, in addition to EBV, the etiological factors proposed for NPC carcinogenesis include genetic susceptibility, immunodeficiency and environmental pollutions. One should also take into account that population in geographic areas at high risk of NPC development displays HLA distribution patterns different from that in areas of low incidence. Dietary, social and environmental factors (hazards), as a rule, are specific for different geographic areas and ethnic groups. [3, 11, 12]. It can be expected that in non-endemic countries, EBV-associated carcinogenesis in patients with uNPC has its own features. Based on the foregoing, the aim of this study was to perform a comparative assessment of the clinical value of two EBV serological markers, the level of VCA/IgG and VCA/IgA antibody titres and plasma EBV DNA load, in NPC patients in Russia, a non-endemic region.

RESULTS

Serological responses to EBV in uNPC patients and patients without EBV associated with other tumours of the oral cavity (OTOC) and blood donors (BDs) are shown in Table 1, from which it follows that antibodies to VCA/IgG were present in 100% of uNPC patients and BDs. In uNPC patients, the percentage of VCA/IgA antibodies was also high, and was observed in 93.8% (30/32) of uNPC patients before treatment, 96.8% (30/31) of patients following treatment who were in remission and 100% (9/9) of patients following treatment who had relapsed; however, the presence of VCA/IgA antibodies was negative in BDs.

Within the 57 OTOC patients, the percentage of seropositive samples was lower. Prior to treatment or following treatment, VCA/IgG antibodies were observed in 93.9% (31/33) and 83.3% (20/24) of samples, respectively, and VCA/IgA antibodies were observed in 15.2% (5/33) and 16.7% (4/24) of samples, respectively.

The geometric mean value (GMT) of VCA/IgG antibody levels of uNPC patients before treatment was 527.2, which decreased to 286.2 in samples of these patients in remission, and significantly increased to 691.3 in patients with tumour recurrence. The same trend was observed for the GMT of VCA/IgA antibody levels, which were found to be 127.1 in plasma samples of uNPC patients before treatment, decreased to 66.4 in patients in remission, and which sharply increased to 172.8 in patients with tumour recurrence. For both serological markers, the differences between these three measurements were highly significant (P=0.01).

As shown in Table 1, both types of EBV-specific antibodies follow the same pattern of clinical characteristics of uNPC patients, and VCA/IgA and VCA/IgG antibodies revealed nearly the same level of sensitivity. Indeed, the GMT of VCA/IgA antibodies in the pre-treatment patient samples (127.1) was 1.91 times higher than that from post-treatment samples from patients in remission (66.4), and this was 2.60 times lower than that in post-treatment samples from patients who had relapsed (172.8). The GMT value of the VCA/IgG antibodies in the pre-treatment samples (527.2) was 1.84 times higher than that from patients in remission (286.2) and this was 2.41 times lower than that in post-treatment samples from patients who had relapsed (691.3). As already mentioned, the differences between these three measurements were statistically significant (P=0.01).

The antibody response to EBV in OTOC patients also differed between the pre- and post-treatment samples. The GMT of VCA/IgG antibodies in the pre-treatment samples (51.2) was 2.1 times higher than that of the post-treatment patients (24.3) and this difference was statistically significant (P=0.04). The GMT of VCA/IgA antibodies in OTOC patient groups was low, and did not differ significantly in patients before and following treatment (1.5 vs 1.65, respectively, P=0.86).

Different levels of serological response to EBV in the studied groups are presented in Fig. 1(a, b). The values of median IgG/VCA titres were: in BDs – 1 : 40 (interquartile range (IQR)–1 : 40–1 : 80); in OTOC patients prior to treatment – 1 : 80 (IQR–1 : 40–1 : 100), and following treatment – 1 : 40 (IQR–1 : 20–1 : 80); in uNPC patients before treatment – 1 : 640 (IQR–1 : 320–1 : 640), at the stage of remission – 1 : 320 (IQR–1 : 160–1 : 640) and with tumour recurrence – 1 : 640
The IgG/VCA titres in uNPC patients were significantly higher than in healthy individuals and OTOC patients ($P=0.01$, respectively). Statistical differences in IgG/VCA titres were also found in OTOC patient groups before and after therapy and between BDs and OTOC patients ($P=0.04$ and 0.05, respectively) (Fig. 1a).

Median values of IgA/VCA titres were: in BDs – 1:10 (IQR–1:0–1:10); in OTOC patients prior to treatment – 1:10 (IQR–1:0–1:10); and following treatment – 1:10 (IQR–1:0–1:10); in uNPC patients before treatment – 1:160 (IQR–1:80–1:320), at the stage of remission – 1:80 (IQR–1:40–1:160) and in patients with tumour recurrence – 1:160 (IQR–1:160–1:160). IgG/VCA titres in uNPC patients were significantly higher than in healthy individuals and OTOC patients ($P=0.01$, respectively). However, no significant difference was found between values in OTOC patients and BDs (Fig. 1b).

---

Table 1. Levels of EBV-specific antibodies in uNPC and OTOC patients and BDs

<table>
<thead>
<tr>
<th>Groups studied</th>
<th>Plasma samples</th>
<th>IgG antibodies to EBV/VCA Positive (%)</th>
<th>GMT</th>
<th>P</th>
<th>IgA antibodies to EBV/VCA Positive (%)</th>
<th>GMT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>uNPC patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before treatment</td>
<td>32</td>
<td>32 (100)</td>
<td>527.2</td>
<td>0.01</td>
<td>30 (93.8)</td>
<td>127.1</td>
<td>0.01</td>
</tr>
<tr>
<td>Subsequent to treatment (remission)</td>
<td>31</td>
<td>31 (100)</td>
<td>286.2</td>
<td></td>
<td>30 (96.8)</td>
<td>66.4</td>
<td></td>
</tr>
<tr>
<td>Subsequent to treatment (relapse)</td>
<td>9</td>
<td>9 (100)</td>
<td>691.3</td>
<td></td>
<td>9 (100)</td>
<td>172.8</td>
<td></td>
</tr>
<tr>
<td>OTOC patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before treatment</td>
<td>33</td>
<td>31 (93.9)</td>
<td>51.2</td>
<td>0.04</td>
<td>5 (15.2)</td>
<td>1.5</td>
<td>0.86</td>
</tr>
<tr>
<td>Subsequent to treatment (remission)</td>
<td>24</td>
<td>20 (83.3)</td>
<td>24.3</td>
<td></td>
<td>4 (16.7)</td>
<td>1.65</td>
<td></td>
</tr>
<tr>
<td>Healthy controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood donors</td>
<td>19</td>
<td>19 (100)</td>
<td>50.0</td>
<td>–</td>
<td>0 (0.0)</td>
<td>1.0</td>
<td>–</td>
</tr>
</tbody>
</table>

---

Fig. 1. Comparative analysis of the serological response to EBV in uNPC and OTOC patients and healthy controls (donors). (a) Comparison of IgG/VCA antibody levels in the groups tested. (b) Comparison of IgA/VCA antibody levels in the groups tested. The categories are on the X-axis. NPC-1 – nasopharyngeal carcinoma patients before treatment; NPC-2 – nasopharyngeal carcinoma patients at the stage of remission; NPC-3 – nasopharyngeal carcinoma patients at the stage of tumour recurrence; OTOC-1 – patients with other tumours of the oral cavity before treatment; OTOC-2 – patients with other tumours of the oral cavity after treatment at stage of relapse; donors – healthy controls. The number of observations is indicated in parentheses. The Y-axis denotes the IgG and IgA antibody titres to the VCA of the EBV. The boxes mark the interval between the 25th and 75th percentiles. The lines inside the boxes denote the medians. $P$-values were evaluated with the Kruskal–Wallis test.
The clinical significance of the biomarkers studied, EBV-specific antibody levels and plasma EBV DNA load, became more evident when monitoring the individual uNPC patients. The behaviour of these markers in uNPC patients reflecting different effects of the therapy in two groups of patients is represented in Figs 3(a–d) and 4(a, b). In particular, Fig. 3(a) shows that in one of four uNPC patients from the group with clinical remission (case 1001), an increased number of plasma EBV DNA copies ml⁻¹ was observed before treatment (33 709 copies ml⁻¹). This value reduced to zero after the first course of chemotherapy and was maintained at approximately the same level after subsequent courses of the therapy. All this time, the patient was in clinical remission. Both the VCA/IgG and VCA/IgA antibody levels in this patient matched the overall dynamics of the patient’s transition into clinical remission. However, the antibody response to viral antigens due to some inertia of the immune mechanism was delayed. Indeed, both antibody responses after the first course of therapy did not change and corresponded to their levels prior to treatment – 1 : 320 and 1 : 160, respectively. After the second course of therapy, in contrast to the decreased plasma EBV DNA load to zero, the anti-EBV antibody titres increased significantly (1 : 1280 and 1 : 640, respectively), which then began to decline after the third course of therapy (1 : 640 and 1 : 80, respectively) and after the fourth course of therapy dropped significantly (1 : 160 and 1 : 20, respectively). Similar dynamics of the EBV DNA load were observed in the other three uNPC patients with positive responses to the therapy (Fig. 3b–d). In all four cases, high concentrations of EBV DNA detected before treatment, after several courses of the chemotherapy were reduced to background levels or even zero. At the same time, the dynamics of detectable amounts of EBV DNA copies and serological responses to virus antigens in each patient were quite individual. It can be noted, however, that dynamics of the EBV markers observed in cases 1001 and 1008 (Fig. 3a, d) are quite similar, although one of these

Importantly, both types of EBV-specific antibody levels (VCA/IgG and VCA/IgA) followed the varying concentrations of EBV DNA in the plasma of uNPC patients. The reduction of plasma EBV DNA load in patients in remission, and its increase in patients who had relapsed, was accompanied, respectively, by the weakening and strengthening of the anti-EBV serological response. The values for plasma EBV DNA loads in the studied groups are presented in Fig. 2. The median and interquartile intervals were used to represent the results. The figure shows that the increased values of EBV DNA load in the plasma of newly diagnosed uNPC patients are in contrast to the low values in OTOC patients and BDs. This was reduced in nNPC patients in remission and increased to its highest value in patients with tumour recurrence.

The analysis of plasma EBV DNA load was conducted in 32 uNPC patients before treatment, 40 uNPC patients following treatment (31 patients in remission and nine who had relapsed), 42 OTOC patients (28 before treatment and 14 after treatment) and 19 BDs (Table 2). We observed a high concentration of viral DNA in the plasma samples of uNPC patients prior to treatment (median 5594 copies ml⁻¹, IQR –559–32 740 copies ml⁻¹), which dropped to background levels in plasma samples of patients in remission (median 11 copies ml⁻¹, IQR –0–448.0 copies ml⁻¹) and increased sharply in plasma samples of patients with tumour recurrence or distant metastasis (median 332 177 copies ml⁻¹, IQR –79 492–2 209 558 copies ml⁻¹). The differences between each of the three measurements of plasma EBV DNA load were highly significant (P=0.001). The plasma EBV DNA concentration in OTOC patients prior to and following treatment did not exceed the background values (median 22 copies ml⁻¹, IQR –0–83 vs median 0 copies ml⁻¹, IQR –0–133, respectively), and the differences between the two measurements were not statistically significant (P=0.43).

Table 2. Plasma EBV DNA copies in NPC and OTOC patients and BDs

<table>
<thead>
<tr>
<th>Groups studied</th>
<th>Plasma samples</th>
<th>Plasma EBV DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (%)</td>
<td>Median (copies ml⁻¹)</td>
</tr>
<tr>
<td>uNPC patients</td>
<td>Prior to treatment</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Subsequent to treatment (remission)</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Subsequent to treatment (relapse)</td>
<td>9</td>
</tr>
<tr>
<td>OTOC patients</td>
<td>Prior to treatment</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Subsequent to treatment remission</td>
<td>14</td>
</tr>
<tr>
<td>Healthy individuals</td>
<td>Blood donors</td>
<td>19</td>
</tr>
</tbody>
</table>

P. The distribution of the plasma EBV DNA levels in pre- and post-treatment groups were compared using the Kruskal-Wallis test.
patients (case 1008, Fig. 3d) has a lower DNA load before treatment. Serological responses in cases 1003 and 1006 (Fig. 3b, c) did not correlate with the dynamics of EBV DNA loads, which were sharply reduced and approached background values after two courses of chemotherapy. Instead, IgG antibodies in case 1003 (Fig. 3b) decreased to a normal level, but in case 1006 (Fig. 3c) – after a slight decrease (1 : 320) increased to a value of 1 : 640. Levels of IgA antibodies in both cases remained at the same levels (1 : 80 and 1 : 160, respectively). From this observation it follows that both IgG/VCA and IgA/VCA antibody titres have a limited prognostic value.

Upon follow-up, two patients developed evidence of tumour recurrence. As shown in Fig. 4(a, b), the initial plasma EBV DNA load in these patients (cases 1005 and 1002) before treatment was high (52 918 and 32 254 copies ml⁻¹, respectively), and accompanied by high VCA/IgG and VCA/IgA antibody titres (1 : 640 and 1 : 80 and 1 : 320 and 1 : 80, respectively). After the first course of chemotherapy, the plasma EBV DNA load in these patients decreased significantly (2189 and 1182 copies ml⁻¹, respectively), which coincided with tumour and cervical lymph node size reduction in both cases. At this stage of the disease VCA/IgG and VCA/IgA antibody titres slightly decreased in case 1005 (Fig. 4a) to 1 : 160 and 1 : 40, respectively, and increased in case 1002 (Fig. 4b) to 1 : 640 and 1 : 160, respectively. In case 1005 (Fig. 4a) after two courses of chemotherapy and one course of chemotherapy in case 1002 (Fig. 4b), a dramatic increase in the number of plasma EBV DNA copies was observed (332 177 and 79 492 copies ml⁻¹, respectively), which coincided with tumour recurrence in case 1005 (Fig. 4a) and the appearance of tumour metastases in the bone in case 1002 (Fig. 4b). By this time, the levels of EBV-specific antibody titres did not change significantly (1 : 640 and 1 : 160 vs 1 : 320 and 1 : 160, respectively). Thus, it is evident that in these patients serological response to EBV did not match the plasma EBV DNA levels and clinical manifestations of the disease.

In this study we also analysed the extent to which EBV-specific antibody levels and concentrations of plasma EBV DNA are correlated with different clinical characteristics of uNPC. We found that (Table 3) both serological markers did not reflect the disease severity. Differences between the GMT of the VCA/IgG and VCA/IgA antibody levels in uNPC patients with different tumour sizes (T1–T4), as well with the sizes of pathologically altered lymph nodes (N0–N3) and the degree of the disease progression (stages I–IV), were statistically insignificant. However, unlike VCA/IgG

![Fig. 2. Comparison of plasma-cell-free EBV DNA loads in NPC patients before treatment (NPC-1), at the stage of remission (NPC-2), at the stage of tumour recurrence, (NPC-3) and in other tumours of the oral cavity (OTOC) before treatment (OTOC-1) and after treatment (OTOC-2) and in healthy controls (donors). The categories are on the X-axis. The number of observations is indicated in parentheses. The boxes mark the interval between the 25th and 75th percentiles. The lines inside the boxes denote the medians. P-values were evaluated with the Kruskal–Wallis test.](image-url)
and VCA/IgA antibody titres, the correlation between plasma EBV DNA load (expressed in median copies ml\(^{-1}\)) and lymph node status was statistically significant (1253.5 in patients with N0–N1 vs 32254 in patients with N2–N3; \(P=0.03\)), while such a correlation between EBV DNA level and tumour size was not established (6459 at T1–T2 vs 21 095 at T3–T4; \(P=0.53\)). Between stages of the disease, on the one hand, and plasma EBV DNA load, on the other one, the correlation was also not detected. It is interesting that the GMT of the VCA/IgG and VCA/IgA antibody titres in patients at stages I–II were higher than that in patients at stages III–IV (640.0 vs 470.3 and 186.6 vs 132.0, \(P>0.48\) and \(P>0.58\), respectively), which can be attributed to the immune deficiency in some patients from the last group. At the same time, the median of plasma EBV DNA load in patients with stages III–IV was two times higher than that in patients with stages I–II (9643.5 vs 4729). Although the difference was not statistically significant (\(P=0.81\)), this marker tends to reflect the gravity of the disease more correctly than serological ones. These findings allow us to

---

**Fig. 3.** (a, b, c, d) EBV markers in uNPC patients who responded positively to therapy. IgG/VCA and IgA/VCA – IgG and IgA antibody titres against the EBV capsid antigen (left ordinate scale); for demonstration of plasma EBV DNA load in the number of DNA copies ml\(^{-1}\) the right ordinate scale was used. Designations on top of the figures are: patient codes, ages, tumour parameters according to the tumour, node and metastasis (TNM) classification and the stage of the disease.
suggest that plasma EBV DNA loads, in contrast to serological markers, may have, although rather limited, use to assess clinical status of uNPC patients in non-endemic regions. We assume that the type of serological response to EBV and levels of plasma EBV DNA load in Russian NPC patients is largely dependent on their specific HLA pattern, which differ from that in NPC patients from endemic regions [3, 11, 12]. The character of EBV replication in uNPC patients is likely to also be influenced by the impact of different harmful environmental factors, dietary peculiarities and possibly by the persistence of EBV strains, which genetically are not identical to those in endemic regions. ([11, 16], Seniuta et al. unpublished data). However, additional study is required to make a final conclusion.

**DISCUSSION**

The testing of tumour-associated nucleic acids in the blood plasma has recently become of increasing interest due to its application in the early detection and monitoring of neoplastic processes. The presence of cell-free DNA in the bloodstream, released from dying tumour cells, has opened new possibilities for the non-invasive diagnosis and monitoring of a number of malignancies [33–36].

Previous studies have shown that EBV is closely associated with uNPC [2, 14]. These studies have also shown that patients with uNPC have characteristically high levels of serum VCA/IgG and VCA/IgA antibody titres [16], as well as an increased plasma EBV DNA load [28, 29, 31]. Patients who are seropositive for VCA/IgA represent a group with a high risk for the development of NPC, while levels of plasma EBV DNA have been correlated with the clinical manifestations of this disease [29]. Both markers have been effectively used for the diagnosis and monitoring of NPC patients in endemic regions, however, their clinical significance in non-endemic regions remains unclear.

This study was conducted in a non-endemic region (Russia), whose population is genetically different from the inhabitants of endemic regions and appears to be exposed to other EBV strains and harmful environmental factors. In our study we have investigated the clinical significance of EBV serological markers, VCA/IgA and VCA/IgG, and plasma EBV DNA load, in uNPC patients prior to and after treatment, in either a state of remission or relapse.

The data obtained are generally consistent with the results of investigations carried out in NPC endemic regions [37, 38], where plasma EBV DNA and VCA/IgA serology testing has become widely used as a diagnostic and prognostic tool for determining the relapse and survival of NPC patients [39–41]. In our study, we were also able to demonstrate the high diagnostic value of plasma EBV DNA load and VCA/IgA, but not VCA/IgG, antibody levels. Most of the pretreatment samples were characterized by a high plasma EBV DNA load (<1300 copies ml⁻¹) and increased titres of VCA/IgA antibodies. Post-treatment plasma samples of patients in remission or the disease stabilization were characterized by a significant decrease in EBV DNA load, which was found to be 0 copies ml⁻¹ in 15 of 31 patients, and only
four samples of the remaining 16 exceeded 1000 copies ml\(^{-1}\). Conversely, the post-treatment samples in patients with tumour recurrence or metastasis were characterized by high plasma EBV DNA loads (sometimes by an order of magnitude) which exceeded that of the pre-treatment samples, while VCA/IgA antibody levels did not always react in the same manner. The VCA/IgA antibody levels did not change significantly between different clinical states, as observed for plasma EBV DNA loads, as the antibody response is usually delayed and in cases of patients with heavy immunodeficiency, the ‘paralysis’ of serological responses could occur (Table 3). The discrepancy between values of the two biomarkers can also be explained by the consequences of different events occurring in the process of tumour formation. The EBV DNA copies in blood plasma are likely to be apoptotic tumour fragments, whereas the IgA antibodies, formed against VCA, occurs in plasma as a result of immune response to EBV replication [32].

While plasma EBV DNA load and VCA/IgA antibody levels are approximately equal as screening markers, the plasma EBV DNA load responds better to the different manifestations of the disease (Figs 3 and 4). Therefore, VCA/IgA antibody testing is technically more suitable for primary diagnostics and screening of the population for NPC, while the plasma EBV DNA load is more useful for monitoring the disease. Simultaneous testing for both markers may be useful in establishing the clinical status of the patient during his examination. In particular, high values of both measures would be expected to be observed in pre-treatment uNPC patient. Negative or low plasma EBV DNA load in the presence of high VCA/IgA antibody titres, may confirm the uNPC diagnosis or indicate an improvement (clinical remission) after previously conducted therapy. A high EBV DNA load in the absence or low levels of VCA/IgA antibodies may indicate patients at an advanced stage of the disease, in addition to severe immunodeficiency of this patient. Low values of both markers in patients are characteristic of keratinizing squamous cell NPC, which is not associated with EBV and may also indicate the presence of other pathology than NPC.

Thus, our study, which is one of the first carried out in a non-endemic region (Russia), clearly demonstrated that plasma EBV DNA load and IgA antibody levels are indispensable uNPC markers, combination of which can be effectively used in such a region not only for the diagnosis of uNPC, but also to evaluate the effect of therapy and prognosis of the disease. However, an IgA/VCA marker does not, and the plasma EBV DNA load has limited use for assessing a patient’s clinical status. In Russian NPC patients, the levels of EBV DNA concentration correlated only with the size of pathologically altered lymph nodes (N), but not with the tumour size or clinical stage of the disease. This may be explained by Russian NPC patients having a specific HLA pattern or by the influence some specific environmental factors has on them or, possibly, may be due to introducing the EBV strains into NPC carcinogenesis in Russia other than in the endemic regions (Seniuta et al. unpublished data).

### METHODS

**Patient characteristics and clinical samples**

A total of 45 patients with diagnosis of undifferentiated nasopharyngeal carcinoma (uNPC) proven on the WHO criteria [7], and 52 patients with other tumours of the oral cavity (OTOC), in which EBV has not been shown to play a role in pathogenesis, and 19 blood donors (BDs) were included in this study. For uNPC patients, the ratio of males to females was 1.6 : 1 and the average patient age was 45.6 years. The OTOC were represented by cancers of the mucous membrane of the tongue, floor of the mouth, cheek, retro molar area, lower jaw and palate. The ratio of males to females for OTOC patients was 2.4 : 1 and the average patient age was 48.8 years. The average age in the group of BDs was 48.7 years and the ratio of males to females was 1.4 : 1. The objects of the study were plasma samples collected from the above representatives of the three test groups. Plasma samples were obtained from 32 NPC patients prior to treatment with concurrent chemoradiotherapy, 31 samples were from patients in a state of remission or tumour stabilization and nine samples were from patients who had relapsed or for whom the tumour

### Table 3. EBV antibody levels, plasma EBV DNA load and TNM characteristics of NPC patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>No. of patients</th>
<th>GMT antibodies to EBV</th>
<th>VCA/IgG Median copies ml(^{-1})</th>
<th>IQR copies ml(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1–T2</td>
<td>9</td>
<td>640.0</td>
<td>217.7</td>
<td>6459</td>
</tr>
<tr>
<td>T3–T4</td>
<td>16</td>
<td>484.5</td>
<td>111.6</td>
<td>21095</td>
</tr>
<tr>
<td>N0–N1</td>
<td>16</td>
<td>452.5</td>
<td>140.5</td>
<td>1253.5</td>
</tr>
<tr>
<td>N2–N3</td>
<td>13</td>
<td>491.4</td>
<td>92.3</td>
<td>32254</td>
</tr>
<tr>
<td>Stages I–II</td>
<td>9</td>
<td>640.0</td>
<td>186.6</td>
<td>4729</td>
</tr>
<tr>
<td>Stages III–IV</td>
<td>18</td>
<td>470.3</td>
<td>132.0</td>
<td>9643.5</td>
</tr>
</tbody>
</table>

EBV, Epstein–Barr virus; IgG/VCA, IgA/VCA, IgG and IgA antibody titres to the capsid antigen of EBV; tumour parameters studied: T, tumour size; N, lymph node status; stages I–II, III–IV, clinical stages of the disease; GMT, geometrical mean titres; IQR, interquartile range; P, distribution differences in the tumour parameters and plasma EBV DNA load in uNPC patients were evaluated by the Kruskal–Wallis test.
had become metastatic. The Ethics Committee of the N.N. Blokhin Cancer Research Centre (Russia) approved the study, which included randomly selected patients with uNPC and OTOC who had given their informed consent.

**Immunofluorescence test**

All plasma samples were titrated for IgG and IgA antibodies against the EBV VCA by indirect immunofluorescence. In order to evaluate anti-VCA activity, the P3HR-1 cell line which expresses VCA was used. To prepare approximately 10% of the antigen-containing cells, the P3HR1 cells were treated with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) at a concentration of 20 ng ml\(^{-1}\) for 3–6 days. The treated cell suspension was dropped onto glass slides (six drops per slide), and after air drying and fixing with cooled acetone, the target-cell smears were used as the VCA-containing preparations. Serial twofold dilutions of each plasma sample were prepared for the testing of IgG and IgA VCA-specific antibodies. The range of IgG titres was 1 : 10–1 : 5120 and the range of IgA/VCA titres was 1 : 10–1 : 1280. The controls used for each test were highly positive and negative plasma samples at a dilution of 1 : 20. For the detection of EBV-specific antibodies, the cell preparations were firstly exposed to the tested plasma at the previously mentioned dilutions, and were then overlaid with FITC-conjugated goat anti-IgG or anti-IgA globulins (Sigma-Aldrich, USA). The reaction was assessed using a fluorescence microscope (Nikon TS100-F, Germany). The presence of antibodies to the VCA/IgG or VCA/IgA was determined by bright green fluorescence of the tested cells against a background of negative cells. The antibody titre was considered to be the last dilution of plasma, which contained at least 5% of specifically fluorescing cells (with clear positive and negative controls). The indirect immunofluorescence staining was performed as previously described [16, 42]. Antibody titres are expressed as their geometric mean value.

**Quantitative determination of the number of EBV DNA copies**

The amount of viral DNA copies in 1 ml of plasma was determined by real-time PCR. DNA from Namalwa diploid cells (containing two integrated viral genomes) was used for the construction of calibration curves, which was based on the ratio of 3.3 pg genomic DNA to 1 copy of viral DNA. For real-time PCR, the primers for the 76 bp fragment in the BamHIW region of viral DNA (GenBank ID: V01555) were the sense primer W44F (5’-CACCAACTCTCCACA-CACC), antisense primer W-119R (5’-CTT TAGGACGTGTCGCCAGGG) and fluorescent probe W-67T (5’-FAM-CACACACTACA ACACCCACCACGCTTC-RTQ1) [20]. The reaction was conducted in 96-well plates using a CFX96 instrument (Bio-Rad Laboratories, USA) in 50 µl of reaction mixture (Syntol, Russia) containing 0.5 µM of each primer, 25 nM fluorescent probe, 4 mM MgCl\(_2\), 200 µM of each dNTP, 1 unit of Taq polymerase and 10 µl of DNA solution in a 10 mM Tris HCl buffer (pH 8.0) with 1 mM EDTA (corresponding to 50 µl of plasma). Two negative controls (samples that did not contain DNA) were included in each analysis. The PCR conditions were as follows: denaturation at 95°C for 5 min, 40 cycles at 95°C for 15 s and 56.5°C for 30 s. The real-time PCR data were analysed using the CFX Manager software (Bio-Rad).

**Statistical analyses**

Statistical significance of the distribution differences of various measurements (GMT of EBV-specific antibody titres and the values of the EBV DNA load) in the plasma of uNPC and OTOC patients before treatment, subsequent to treatment in remission and in relapse, as well as those of different TNM parameters of the NPC manifestation, was evaluated with the Kruskal–Wallis test. The obtained P-values were considered significant at P<0.05.

**Funding information**
The authors received no specific grant from any funding agency.

**Acknowledgements**

Authors are grateful to Professor M. A. Krasilnikov, the director of the Institute of Carcinogenesis, deputy director for scientific work of the N. N. Blokhin Cancer Research Center of the Ministry of Health of Russian Federation, for his attention and support of the investigations aimed at studying the role of Epstein–Barr virus (EBV) in the development of EBV-associated pathologies in the country.

**Conflicts of interest**
The authors declare that there are no conflicts of interest.

**Ethical statement**
The Ethical Committee of the Russian N.N. Blokhin Cancer Research Centre in which this investigation was done approved it, and randomly selected patients with nasopharyngeal carcinoma (NPC) and OTOC who had given their informed consent to the work.

**References**