Increased frequency of micronuclei in the lymphocytes of patients chronically infected with hepatitis B or hepatitis C virus

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In this study, we analysed the frequency of micronuclei (MN), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) and evaluated mutagen-induced sensitivity in the lymphocytes of patients chronically infected with hepatitis B virus (HBV) or hepatitis C virus (HCV). In total, 49 patients with chronic viral hepatitis (28 HBV-infected and 21 HCV-infected patients) and 33 healthy, non-infected blood donor controls were investigated. The frequencies (%) of MN, NPBs and NBUDs in the controls were 4.41 ± 2.15, 1.15 ± 0.97 and 2.98 ± 1.31, respectively. The frequencies of MN and NPBs were significantly increased (p < 0.0001) in the patient group (7.01 ± 3.23 and 2.76 ± 2.08, respectively) compared with the control group. When considered separately, the HBV-infected patients (7.18 ± 3.57) and HCV-infected patients (3.27 ± 2.40) each had greater numbers of MN than did the controls (p < 0.0001). The HCV-infected patients displayed high numbers of NPBs (2.09 ± 1.33) and NBUDs (4.38 ± 3.28), but only the HBV-infected patients exhibited a significant difference (NPBs = 3.27 ± 2.40, p < 0.0001 and NBUDs = 4.71 ± 2.79, p = 0.03) in comparison with the controls. Similar results were obtained for males, but not for females, when all patients or the HBV-infected group was compared with the controls. The lymphocytes of the infected patients did not exhibit sensitivity to mutagen in comparison with the lymphocytes of the controls (p = 0.06). These results showed that the lymphocytes of patients who were chronically infected with HBV or HCV presented greater chromosomal instability.

Key words: hepatitis B - hepatitis C - lymphocytes - micronucleus

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections affect millions of individuals worldwide. Approximately 10-15% of HBV-infected patients and 70-80% of HCV-infected patients develop the chronic form of their respective diseases. The primary problem associated with persistent HBV and HCV infections is the development and progression of liver disease, which ranges in severity from minimal lesions throughout the liver parenchyma to severe fibrosis, cirrhosis and hepatocellular carcinoma (HCC) (Szabó et al. 2004).

Both HBV and HCV may promote potentially mutagenic cellular processes, such as integration into the host DNA (in the case of HBV) and the generation of oxidative stress as a consequence the immune response (Farinati et al. 1995, Bartsch & Nair 2004, Saigo et al. 2008, Sung et al. 2012).

The mutagenic effects of HBV and HCV infections are visible in hepatocytes and lymphocytes, as demonstrated by the presence of the oxidative promutagenic DNA lesion 8-oxo-7,8-dihydro-2’-deoxyguanosine (8-oxodG), which is primarily found in the lymphocytes of HCV-infected patients (Hagen et al. 1994, Farinati et al. 1995, 1999, Cardin et al. 2001). In addition, the leukocytes of HCV-infected patients and HBV-infected patients have been demonstrated to exhibit increased chromosomal instability (Machida et al. 2010).

The DNA damage response (DDR) includes various downstream pathways that coordinate cell cycle arrest with the repair of damaged DNA. Alternatively, the DDR can mediate the clearance of affected cells that are beyond repair through apoptosis or senescence (Heijink et al. 2013). Cells that are inefficient in these mechanisms generally exhibit higher sensitivity to mutagenic agents and it has been suggested that these cells may be preferentially selected during tumour development (Jackson & Bartek 2009). The in vitro mutagen sensitivity assay in lymphocytes has been used to examine the individual response to mutagen in comparison with the lymphocytes of the controls (p = 0.06). These results showed that the lymphocytes of patients who are chronically infected with HBV or HCV presented greater chromosomal instability.

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SUBJECTS, MATERIALS AND METHODS

Study population - In total, 49 patients with chronic viral hepatitis, consisting of 28 HBV-infected patients and 21 HCV-infected patients, and 33 healthy, non-infected controls were included in the study. The patients were recruited from the Clinic of Infectious Diseases of Julio Muller Hospital (Federal University of Mato Grosso, Cuiabá, state of Mato Grosso, Central-West Brazil). Chronic HBV infection was confirmed by persistent HBV surface antigenemia lasting more than six months. Chronic HCV infection was confirmed by the presence of HCV RNA in blood tests. Cirrhosis was diagnosed by liver biopsy or based on clinical observations, laboratory tests or ultrasonographic evidence. Non-infected subjects were recruited from a group of healthy blood donors at the Public Blood Bank of Mato Grosso State. The absence of infection in the control group was confirmed by laboratory tests. Information about alcohol consumption, tobacco use, ethnicity and age was obtained from medical records and from an interviewer-administered questionnaire, which also included questions about exposure to mutagens and any history of cancer in the individual.

Cytokinesis-block MNs (CBMN) assay - The CBMN assay was performed as described by Fenech and Morley (1985), with minor modifications. In total, 5 µL of venous blood was collected in heparin-Vacutainer tubes (Becton & Dickinson, Franklin Lakes, NJ, USA) and lymphocyte cultures (2 per subject) were established using 0.3 mL of whole blood added to RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) at 400X magnification. For each analysis was performed with a light microscope (Nikon, Melville, NY, USA) supplemented with 20% foetal calf serum (Cultilab, Campinas, SP, Brazil), 0.001% penicillin (Vetc, Duque de Caxias, RJ, Brazil), 0.0005% streptomycin (Sigma-Aldrich) and 2% phytohemagglutinin (Cultilab). After the cultures were incubated for 44 h at 37°C in a BOD incubator (Eletrolab, São Paulo, SP, Brazil), cytochalasin B (Sigma-Aldrich) was added to the cultures (6 µg/mL). The cells were harvested by centrifugation at 72 h after the culture was initiated. The lymphocytes were treated with a hypotonic solution (1% sodium citrate w/v) and fixed in a solution of methanol:acetic acid (3:1 v/v); in both cases, the solutions were ice cold and freshly prepared. The cell suspension was dropped onto a pre-cooled microscope slide and air dried before being stained for 5-7 min with 5% Giemsa in Sorensen phosphate buffer (0.06 M Na2HPO4 and 0.06 M KH2PO4, pH 6.8). Microscopic analysis was performed with a light microscope (Nikon, Melville, NY, USA) at 400X magnification. For each individual, 2,000 binucleated cells were analysed for the presence of MN, NPBs and NBUDs in accordance with previously established criteria (Fenech et al. 2003). We calculated the frequency of each biomarker (number in 1,000, %) using the following formula: % of X = (number of X/2,000) x 1,000, where X is MN, NPBs or NBUDs (Montero et al. 2006).

Mutagen sensitivity evaluation - To determine mutagen sensitivity, the well-established mutagen DXR was used to treat lymphocytes from patients and non-infected subjects at 44 h after the culture was initiated. The cells were treated with 0.15 µg/mL DXR (Bergamo, Taboão da Serra, SP, Brazil) diluted in sterile distilled water for 28 h, which brought the total culture time to 72 h. The experimental conditions for the DXR treatment were previously established in preliminary experiments. The conditions for cell harvesting and slide preparation were described in the previous section. Sensitivity to DXR was expressed as induced DNA damage: [(mean MN after DXR)-(basal mean MN)].

Statistical analysis - An age comparison between groups was performed using one-way ANOVA and the Bonferroni post-hoc test. When the data exhibited unequal variance, the median numbers of MN, NPBs and NBUDs were compared between the groups using the non-parametric Kruskal-Wallis test followed by Dunn’s post-hoc test. Similarly, the median numbers of MN obtained in the mutagen sensitivity test were compared between the groups using the Mann-Whitney U test. The mean measurements of the induced DNA damage were compared using the Student’s t test. The G-test or the χ2 test was used to compare ethnicity, sex, alcohol intake and tobacco intake between the groups. A linear regression model was constructed using the Stata 8.2 software programme (StataCorp, College Station, TX, USA) to verify the independence of the frequencies of MN, NPBs and NBUDs from the virus type, sex, age, alcohol intake, tobacco intake and the use of antiviral drugs. A value of p ≤ 0.05 was used as the criterion for significance. The statistical analyses were performed using the statistical software programme Bioestat 5.0 (Ayres et al. 2012).

Ethics - This study was approved by the Ethical Research Board of Julio Muller Hospital (protocol 439/CEP-HUJM/07) and informed consent to voluntarily participate was given by all of the subjects.

RESULTS

Of the 49 patients included in the study, 28 were chronically infected with HBV (21 males and 7 females) and had a mean age of 36.1 years (ranging from 21-56 years), while 21 were chronically infected with HCV (13 males and 8 females) and had a mean age of 44.1 years (ranging from 27-55 years). The non-infected control group consisted of 23 males and 10 females with a mean age of 37.4 years (ranging from 20-52 years). No differences were observed between the non-infected group and the HBV or HCV-infected patients with regard to their sex (p = 0.97) and smoking habits (p = 0.16). The mean age was similar between the infected patients and the non-infected control subjects; however, the mean age of the HCV-infected patients was significantly higher than that of either the chronically HBV-infected group or the controls (p < 0.05). Alcohol consumption among the study subjects ranged from 0-8 g/day and was more frequently reported by the non-infected control subjects (45.4%) than by the HBV-infected patients (17.86%) or the HCV-infected patients (14.28%) (p = 0.003). Cirrhosis was present in eight HBV-infected patients (28.6%) and three HCV-infected patients (10.71%) (p = 0.013) (Table I). All patients with cirrhosis were males. Seven patients (6 HBV-infected and 2 HCV-infected patients) were undergoing antiviral therapy at the time of the study (1 lamivudine and tenofovir, 2 ribavirin and α-interferon and 3 tenofovir).
TABLE I
Demographic and clinical characteristics of patients chronically infected with hepatitis B virus (HBV) or hepatitis C virus (HCV) and of control subjects

| Demographic and clinical parameters | Controls (n = 33) | Total patients (n = 49) | HBV (n = 28) | HCV (n = 21) |
|-------------------------------------|------------------|------------------------|--------------|--------------|
| Males                              | 23 (69.7)        | 36 (69.4)              | 21 (75)      | 13 (61.9)     |
| Females                            | 10 (30.3)        | 15 (30.6)              | 7 (25)       | 8 (38.1)      |
| Age (years)                        | 37.4 ± 10.4      | 37.4 ± 10.3            | 36.1 ± 10.36 | 37.4 ± 7.7 |
| Range                              | (20-52)          | (20-55)                | (27-55)      | (20-52)      |
| Smoking habit                       | 1 (3.03)         | 8 (16.3)               | 4 (14.3)     | 4 (19.04)    |
| Alcohol consumption                | 15 (45.4)b       | 8 (16.3)               | 5 (17.9)     | 3 (14.3)     |
| Cirrhosis                          | -                | 11 (22.4)              | 8 (28.6)     | 3 (14.3)     |

a: ANOVA and Bonferroni post-hoc test, p < 0.05; b: G test, p = 0.004.

The numbers of MN, NPBs and NBUDs observed in the patient group were 7.01 ± 3.23, 2.76 ± 2.08 and 4.57 ± 2.98, respectively. The frequencies of MN and NPBs, but not NBUDs, were significantly increased (p < 0.0001) compared with the frequencies in the controls (4.41 ± 2.15 and 1.15 ± 0.97 for MN and NPBs, respectively, in the controls). Considering the HBV-infected patients and the HCV-infected patients separately, the numbers of MN in the HBV-infected patients (7.18 ± 3.57) and HCV-infected patients (6.78 ± 2.80) were significantly higher than in the non-infected group (4.41 ± 2.15) (p < 0.0001). The numbers of NPBs and NBUDs in the HBV-infected patients (3.27 ± 2.40 and 4.71 ± 2.79, respectively) and in patients infected with HCV (2.09 ± 1.33 and 4.38 ± 3.28, respectively) were increased relative to those of the control group (1.15 ± 0.97 and 2.98 ± 1.31, respectively). However, only the difference between the HBV-infected group and the control group was statistically significant (p = 0.03) (Table II). The results were independent of age, sex, alcohol consumption, tobacco intake and the presence of cirrhosis.

The frequency of MN was significantly influenced by sex only for males aged < 40 years in the control group, who showed a significantly lower frequency of MN (3.46 ± 1.12) than did females (5.63 ± 1.60) of the same age (p = 0.01). The frequency of MN and NPBs was significantly higher in males, among all patients and within the HBV-infected group in comparison with the controls (p < 0.01) (Table III). The frequency of NPBs was significantly higher in females aged > 40 years in comparison with the controls (p < 0.01). The frequency of MN, NPBs and NBUDs was not significantly higher in male patients with cirrhosis in comparison with male patients without cirrhosis (Table IV).

Cells from 15 chronically infected patients (9 HBV-infected and 6 HCV-infected patients) and 14 non-infected individuals were tested for DXR sensitivity. The numbers of MN were not significantly different between the HCV-infected and the HBV-infected patients; therefore, the patients were grouped together for comparison with the non-infected individuals to improve the statistical analysis. The patients exhibited significantly higher numbers of MN in both untreated (6.76 ± 3.03, p = 0.03) and treated (15.03 ± 4.94, p = 0.001) cells compared with the cells of non-infected individuals (4.25 ± 1.45 and 9.39 ± 3.39 in untreated and treated cells, respectively). DXR treatment significantly increased the number of MN in both the control group (p = 0.0002) and the patient group (p < 0.0001). The mean measurements of the induced DNA damage were higher in the patient group (8.30 ± 5.40) than in the non-infected control group (5.18 ± 3.45, p = 0.03) and treated cells, respectively.

**DISCUSSION**

Chronic hepatotropic virus (HBV and HCV) infections are characterised by potentially mutagenic cellular events, such as an increase in oxidative stress and viral integration into the DNA of the host cell (Farinati et al. 1995, Bartsch & Nair 2004, Saigo et al. 2008, Sung et al. 2012). In the present study, we demonstrated that the lymphocytes of HBV-infected patients and HCV-infected patients exhibited an increased frequency of MN compared with lymphocytes from healthy, non-infected individuals. This result is in contrast to findings previ-
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Previously reported by Ozkal et al. (2005). However, these authors reported a higher frequency of chromosome breaks, leading to the formation ofacentric chromosome/chromatid fragments that ultimately contributed to the formation of MN. Furthermore, the MN may have resulted from an inability of whole chromosomes to travel to the spindle poles during mitosis (Fenech et al. 2011). A higher frequency of chromosome gaps, aneuploidy and polyploidy in the peripheral blood mononuclear cells (PBMCs) of HCV-infected patients was reported previously (Machida et al. 2010). The present results suggest that chronic infection with HBV or HCV accounts for chromosomal instability in lymphocytes and this phenomenon is characterised by the formation ofacentric fragments and/or aneuploidy.

We also observed increased numbers of NPBs and NBUDs in HBV-infected patients. NPBs may occur when dicentric chromosomes originating from chromosome breaks or telomere-to-telomere end fusions are pulled to opposite poles of the cell during mitosis. NBUDs are primarily considered to be formed from amplified DNA that is being eliminated from chromosomes (Fenech et al. 2011). Although gene amplifications (MYC and ERBB2) were demonstrated in HCC tissue samples that were infected with HBV or HCV (Al-Qahtani et al. 2010), no reports have described this genetic alteration in PBMCs from individuals with viral hepatitis. Dicentric Y chromosomes have also been reported in the PBMCs of HCV-infected patients (Machida et al. 2010). Together with our results, these data suggest that the chromosomal instability in lymphocytes that results from HBV infection (and likely also from chronic HCV infection) is characterised by the presence of dicentric chromosomes and gene amplification.

### TABLE III

The effect of age and sex on frequency of micronucleus (MN), nucleoplasmatic bridges (NPB) and nuclear buds (NBUDs) in hepatitis B virus (HBV) or hepatitis C virus (HCV)-infected patients and controls

| Groups                  | MN (mean ± SD) | NPB (mean ± SD) | NBUDs (mean ± SD) |
|-------------------------|----------------|-----------------|-------------------|
| Controls (n = 33)       |                |                 |                   |
| Males < 40 years (n = 14) | 3.46 ± 1.12a  | 1.32 ± 1.31     | 2.86 ± 2.67       |
| Males ≥ 40 years (n = 9)  | 4.06 ± 1.59    | 0.83 ± 0.5      | 2.67 ± 1.17       |
| Females < 40 years (n = 4) | 5.63 ± 1.60    | 1.50 ± 0.82     | 4.13 ± 0.85       |
| Females ≥ 40 years (n = 6) | 6.33 ± 3.54    | 1.0 ± 0.63      | 3.0 ± 1.26        |
| Total patients (n = 49) |                |                 |                   |
| Males < 40 years (n = 14) | 6.79 ± 4.49b   | 3.5 ± 2.97b     | 4.03 ± 2.45       |
| Males ≥ 40 years (n = 20) | 7.25 ± 2.92b   | 3.08 ± 1.45b    | 5.10 ± 3.72       |
| Females < 40 years (n = 6) | 6.92 ± 2.85    | 3.0 ± 2.34      | 4.0 ± 2.61        |
| Females ≥ 40 years (n = 9) | 6.89 ± 2.10    | 3.50 ± 1.44b    | 4.61 ± 2.25       |
| HBV (n = 28)            |                |                 |                   |
| Males < 40 years (n = 12) | 6.71 ± 4.82b   | 3.27 ± 3.14b    | 4.42 ± 2.45       |
| Males ≥ 40 years (n = 9)  | 7.33 ± 2.03b   | 3.27 ± 1.51b    | 5.89 ± 3.46       |
| Females < 40 years (n = 4) | 7.0 ± 3.63     | 3.25 ± 2.96     | 3.25 ± 2.90       |
| Females ≥ 40 years (n = 3)  | 8.83 ± 1.53    | 3.12 ± 0.48     | 4.33 ± 0.76       |
| HCV (n = 21)            |                |                 |                   |
| Males < 40 years (n = 2)  | 7.25 ± 2.47    | 5.0 ± 0         | 1.75 ± 0.35       |
| Males ≥ 40 years (n = 11) | 7.18 ± 3.59b   | 2.67 ± 1.37b    | 4.45 ± 3.97       |
| Females < 40 years (n = 2) | 6.75 ± 1.06    | 2.50 ± 0.71     | 5.50 ± 1.41       |
| Females ≥ 40 years (n = 6) | 5.92 ± 1.66    | 3.80 ± 1.92b    | 4.75 ± 2.79       |

a: statistically significant in comparison to females of the same age-class (p = 0.01, Mann-Whitney U test); b: statistically significant in relationship to controls of the same age class (p < 0.01, ANOVA, post-test Student t or Kruskal-Wallis, post test Student-Newman-Keuls); c: groups with n < 4 were not included in statistical analysis; SD: standard deviation.

### TABLE IV

The effect of cirrhosis on frequency of micronucleus (MN), nucleoplasmatic bridges (NPB) and nuclear buds (NBUDs) in males hepatitis B virus (HBV) or hepatitis C virus (HCV)-infected patients

| Groups | Cirrhosis | MN (mean ± SD) | NPB (mean ± SD) | NBUDs (mean ± SD) |
|--------|-----------|----------------|-----------------|-------------------|
| Total patients | No (23) | 6.82 ± 4.10 | 2.98 ± 2.53 | 3.67 ± 1.75 |
|          | Yes (11) | 7.54 ± 2.27 | 2.68 ± 1.58 | 5.00 ± 2.59 |
| HBV     | No (15)  | 6.56 ± 4.36 | 3.26 ± 2.91 | 4.13 ± 1.72 |
|          | Yes (8)  | 8.25 ± 2.23 | 3.06 ± 1.68 | 5.25 ± 2.39 |
| HCV     | No (9)   | 7.31 ± 3.80 | 2.43 ± 1.63 | 2.81 ± 1.53 |
|          | Yes (3)  | 7.00 ± 2.90 | 1.66 ± 0.76 | 4.33 ± 3.54 |

SD: standard deviation.

We also observed increased numbers of NPBs and NBUDs in HBV-infected patients. NPBs may occur when dicentric chromosomes originating from chromosome breaks or telomere-to-telomere end fusions are pulled to opposite poles of the cell during mitosis. NBUDs are primarily considered to be formed from amplified DNA that is being eliminated from chromosomes (Fenech et al. 2011). Although gene amplifications (MYC and ERBB2) were demonstrated in HCC tissue samples that were infected with HBV or HCV (Al-Qahtani et al. 2010), no reports have described this genetic alteration in PBMCs from individuals with viral hepatitis. Dicentric Y chromosomes have also been reported in the PBMCs of HCV-infected patients (Machida et al. 2010). Together with our results, these data suggest that the chromosomal instability in lymphocytes that results from HBV infection (and likely also from chronic HCV infection) is characterised by the presence of dicentric chromosomes and gene amplification.
In this study, females and subjects aged ≥40 years exhibited more MN than did males and subjects <40 years, respectively, among both controls and HBV-infected patients. Although these differences were not statistically significant, the results are in accordance with the well-known influence of sex and age on MN frequencies, as reported before (Fenech 1998, Bonassi et al. 2001, Fenech & Bonassi 2011). Reports on the effects of sex and age on NPBs and NBUDs are conflicting in the literature (Donmez-Altuntas & Bitgen 2012, Nefic & Handzic et al. 2013).

Considering the patient group, we found statistically significantly higher frequencies of MN in males than in females; however, females did not present cirrhosis in our sample. It is known that DNA damage levels in the leukocytes of HBV-infected patients and HCV-infected patients significantly correlate with the presence of liver lesions (Farinati et al. 1999, Cardin et al. 2001, Grossi et al. 2008, Hoare et al. 2013). In fact, in the current study, the frequencies of MN (in HBV-infected patients) and NBUDs (in HBV-infected patients and HCV-infected patients) were increased in cirrhotic males, but this difference was not significant. Whether the extension of liver lesions in male patients is correlated with the frequency of MN found in lymphocytes is not completely clear in the sample investigated here. Even considering that the statistical analysis may have been influenced by the small size of our sample, these results should be considered with caution, especially because chromosome damage detected by the MN assay is an important biomarker for cancer prediction (Bonassi et al. 2007).

HBV and HCV may contribute to increased chromosomal aberrations in infected cells by direct and indirect pathways. Regarding the indirect pathway, it has been shown that the presence of reactive oxygen species (ROS) resulting from cytokine activity during chronic inflammation has a potent mutagenic effect (Yan et al. 2006). A relationship between infection with HBV or HCV and increased production of ROS, chromosomal aberrations and other DNA damage has been reported previously (Hagen et al. 1994, Machida et al. 2010). Furthermore, leukocytes from patients who are chronically infected with HBV or HCV exhibit higher levels of 8-OHdG, which is the most frequent ROS-induced base lesion (Farinati et al. 1999, Cardin et al. 2001).

Viral integration is considered to have direct mutagenic potential in hepatocytes. The integration of HBV into the human genome affects the expression of genes located near the site of insertion and also causes more widespread alterations of chromosomal stability (Saigo et al. 2008, Hoare et al. 2013). The integration of HBV and HCV interact with the proteins of the DNA repair machinery and inhibit their functions in host cells (Chen et al. 2008, Machida et al. 2010, Pal et al. 2010). Therefore, it is possible that the cells of infected patients exhibit less efficient DNA repair mechanisms due to the effects of the viral proteins, which may contribute to the elevated frequency of DNA damage detected in this study.

Au et al. (2010) reported that when the cells of exposed populations (in this case, HCV-infected patients or HBV-infected patients) are challenged with a DNA-damaging agent in vitro, the in vivo exposure-induced repair deficiency is dramatically amplified. Additionally, the deficiency will be detectable in a challenge assay as an increase in the number of chromosomal aberrations, MN or unaired DNA strand breaks.

Less effective DNA repair may also result in a higher sensitivity to mutagens. It has been shown that B lymphocytes infected with HCV in vitro exhibit increased sensitivity to bleomycin due to the action of the NS3 protein and the core viral protein (Machida et al. 2010). Furthermore, it was demonstrated that lymphocytes from HBV or HCV-infected HCC patients exhibit an increased sensitivity to bleomycin and benzo(a)pyrene-diol-epoxide, which is associated with an increased risk of cancer development (Wu et al. 1998). The results obtained in the present study showed that the lymphocytes of HBV-infected patients and HCV-infected patients are not more sensitive to DXR than the lymphocytes of non-infected subjects. However, a challenge assay with other substances, such as bleomycin, needs to be performed to allow more definite conclusions about mutagen sensitivity in these patients.

In summary, the present study demonstrated that the lymphocytes of patients who are chronically infected with HBV or HCV exhibit greater chromosomal instability, characterised by the presence of MN, NPBs and NBUDs. Although we did not observe a statistically significant result, a possible influence of cirrhosis on these parameters should be considered for further investigation.

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