# Diagnosis of Primary Vitreoretinal Lymphoma

Subjects: Hematology

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Intraocular lymphomas (IOLs) include vitreoretinal lymphomas (VRLs) and primary uveal or choroidal lymphomas. VRLs are further subdivided into primary VRLs and secondary VRLs, the latter deriving from systemic lymphomas. Primary uveal or choroidal lymphomas are usually low-grade neoplasms and are frequently extranodal marginal zone lymphomas with very good outcomes, unlike primary vitreoretinal lymphomas (PVRLs) which are high-grade diseases with poor outcomes. Secondary IOLs derive from ocular involvement by systemic lymphomas through haematogenous spread. Systemic lymphomas mainly disseminate to the uvea, due to its rich blood flow. PVRL represents a diagnostic challenge for both clinicians and pathologists, and it is critical, for the patient's life, to shorten the time between the onset of symptoms often mistaken for chronic uveitis and correct diagnosis. Different laboratory methods are in use to diagnose PVRL. The main employed techniques are described, highlighting the principal diagnostic issues with the different laboratory methods.

lymphoma vitreoretinal IL-10

## 1. Cytology plus Immunohistochemistry

Cytological examination of vitreous fluid obtained through vitrectomy is considered the mainstay for primary vitreoretinal lymphomas (PVRLs) diagnosis [1].

Decreasing the cut rate during vitrectomy is essential for obtaining a sufficient number of cells and further improving the diagnostic rate of cytological examination. Regarding this issue, Jiang et al. recommended a cut rate of 600 cpm or less during diagnostic vitrectomy to confirm vitreoretinal lymphomas (VRLs), because cell viability began to decrease at 600 cpm in the in vitro experiments [2].

The morphological assessment of the vitreous sample is complicated, requiring a pathologist with expertise in dealing with this kind of specimen.

The main difficulty is due to the small volume of vitreous sample, which often contains a low number of lymphoma cells. Lymphoma cells are known to be fragile and easily degenerate; therefore, the undiluted and refrigerated vitreous sample should be promptly transported within 1 h from the surgery room to the pathology laboratory and immediately processed.

Prior steroid treatments, performed in clinical suspicion of chronic uveitis, may not only delay diagnosis for a transient beneficial effect but compromise the morphological evaluation due to degenerative changes of lymphoma cells [3].

Diffuse large B cell lymphomas (DLBCLs) cells are large-sized atypical elements with large irregular nuclei, evident nucleolus, and scanty cytoplasm (**Figure 1** and **Figure 2**).

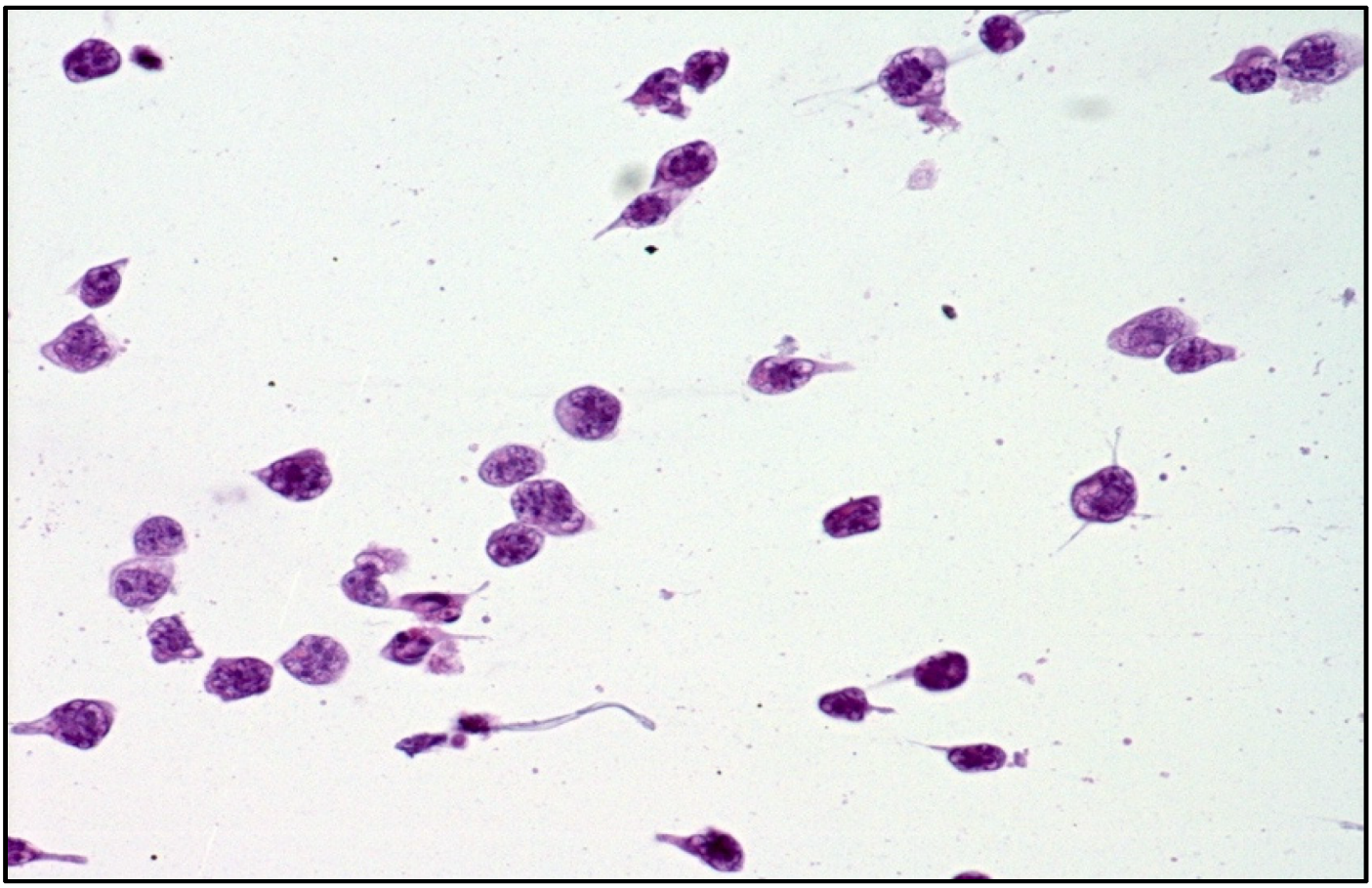

**Figure 1.** Cytology of vitreous sample with a discrete number of atypical lymphoid cells with a high nuclear–cytoplasmic ratio (haematoxylin and eosin, 200× magnification; original image from Dr M. Zanelli).

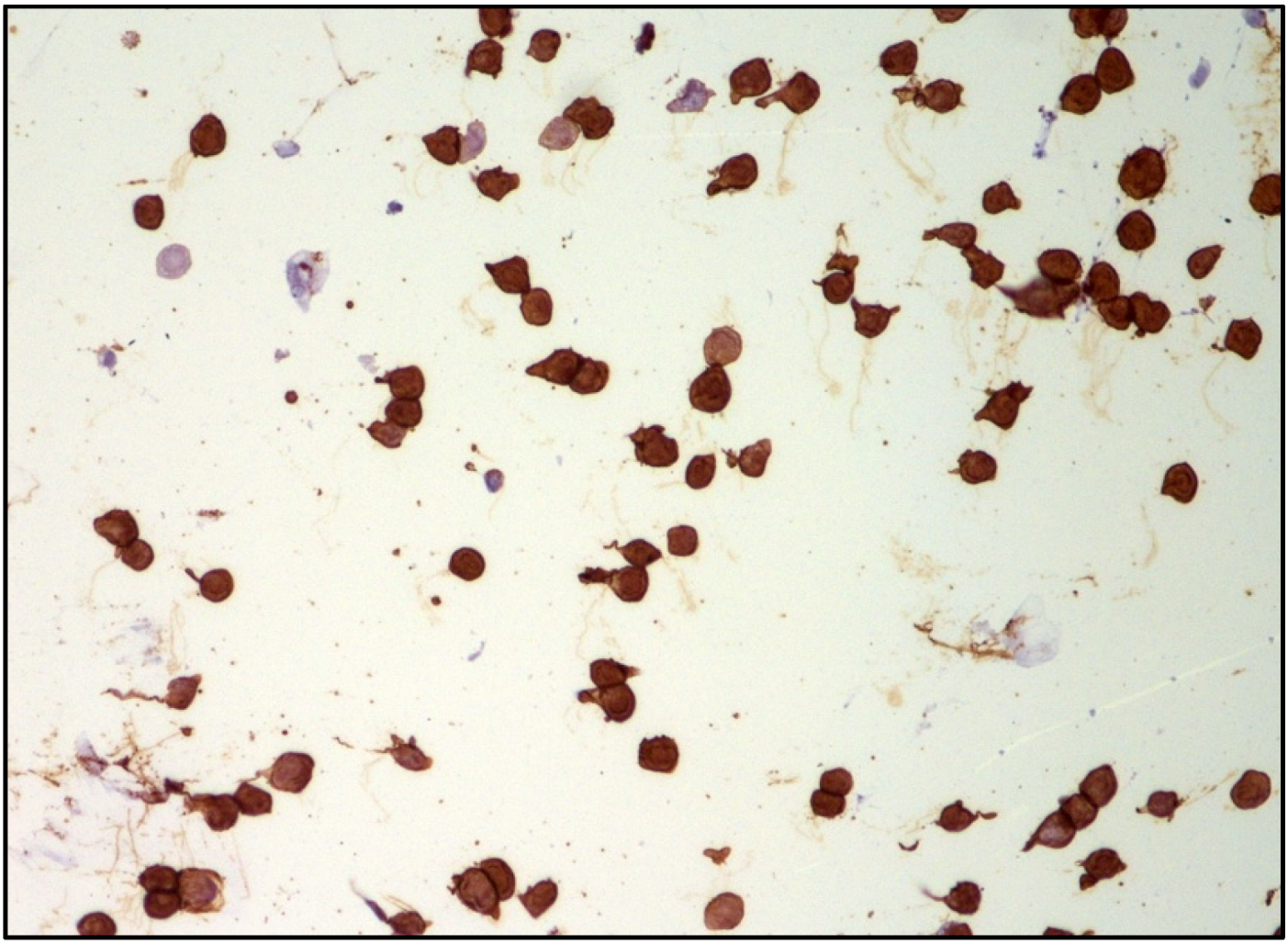

**Figure 2.** Immunohistochemistry performed on cytological sample of vitreous fluid: CD20 highlights the B cell phenotype of the majority of atypical cells (immunostaining; 200× magnification; original image from Dr M. Zanelli).

However, the morphological assessment of vitreous fluid can be complicated by the presence of a background rich in inflammatory cells (T lymphocytes and histiocytes) admixed with only rare lymphoma B cells (**Figure 3**, **Figure 4** and **Figure 6**).

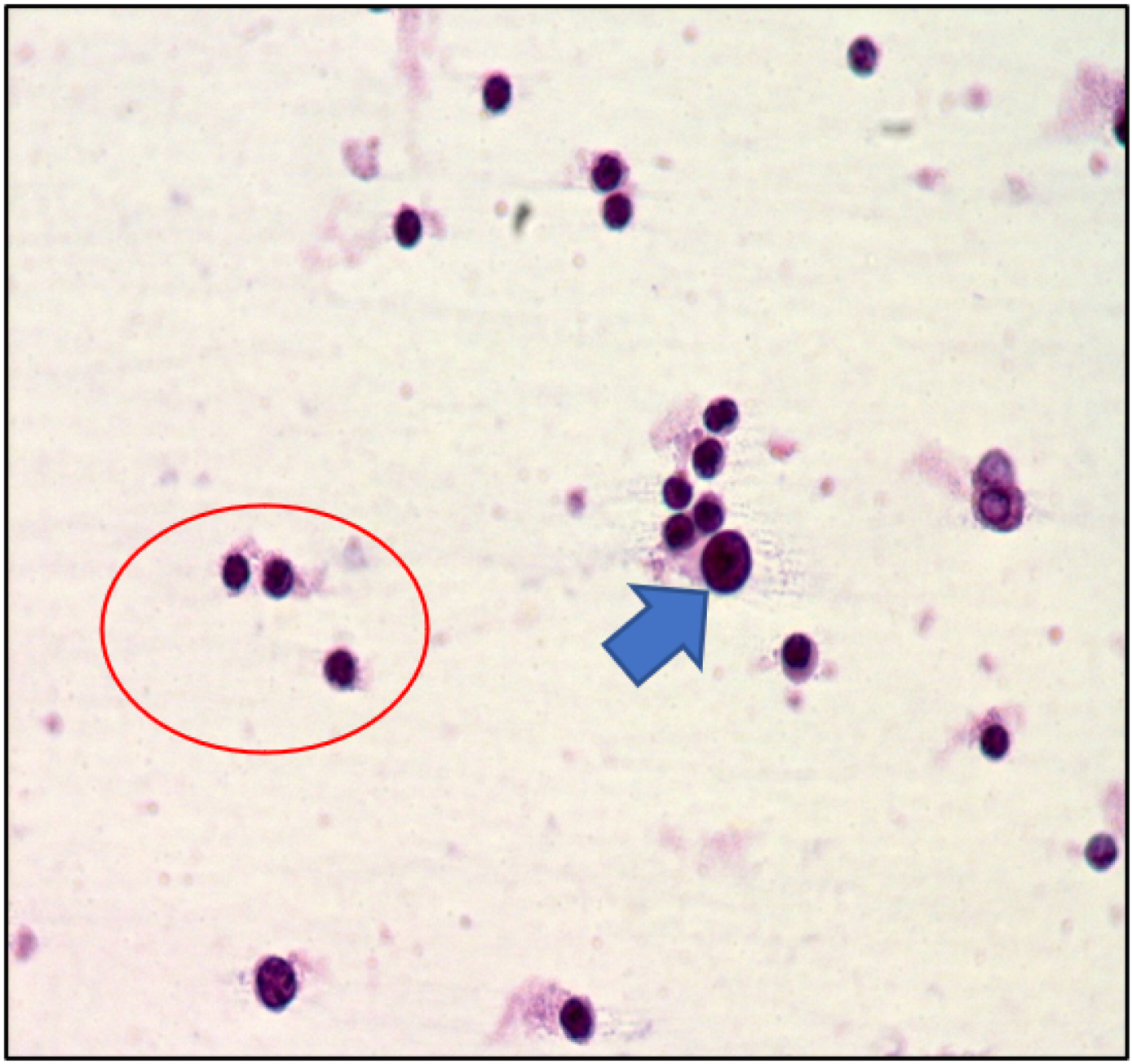

**Figure 3.** Cytology of vitreous sample with only rare large lymphoid cells and a discrete number of small lymphocytes (blue arrow pointing toward a large lymphoid cell; red circle highlighting small lymphoid cells) (haematoxylin and eosin, 200× magnification; original image from Dr M. Zanelli).

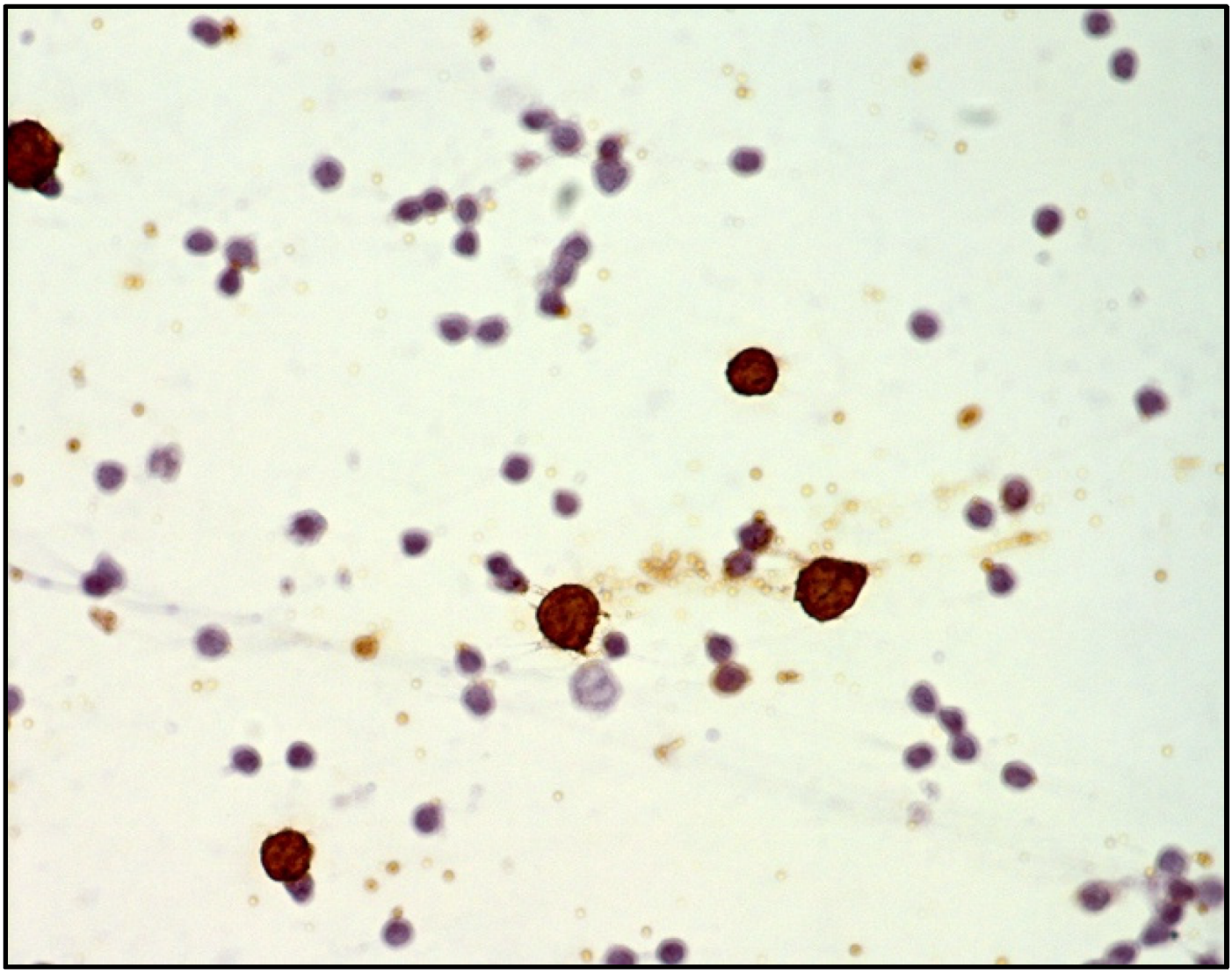

**Figure 4.** Immunohistochemistry performed on cytological sample of vitreous fluid showing only sparse CD20-positive atypical B cells (immunostaining; 200× magnification; original image from Dr M. Zanelli).

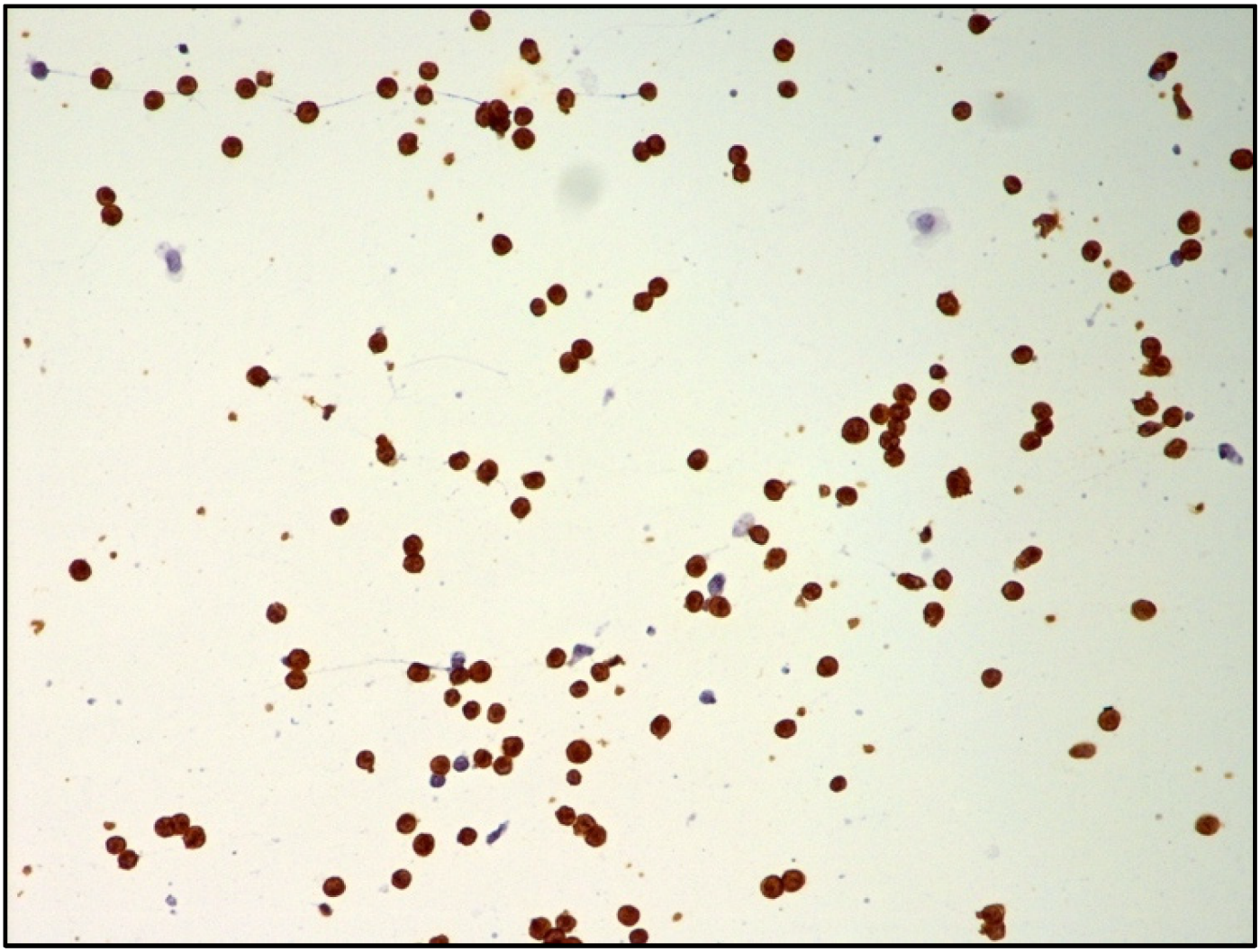

**Figure 5.** Immunohistochemistry performed on cytological sample of vitreous fluid showing numerous small-sized reactive CD3-positive T cells (immunostaining; 200× magnification; original image from Dr M. Zanelli).

Immunohistochemistry with B cell (CD20) and T cell markers (CD3) is often used in adjunct to cytology. However, the paucity and fragility of lymphoma cells often reduce the diagnostic power of immunophenotyping analysis. For the above-mentioned reasons, the sensitivity of cytology is variable (31–87.5%) [4][5] and further tests are needed in the case of a negative cytological result.

## 2. IL-10 and IL-6

The levels of both IL-10 and IL-6 may be measured in vitreous fluid using enzyme-linked immunosorbent assays (ELISA), multiplex bead-based assays, and cytometric bead array assays. IL-10 is a growth and differentiation factor for B lymphocytes, whereas IL-6 is produced by different types of cells, including inflammatory cells [6][7][8]. Unlike IL-6, which is a marker of inflammatory diseases, IL-10 is high in the intraocular fluid of PVRL patients, and an IL-10: IL-6 ratio over 1.0 is highly suggestive of lymphoma [1][9].

Recently, the ‘Interleukin Score for intraOcular Lymphoma Diagnosis’, or ISOLD, was developed [9]. Aqueous or vitreous IL-10 and IL-6 levels are inserted into a mathematical formula, resulting in a probability score for PVRL diagnosis. In the recent consensus recommendation paper for PVRL diagnosis by Carbonell et al., the IL-10 level or the IL-10:IL-6 ratio are considered useful parts of the diagnostic repertoire for PVRL diagnosis [1].

However, it needs to be underlined that cytokine production may be influenced by previous steroid or immunosuppressive therapies or certain systemic diseases, and hence, the diagnostic power of IL-10 to IL-6 ratio can be reduced [10]. Taking these limitations into consideration, cytokine analysis is currently considered a valuable adjunctive tool for screening patients suspected of PVRL.

## 3. Clonality Analyses

The majority of PVRLs are aggressive B cell lymphomas, mainly DLBCL, and a minority of cases are T cell lymphomas.

The determination of clonality when evaluating *IGH* and *TCR* gene rearrangements is considered a valuable adjunct for lymphoma diagnosis. PCR analysis targeting rearranged *IG* genes gives multiple amplicons in the case of polyclonal cells, such as in inflammatory conditions (**Figure 6**), and a single amplicon if the cells are monoclonal and neoplastic, such as in lymphomas (**Figure 7**).

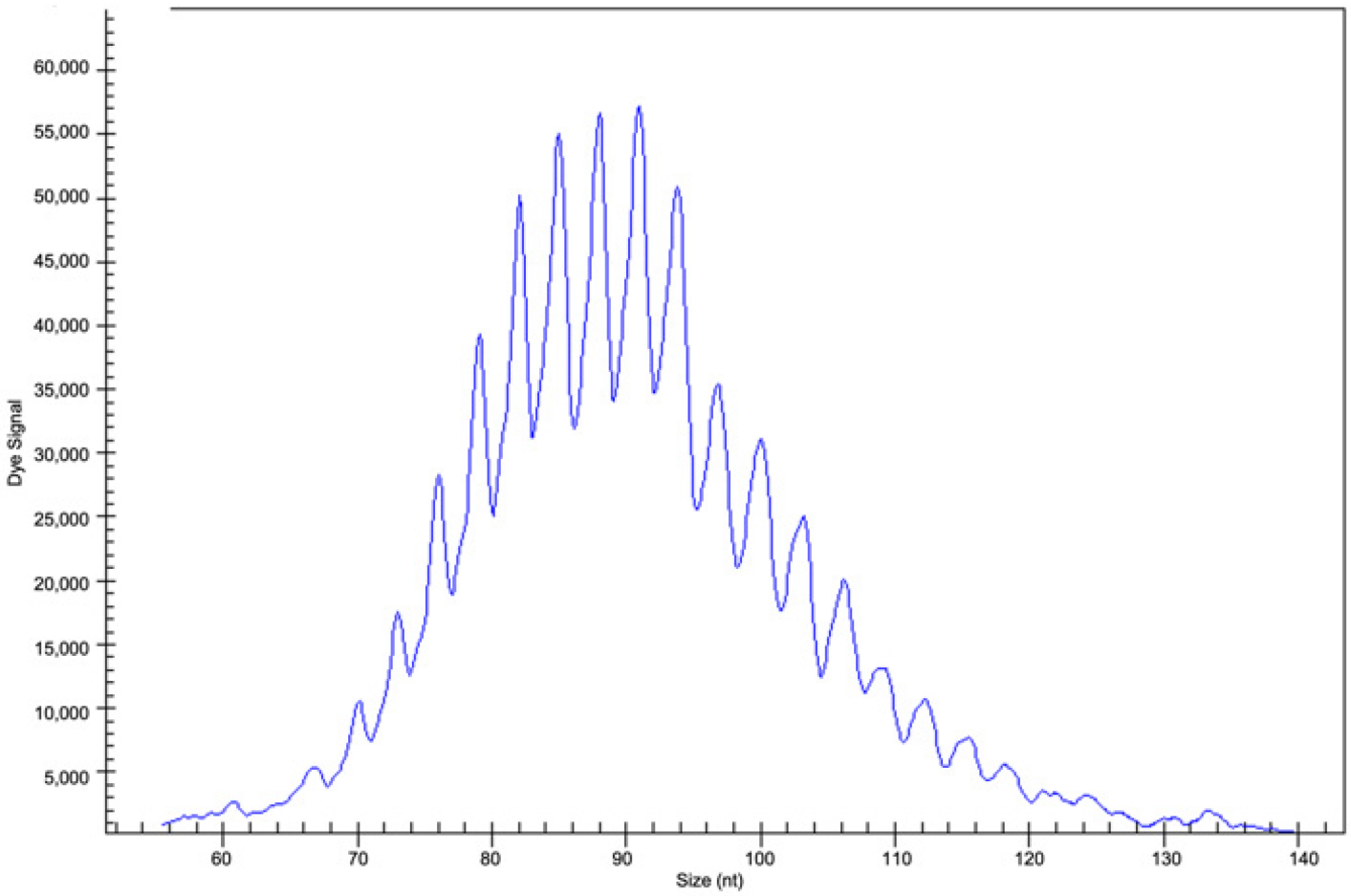

**Figure 6.** Fragment analysis by capillary electrophoresis: case analysed in clinical suspicion of PVRL, not confirmed by clonality analysis showing a polyclonal pattern suggestive of an inflammatory condition (previously unpublished image).

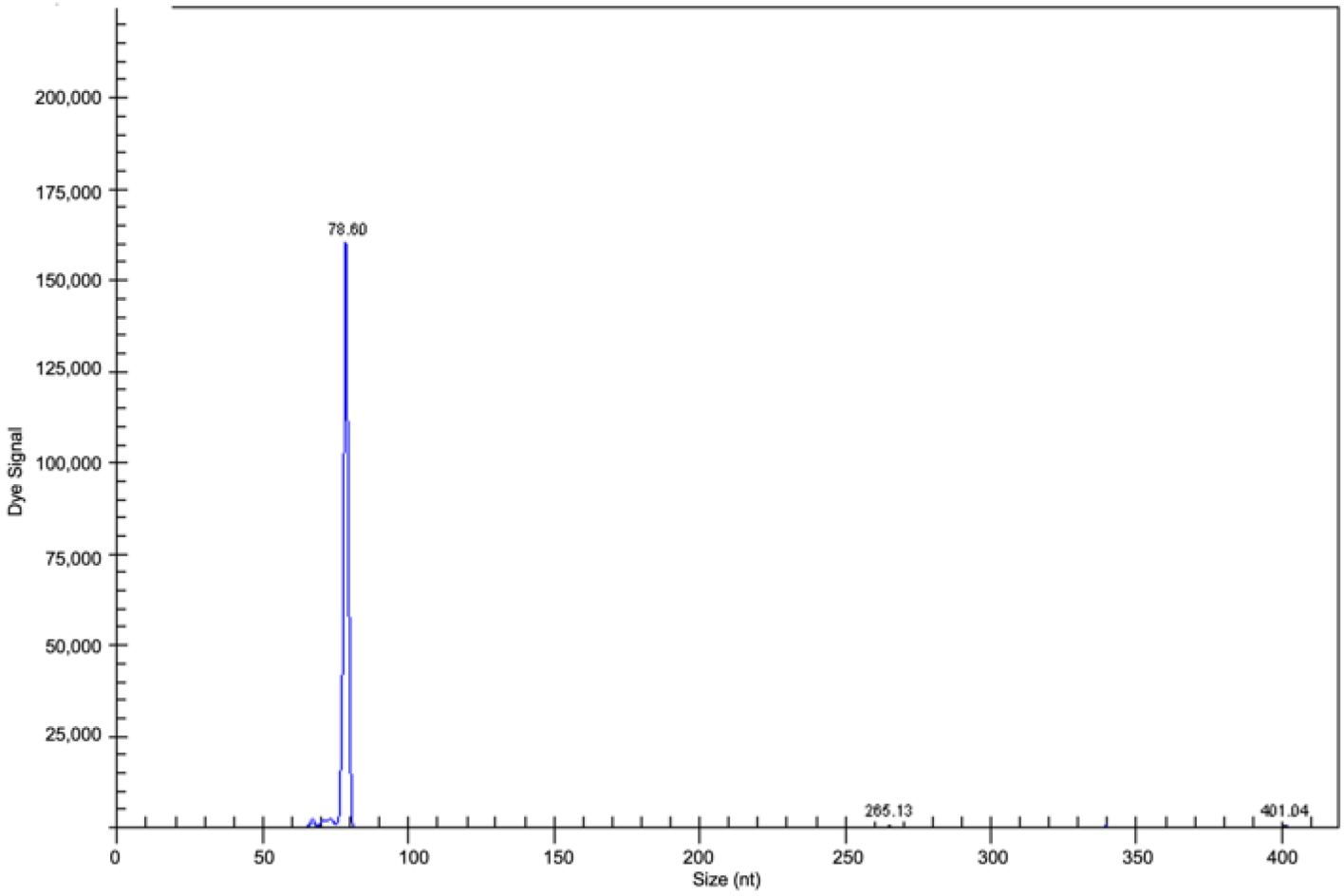


**Figure 7.** Fragment analysis by capillary electrophoresis: clonal gene rearrangement in CDR3 in the range of positivity 70–100 nt in a PVRL case (previously unpublished image).

The accuracy of PVRL diagnosis is improved by the molecular analysis of DNA obtained by PCR, particularly in samples with low cellularity, poorly preserved neoplastic cells, or a prevalence of non-neoplastic T lymphocytes, in which cytology may give a negative result [10].

However, in vitreous samples, there is the potential risk of false negative or false positive results, even by clonality tests.

False negative results may occur because of the high frequency in PVRL of somatic hypermutation, potentially abrogating primer binding [11].

False positive results may be due to the detection of pseudoclonal/oligoclonal B cells by PCR due to the low cellularity of vitreous sample; this event may occur even in benign/inflammatory conditions, making the diagnosis of

PVRL even more difficult [12][13].

## 4. MYD88 Mutation Analysis

The *MYD88* gene is on chromosome 3p22.2. The MYD88 protein, the gene product, is involved in signalling within the immune system. It is a cell membrane-associated protein acting as an adaptor molecule involved in Toll-like receptors (TLRs) and the interleukin-1 receptor (IL-1R) signalling pathway. Following a TLR stimulus, MYD88 activation causes intracellular signalling cascades, such as nuclear factor (NF)-kB activation, favouring the survival of tumour cells [14][15][16][17].

The change of adenine by guanine in the DNA sequence of *MYD88* results in the substitution in MYD88 protein of the amino acid lysine by proline at position 265; this determines the activation of B cells in various diseases [18][19].

DLBCLs arising in immune privileged sites are frequently associated with *MYD88* mutation, predominantly *L265P*. *MYD88-L265P* mutation is commonly associated with DLBCLs of activated B cell (ABC) phenotypes, such as PCNSL, in which the mutation is detected in approximately 75% of cases [20].

Bonzheim et al. retrospectively evaluated the frequency of *MYD88* mutation in PVRLs, analysing 75 vitrectomy specimens of 69 patients, and identified *MYD88* mutations in 69% of cases [21].

The high frequency of *MYD88* mutations, mainly *L265P*, identified in PVRL further supports the concept that PVRL and PCNSL represent the same disease [21]. Narasimhan et al. suggested that *MYD88* mutation analysis has a high diagnostic profile in terms of sensitivity, specificity, and accuracy and that detection of *MYD88* mutation significantly improves the diagnostic yield of vitrectomy samples [17][21].

Real-time PCR is a variation of the standard PCR technique commonly used to quantify DNA or RNA in a sample. Using sequence-specific primers, the number of copies of a DNA or RNA sequence can be determined. By measuring the amount of amplified product at each stage during the PCR cycle, quantification is possible. The threshold of the real-time PCR reaction is the level of signal that reflects a statistically significant increase over the calculated baseline signal, as shown in the detection of *MYD88 L265P* mutation (**Figure 8**).

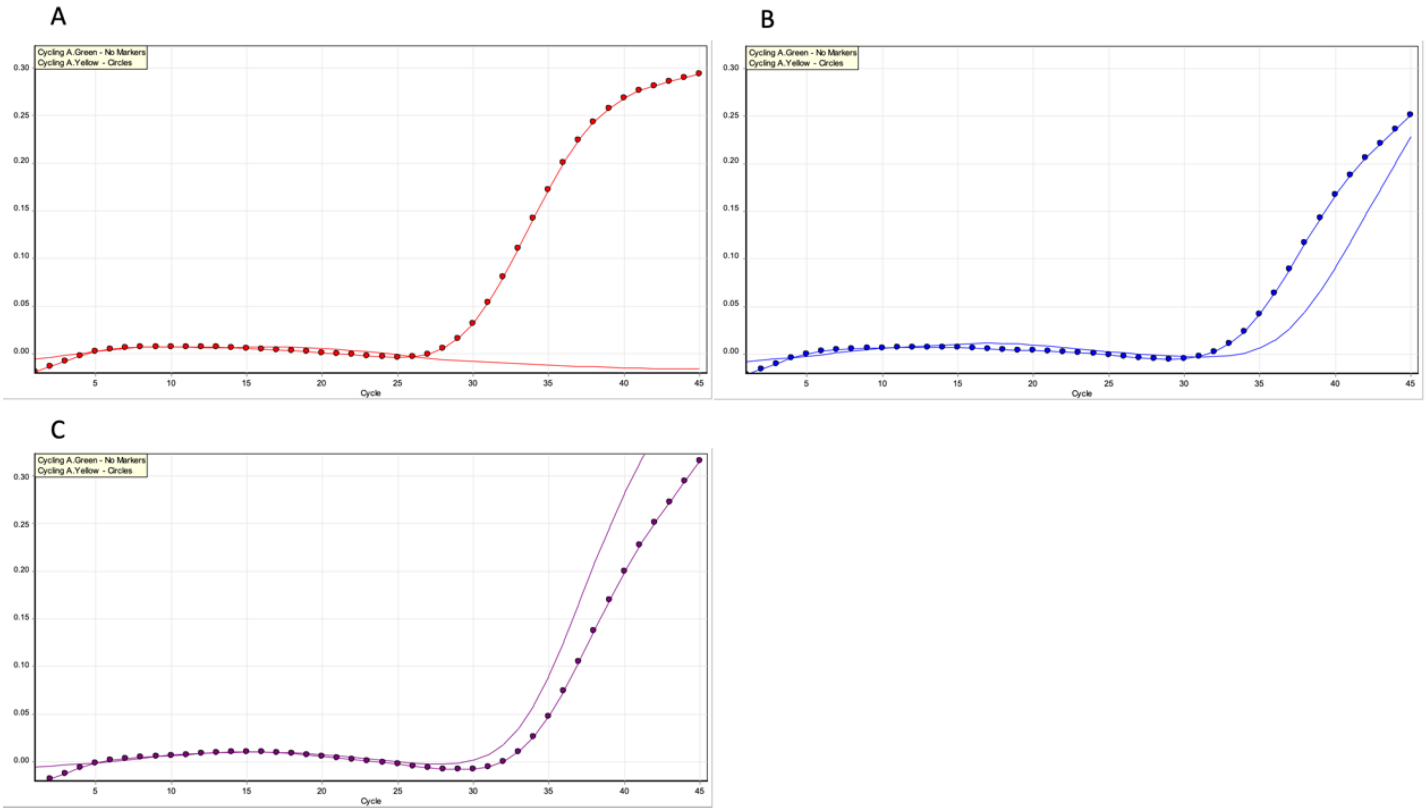


**Figure 8.** Real-time PCR analysis for *MYD88 L265P* mutation. Real-time PCR cycler with 2 channels (green, yellow); test performed through CORBETT/QIAGEN ROTOR-GENE RG-6000 REAL-TIME PCR. (**A**) Wild-type sample with one-channel amplifications; (**B**) mutated sample with two-channel amplification; (**C**) amplification of reference (previously unpublished image).

Several studies report that the PCR-based *IgH* rearrangement assay has some limitations, requiring a larger quantity of cells and having a higher limit of detection (10–20% of clonal B cell population) compared to *MYD88* mutation analysis, which may detect 5% or less of mutant cells [16][17][22][23][24].

## 5. Flow Cytometry

Flow cytometry is a technique used to identify phenotype cells by fluorescent antibodies or dyes. The use of flow cytometry in the diagnosis of lymphoma of B cell origin is based on the criteria that detecting the expression of either immunoglobulin kappa (IGK) or lambda (IGL) light chains may be suggestive of clonality.

In 1997, Davis et al. used flow cytometry for the first time in VRL diagnosis, with good results compared to cytology alone [5][25].

Despite being considered a valuable tool for VRL detection, with elevated sensitivity (82.4%) and specificity (100%) [26], it has to be taken into account that flow cytometry requires a large number of viable cells for diagnosis [22][27].

Hence, the low number of intact neoplastic cells in vitreous fluid, which is often combined with the presence of numerous inflammatory/reactive cells, unfortunately represents a critical limitation for the use of this technique in PVRL diagnosis [22][27].

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