REVIEW ARTICLE

The Vitreous Body and Its Role in the Diagnosis of Eye Pathologies

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Abstract

Vitreous humour is a biologically weak immune cavity of the eye, and is in some way isolated from pathogens of the rest of the organism due to a series of special immunological privileges. However, it is a place where processes of varied ethiology (immune, infectious or neoplastic) can develop indirectly due to leakage or release of molecules or even cells into the vitreous chamber, or may be the receptacle of molecules derived from processes developed in other organs in close contact with the vitreous (such as the retina or other underlying layers such as the choroid), which makes the vitreous body an accessible place for diagnosis and with less potential for side effects for analysis than other adjacent eye structures. This article reviews the most frequent pathologies that requires vitreous diagnosis, the most commonly used approaches for sampling and the diagnostic tests that can be performed on vitreous samples.

Key words: masquerade syndrome, uveitis, lymphoma, vitritis, vitrectomy.

1. Introduction.

Vitreous humor or vitreous body is the transparent gel that occupies the posterior chamber (PC) of the eyeball, located between the lens and the posterior eye wall, whose inner layer is the neurosensory tissue that receives and transmits the image to the central nervous system (CNS) called the retina. Its functions are to support the retina, provide volume to the eyeball, and maintain its transparency to allow light rays to enter the retina. This gel is avascular and weakly antigenic, containing only cells, mostly phagocytes, whose function is to remove unwanted cellular waste from the visual field.[1,2] This fact, coupled with the so-called "immune privilege" (physiological mechanism characteristic of the inner compartments of the eye, which prevents local expression of existing systemic immunity, which gives it the ability to protect against pathogens); and the presence of the internal blood-retinal barrier, make it an organ a priori isolated from the rest of the body.[3,4] However, in the vitreous humor, processes of varied etiology (immune, infectious or neoplastic) may develop or may be the receptacle of molecules indirectly derived from processes developed in other organs in close contact with the vitreous (such as the retina or other underlying layers like the choroid), which makes the vitreous body an accessible place for diagnosis and with less potential for side effects for analysis than other adjacent eye structures.

In this article we try to make a review of eye pathologies in which the diagnosis of vitreous is more cost-effective, detailing both current surgical and non-surgical techniques that allow us to access the vitreous body, and to detail the different diagnostic techniques involving multidisciplinary collaboration between ophthalmologists and other specialists such as pathologists, hematologists, medical and radiation oncologists, and other specialists. This has led to the standardization of diagnostic criteria, classification, staging, and treatment strategies to provide patients with optimal comprehensive care.[5]

2. Eye diseases with clinical repercussions on the vitreous body.

Vitreous plays an important role in the origin and activation of various ocular pathologies. The subsequent vitreous liquefaction developed over the years by dissolving collagen fibers gives rise to several primary degenerative pathologies at the vitreoretinal junction and which subsequently affect the retina. And in contrast, there are primary retinal diseases that affect vitreous in later stages, such as diabetic retinopathy and other vitreoretinal proliferations. And also, certain eye diseases have their beginnings in other layers of the eye that are not in immediate contact with the vitreous, but can cause leakage or release of molecules or even cells into the vitreous chamber, causing symptoms and this, may be useful for making diagnosis because vitreous is more accessible.

2.1. Masquerade syndromes.

The latter is the case the most important pathology for vitreous diagnosis: masquerade syndromes (MS). This is a type of idiopathic chronic uveitis, which comprises a vast group of diverse diseases that can affect the retina, uvea, optic nerve and also the vitreous compartment, and can have devastating effects on vision. Uveitis means

inflammation of the uveal tract, which lines the inside of the eye behind the cornea and between the retina and the sclera. The disease is categorized according to the part of the affected uveal tract: anterior, intermediate, or posterior. It can be classified according to the duration of symptoms in acute or chronic, and also as granulomatous or non-granulomatous, depending on the type of inflammation.[6] The frequency of MS among patients with uveitis in a tertiary ophthalmic center was 5%.[7]

2.1.1. Pathogenesis of vitreous inflammation in MS.

Most of these non-infectious or "autoimmune" uveitis are limited to the eye (organ- specific), while the rest are included within more widespread connective tissue diseases and multisystem granulomatous disorders. Inflammation causes rupture of the blood-retinal barrier, and the escape of white blood cells to the eye, triggering a spectrum of responses mediated by T cells, predominantly CD4+ T cells through the phenotype Th1.[8,9] The same mechanism has also been proposed for autoimmune uveitis triggered by infectious agents.[10]

T-cells then recognize antigens (autoantigen or foreign antigen) presented on the cell surface of cells presenting antigens (APCs, such as dendritic cells or macrophages), initiating the clonal activation and expansion cascade, resulting in the production of IL2, interferon-γ and TNF-α.[11] Only a few responses are able to overcome the condition of immune privilege of the eye and trigger inflammatory cell accumulation and tissue damage. Other cells present are neutrophils, predominant in cases of acute uveitis, and mononuclear cells, more prevalent in chronic cases.

In most of the cases, the clinical aspect is sufficient for the diagnosis of uveitis, but most have an unknown etiology of intraocular inflammation, which could be either infectious, inflammatory or tumor, so the correct diagnosis is mandatory. Intraocular inflammation is associated with increased expression and action of various cytokines and growth factors, which can be determined in the vitreous which may help the diagnosis. Several molecules have been identified over the last few decades in the vitreous humor, in most cases for research purposes, in others, it is more of a complement for the prognosis and in a minority of cases its finding constitutes an indispensable diagnostic tool.

2.2. Intraocular Lymphoma.

Although both Hodgkin lymphoma and non-Hodgkin lymphoma (NHL) can occur as intraocular inflammation, ocular involvement of Hodgkin lymphoma is usually rare and often occurs in the course of the disease, while NHL most commonly affects the eye. Both can manifest as any form of uveitis, and suspicion is very important given the potential lethality if an incorrect diagnosis is made or an inadequate systemic treatment is applied. NHL can be divided into two clinically different entities: ocular secondary systemic NHL and central nervous system NHL (NHL-CNS) or primary intraocular lymphoma.

2.2.1. Primary Intraocular Lymphoma or Primary Vitreoretinal Lymphoma (PVRL).

It is the variant of lymphoma with the highest ophthalmic impact, a subtype of

primary central nervous system lymphoma (CNS) that develops most frequently in patients aged 50-70 years. More than 15% of patients with primary CNS lymphoma (PCNSL) have intraocular lymphoma, which usually occurs in the retina and/or vitreous, and in contrast, 65% to 90% of patients with PVRL eventually have CNS lymphoma.[12] Consequently, PVRL is often fatal due to the definitive association with the CNS, which can happen between 1 month to 10 years later.[13,14] Typical clinical findings include thin vitreous cell infiltrates (lymphoma and inflammatory cells) and subretinal yellow infiltrates. **Figure 1.**

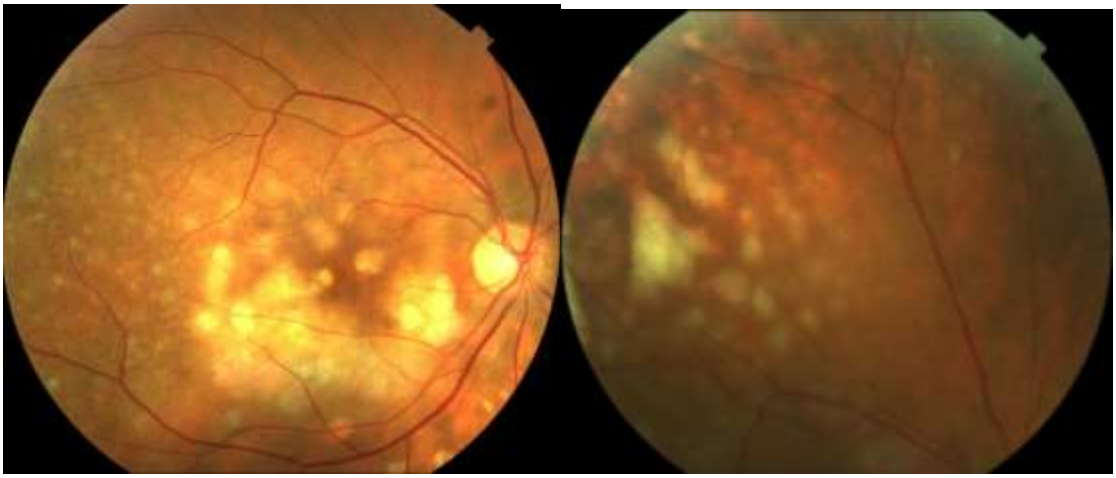

Figure 1. Primary vitreoretinal lymphoma. Left: Posterior pole of patient with PVRL showing typical multiple subretinal infiltrates located in equator and macula. Right: Same patient and same eye showing the same infiltrates located in the peripheral retina, with no vasculitis associated.

Due to its potential lethality, side effects associated with aggressive treatment and subtle and non-specific eye presentation, sampling for confirmatory diagnosis is mandatory. The diagnosis of intraocular lymphoma from vitreous samples relies on the proper management of samples, methods of aspiration, concentration, fixation, and staining. Previous treatment with steroids reduces the number of viable lymphoma cells, which are known to be cytolytic, so it is recommended to discontinue systemic and topical steroids prior to biopsy to increase the profitability of these cells.[15]

2.3. Other lymphoproliferative malignancies.

Leukemia has increased the variability of its related eye presentations, due to improved survival after the new era of effective antileukemia therapy. Leukemia can affect almost all eye tissues, but the retina is the most frequently affected structure, with up to 69% of all patients showing background changes at some point in the course disease. Hemorrhages, infiltrates and aggregates of leukemia cells are found at all levels,[16] and generally the internal limiting membrane acts as a barrier, however, cells occasionally invade the vitreous possibly emerging from the optic nerve head and these cases can be diagnosed by

examination.[17,18] These patients usually present with ocular presentations from multifactorial and/or mixed etiology, which requires a differential diagnosis that includes obtaining ocular samples.

2.4. Uveal melanoma.

Uveal melanoma (UM) is the most common intraocular tumor in adulthood. Uveal melanoma is a cancer of the eye involving the iris, ciliary body, or choroid (collectively referred to as the uvea). Funduscopy combined with ultrasonography with the finding of a solid choroidal mass in most cases pigmented, actually give an accurate diagnosis in almost 95% of patients. There are, however, some cases difficult to diagnose due to atypical eye manifestations or accompanying intraocular changes, such as extensive retinal detachment, vitreous hemorrhage or others. In these cases and recently for prognosis purposes, access to a sample becomes necessary.

2.5. Retinoblastoma

Retinoblastoma is the most common pediatric intraocular malignancy with a prevalence of 1/15.000 new borns. It develops from immature cells in the retina and may or may not be inherited. The inherited form can occur in one or both eyes and usually affects younger children. Retinoblastoma present in only one eye is nonhereditary and affects predominantly older children. The development of retinoblastoma is due to the inactivation of both alleles of the gene of susceptibility to retinoblastoma RB1. The RB1 gene was the first tumor suppressor gene identified and cloned, recognized as a key cell cycle regulator that has been shown to be involved in many different types of cancer. 17% to 83% of patients have some type of RB1 alteration. Recently, additional mutations in genes other than RB1 are thought to be required for malignant transformation into retinoblastoma, as both copies of the RB1 gene have been found to be lost in benign tumor retinocitoma.[19]

One of the most characteristic clinical findings is leucocoria; in fact, it is the second most frequent cause of leucocoria in children after congenital cataract. Strabismus is also present due to involvement of the macular area. Usually presents as a single or multiple, uni or bilateral, endophytic retinal mass that may be accompanied by subretinal or vitreous seedings. Diagnosis is made by funduscopy and ultrasound, sometimes aided by other cranial imaging tests. Because of the well known high risk of metastasis from retinoblastoma, there are very few exceptions to the need for confirmatory histological diagnosis, and this is reserved only in cases where therapeutic enucleation is required. However, histopathology may be supplemented with cytogenetic studies evaluating the prognosis of these patients.

2.6. Vitreous metastasis from other sources.

Ocular metastases of cancer of another origin are generally more frequent in the choroid, with cases of vitreous metastasis being extremely rare. Isolated cases of metastasis of primary lung, breast, liver, cervix, gastric and melanoma and lymphoma have been reported.[20-26] The symptoms are nonspecific, which makes the diagnosis difficult and requires a high level of suspicion and an extension study aimed at the most frequent neoplasms in the patient's age range. Therapeutic vitrectomy and diagnostic biopsy of vitreous aspirates with cytologic to

immunohistochemical cell identification are sometimes required.

3. Vitreous sampling techniques.

Masquerade syndrome or idiopathic chronic uveitis is the most frequent cause for the need of vitreous diagnosis. Generally, are patients with uveitis and ophthalmic and/or systemic characteristics that are atypical or do not respond to conventional anti-inflammatory therapies. Several approaches can be used in the evaluation of vitreous body: aqueous humor aspiration, vitreous humor aspiration, diagnostic vitrectomy, fine needle aspiration biopsy (FNAB), controlled subretinal fluid aspiration, chorioretinal incisional biopsy and diagnostic enucleation.

3.1. Aspiration of aqueous humor

Indications of anterior chamber aspiration may vary, but the most common situations are: patients with anterior chamber inflammation with suspicion of masquerade syndrome, a hypopion suspected of infection, endophthalmitis and lens-induced uveitis and cytopathological examination for suspected malignancy.[27] The technique consists in inserting a fine needle (30, 27 or 25 gauge) with the bevel upwards through the transparent cornea over the stroma of the iris, with an optimal visualization by slit lamp or surgical microscope, taking care to avoid the lens. A 0,1-0,2 ml of aqueous humor is removed in a 3 ml syringe in a sterile technique and then, a balanced saline solution can be used to reform the compartment.[28] The cytospin or other technique can be used to increase the sensitivity of cytology specimen.[29]

The advantage of the anterior chamber aspiration is that it can be performed in an outpatient setting, but the disadvantage is that it only achieves a limited sample volume of 100 to 200 µL per procedure,[30] which limits the number of molecular tests that can be performed on the sample.

3.2. Vitreous aspiration tap.

The indications for this technique are the same as the previous case, especially when it is necessary to obtain a larger sample such as the need to rule out malignancy or when the infection is considered the main cause of intraocular inflammation.[31,32] The primary indication for vitreous aspiration would be the search for intraocular lymphoma. The technique is similar to that of anterior chamber paracentesis, and is currently performed through the pars plana under local anesthesia with a needle of larger caliber, such as 21-gauge (G) hollow needle or fine, like 23 or 25-G mounted on a 1 ml syringe as an aspiration device, allowing a volume of 100 to 250 µl of vitreous humor to be aspirated while the needle is radially directed towards the optic nerve head.

The main complications associated with the technique are retinal tears and endophthalmitis, and although the risk is low, it is more common than after vitrectomy.[33] Other advantages of vitreous biopsies over vitrectomy are speed, since it can be performed without hospital admission, is repeatable and less traumatic for the eye.

Another variation of the technique is *fine-needle aspiration biopsy* (FNAB), in which a fine-needle gauge is directed to the suspected localized areas of tumor or intraocular lesion. The aspiration is done automatically or manually using a 25-G

to 30-G needle connected to an aspiration syringe. This aspirated block is likely to have a higher concentration of neoplastic cells than any of the adjacent intraocular fluids (i.e. vitreous, aqueous), decreasing the possibility of inconclusive cytological diagnoses, that often occur in vitrectomy samples, and in general is able to obtain a sufficient amount of cells (100 to 500 µl of eye fluid), to perform routine analyses such as microbiology, cytmorphological evaluation of Papanicolau or hematoxylin and eosin staining, immunocytochemical analysis and other applications.[34] FNAB appears to be less invasive and complicated than other methods,[35] being the preferred method for diagnosis in some centers, rather than PPV or chorioretinal biopsy, in patients in whom a uveal, retinal, or subretinal lesion is predominant.[36]

3.3. Diagnostic vitrectomy.

This technique may be the best option in selected cases, especially when vitreous removal is required not only for diagnosis but also for therapy (e.g., endophthalmitis, intraocular bleeding with suspicion of malignant origin, and for the treatment of vitreoretinal complications of chronic uveitis), and when the eye is inflamed and therefore patients may experience discomfort during outpatient vitreous aspiration.[37] Some authors recommend that vitrectomy-assisted biopsy may be considered only in cases of FNAB failure,[35] or when multiple punctures are required. A standard diagnostic three-port pars plana vitrectomy (PPV) under local anesthesia provides a large amount of material (vitreous core, retina or choroid, although diluted), but always requires an operating room under sterile conditions and direct visualization of the procedure.

In order to obtain an undiluted vitreous core sample, it is required to start the vitrectomy using the vitreous cutter at a rate of 600 cpm (cuts per minute) but with the infusion cannula closed, and then collect the sample through the aspiration lines of the vitrectomy directly connected to a 5 or 10 ml syringe, and inject retrograde air so that the fluid obtained is collected in a tube through the tip of the vitreous cutter.[38] With this technique, at least 1,5 ml of undiluted vitreous can be reliably obtained. With vitrectomy combined with perfluorocarbon liquid injection, in which the entrance of vitreous aspirated with perfluorocarbon liquid is compensated during vitreous aspiration, other authors were able to obtain an average of 2.24 ml of undiluted vitreous.[39] A recent publication recommended a complete diagnostic vitrectomy (*Full diagnostic vitrectomy*, FDV), which includes the surgical induction of a posterior vitreous detachment (PVD), since it has been observed that the highest density of cells within the vitreous cavity in a vitritis occurs closer to the retina and not in the core of the vitreous, either in the vitreous cortex if there is no PVD or in the posterior hyaloid membrane if PVD is present. This technique showed significantly higher performance especially in uveitis of infectious or neoplastic cause (77% and 80% respectively), with a lower false-negative rate.[40] However, the rate of complications was somewhat higher than conventional vitrectomy. In cases of insufficient sample collection, it is possible the study of the vitrectomy cassette material.

With the most recent 23, 25, or 27 G microincisional vitrectomy systems, the overall diagnostic performance of PPV varies considerably from 14.3% to 61.5%

in different published studies,[41-46] and was higher when intraocular infection was suspected compared to intraocular malignancy,[46] and higher for detecting primary vitreoretinal lymphoma than for detecting metastatic disease.

3.4. Chorioretinal biopsies.

Chorioretinal biopsies have been performed in certain cases of idiopathic uveitis in which the other diagnostic methods failed, in choroiditis and when retinal or choroidal masses are present.[47] The indications also include the patient's request for diagnostic confirmation before treatment. The procedure may be performed transsclerally or by an ab internal approach. The limited performance of intraocular biopsy is explained by the possibility of acellular or false-negative aspirates, the risks of malignant cell spread, and associated eye complications (mainly hemorrhage, retinal detachment, and infection), and fear of misdiagnosis.[48,49] However, on the other hand, several authors have stated that the identification of patients with aggressive disease and with a high risk of spreading in malignant processes should be a priority and the histopathological diagnosis should be mandatory.[50,51]

Several techniques have been developed to minimize the risks mentioned above. In the classic approach of lesions located in the region before the equator of the eye, in the transscleral approach, a scleral flap was created in the area of location of the tumor that was pressutured. A sharp blade then incised the choroid, the biopsy tissue was grasped with forceps, and the procedure was completed by quickly knotting the suture. Subsequently, several modifications have been described to facilitate the biopsy procedure and also to reduce the risk of complications[52]: PPV can be performed prior to the creation of the scleral flap to reduce vitreous pressure; another modification is the use of cyanoacrylate glue to provide greater stability of the scleral flap; and the third consists of performing a FNAB instead of a biopsy after the scleral flap is performed, whether or not using a calibrated needle marked with the maximum elevation of the lesion.[53]

In the transvitreal or ab internal approach, which is recommended for lesions located behind the equator of the eyeball, a retinochoroidectomy is performed up to the sclera after vitrectomy. In this case, the maximum tumor height can be previously marked on the vitrectomy. The risk of complications is high, mainly due to bleeding and retinal detachment. FNAB for choroidal lesions provides the least invasive method of tissue collection.[54,55] The tip of the needle can be bended, facilitating entry into shallow choroid lesions. In addition, the risk of posterior sclerotic perforation decreases.

There is a strong correlation between the thickness of the tumors in the A-scan ultrasound and the diagnostic results of a biopsy (so that the lesions of 1.9-4 mm: have a positive result in 90%; > 4 mm: 98%; but < 1.9 mm: only 40%).[56] False negatives are frequent and occur in 8% of cases and mainly because the material obtained is too scarce. Inadequate or insufficient samples can be obtained even with proper sampling in small, compact and solid tumors,[57] and even with gross lesions (> 4.5 mm), this is mainly due to the heterogeneity of the tumor. Another cause is that tumors that produce a limited material tend to be composed of strongly cohesive cells, which are a relative indicator that the process is benign in nature. In addition, some cases are

difficult to interpret, even for experienced eye pathologists.

4. Diagnostic techniques for vitreous samples.

4.1. Histopathology.

The histopathological diagnosis is usually required from tumor samples obtained by FNAB, choroidal biopsy, internal and external tumor resection or enucleation pieces in case of large tumor mass.

In cases of choroidal tumors like melanoma, histopathologic examination with preservation of the eyeball is the ideal method. The American Joint Committee of Cancer (AJCC) classification identifies three types of choroidal melanoma (CM) cells: fusiform, epithelioid, and mixed cell. Other rare histological variants of CM include: (i) diffuse melanoma; (ii) clear cell melanoma; and (iii) balloon cell melanoma.[58] Epithelioid subtype is considered a variant with bad prognosis.

In the case of eyes from enucleation due to retinoblastoma, cells are small and stain blue with hematoxylin and eosin (H&E) stain. Rings of cells surrounding an empty lumen are known as Flexner-Wintersteiner rosettes. Also large areas of necrosis and multifocal calcifications can be seen. Two histological subtypes are distinguished: well differentiated (if presence of >50% Homer-Wright rosettes) and poorly differentiated (if <50% of Flexner-Wintersteiner rosettes).[59] Some pathologic findings constitute poor prognostic factors: optic nerve invasion, uveal tract or sclera invasion, seeding of vitreous, involvement of anterior segment, extensive ocular tissue and tumor necrosis.[60] Approximately one-third of retinoblastoma eyes contain vitreous seeds, small tumor fragments that detach and float in the vitreous cavity. Usually the presence of vitreous seeds indicated until recently disease or advanced with an ominous eye prognosis.[61] In the presence or absence of vitreous seedings, the histopathological diagnosis, however, is contraindicated in any of its clinical presentations, due to the high rate of mestastasis, which in the case of retinoblastoma can be extended by any route (hematogenous, lymphatic, neural, etc). Diagnostic biopsy can only be used in cases of diagnostic dilemma, but it should be the last option. In this case it is recommended to use the aqueous humor as a subrogate of the vitreous humor or retina or the study of genetic malformations associated with retinoblastoma.[62]

If a choroidal or vitreous metastasis is suspected, stains and immunochemistry oriented according to the patient's history and clinical characteristics, will assist us in the confirmation diagnosis.

4.2. Cytology and immunohistochemistry.

Currently, PVRL is the pathology that benefits most from diagnosis by cytology (*gold standard*), although biopsy using vitrectomy can also be used to identify lymphoma cells in the vitreous or retina.[63] In order to prevent lysis of lymphoma cells, which occurs within about an hour, some authors advocate placing the vitreous specimen in RPMI culture medium (Roswell Park Memorial Institute),[64] or immediate placement in normal saline solution and move it urgently to the laboratory of analysis, taking care to not fixing with alcohol in order to not to alter the identification of the cells. Vitreous samples should be sent

immediately to an experienced cytopathologist to distinguish malignant cells (usually B lymphocytes) of reactive lymphocytes (T cells). PVRL malignant B cells characteristically appear in Papanicolau smears, Giemsa or Diff Quick[41,65] with large round or oval nuclei, often segmented and often containing prominent nuclei, surrounded by scant basophil cytoplasm. Histologically, according to the World Health Organization Classification, most of the PVRL are high-grade malignant non-Hodgkin lymphoma, and may be subclassified in most cases as diffuse large B-cell lymphoma.

Lymphoma cells can be clinically distinguished from uveitis lymphocytes because they are larger (2 to 5 times larger than a small lymphocyte) and increased by an in situ proliferation rather than by amplification and recruitment of inflammatory cells such as uveitis.[66] Ki-67 staining can be used to detect the unusually high (>80%) proliferation rate of malignant cells. B and T cell markers can be used to detect different cell subtypes. The samples may be negative due to poor biopsy sampling or previous treatment with steroids, with a reported effectiveness of only 48.3% of cases of PPV lymphoma, although other authors have found a diagnostic effectiveness in 87.5% of suspected lymphoma cases for FNAB.[31] **Figure 2**.

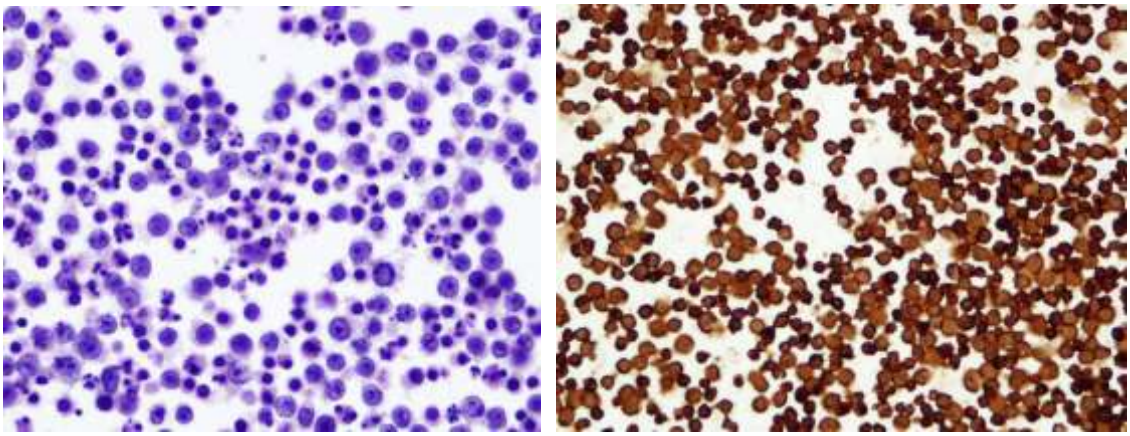

Figure 2. immunocytochemistry stain in primary intraocular Lymphoma. Left: 30 magnification, hematoxilin eosin stain. Great cellularity of monomorphic cells, with round nucleus, and nucleolus, and other smaller, picnotic degenerate cells, in apoptosis. Right: 45 magnification, CD45 and CD20 staining. All cells are positive for leukocyte common antigen and CD20 (cytoplasmic brown staining) that confirms that all of them are B lymphoid.

Malignant melanoma cells are stained with the antigen Human Black Melanoma 45 (HMB45), the S-100 protein, the Melan-A protein (also known as melanoma antigen recognized by T 1/MART-1 cells), the melanocyte-inducing transcription factor (MITF), tyrosinase, vimentin, and the sex-determining region Y-Box 10 (SOX10).[67] SOX10 represents the most specific melanoma cell marker. Cytological tests with Shorr stains or other modified stains have been able to diagnose cells with intracytoplasmic melanin pigment granules from samples obtained in eyes

that harbor metastatic cutaneous or choroid melanomas.[68,69]

Immunohistochemistry can also exclude the diagnosis of melanoma when positive for epithelial markers in tumoral cells.

4.3. Flow cytometric immunophenotyping (FCI)

It allows to analyze diluted vitreous samples, and the analysis of several surface markers, for example of different immune cells simultaneously (B lymphocytes, T lymphocytes, monocytes and macrophages) by marking with fluorescent antibodies or stains, offering a quantitative method to determine the percentage of one cell phenotype over others (monoclonality), which increases the efficiency of a simple biopsy.[41] This technique is widely used in suspected cases of intraocular lymphoma, where markers that can be studied for T lymphocytes are: CD3, CD8, CD4, CD7, CD2, CD25, and CD5283; for B lymphocytes: CD19, CD20, CD22, CD79a, and PAX5; and others for monocytes/macrophages, and lymphocyte activation. The test is based on the finding that most PVRL have restricted expression of either the k-chains or the λ-chains, the most sensitive marker being the relation k : λ 3 or 0.6 (80%). The advantage of flow cytometry is the ability to simultaneously immunophenotype subsets of B and T cells, such as CD4+ and CD8+ T cells with a reported sensitivity of 82% and 100% specificity,[70] which is even more sensitive than CD22 and CD20 markers (50% and 33%, respectively),[46] and comparable in terms of sensitivity to immunoglobulin heavy chain rearrangement analyses, and to the cytokine quotient IL-10:IL-6. However, flow cytometry has a limited ability to discriminate PVRL from uveitis when there are reactive infiltrates of B and T cells and since it requires a large number of cells (in relation to cytology), priority should be given to cytological examination for paucicellular eye biopsies.[71] **Figure 3a and Figure 3b**.

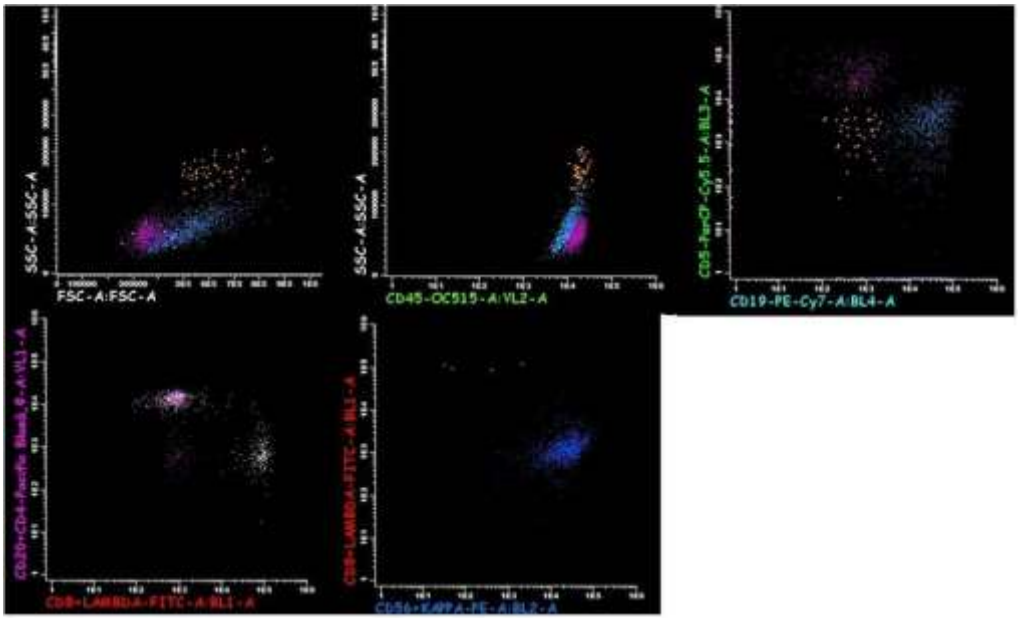

Figure 3a. Flow cytometric immunophenotyping of indiluted vitreous humor. Sample infiltrated by LNH-B DCG Kappa. Predominant B lymphocytes (blue) with kappa monoclonality. In pink: T-lymphocytes, in orange: macrophages and green: monocytes.

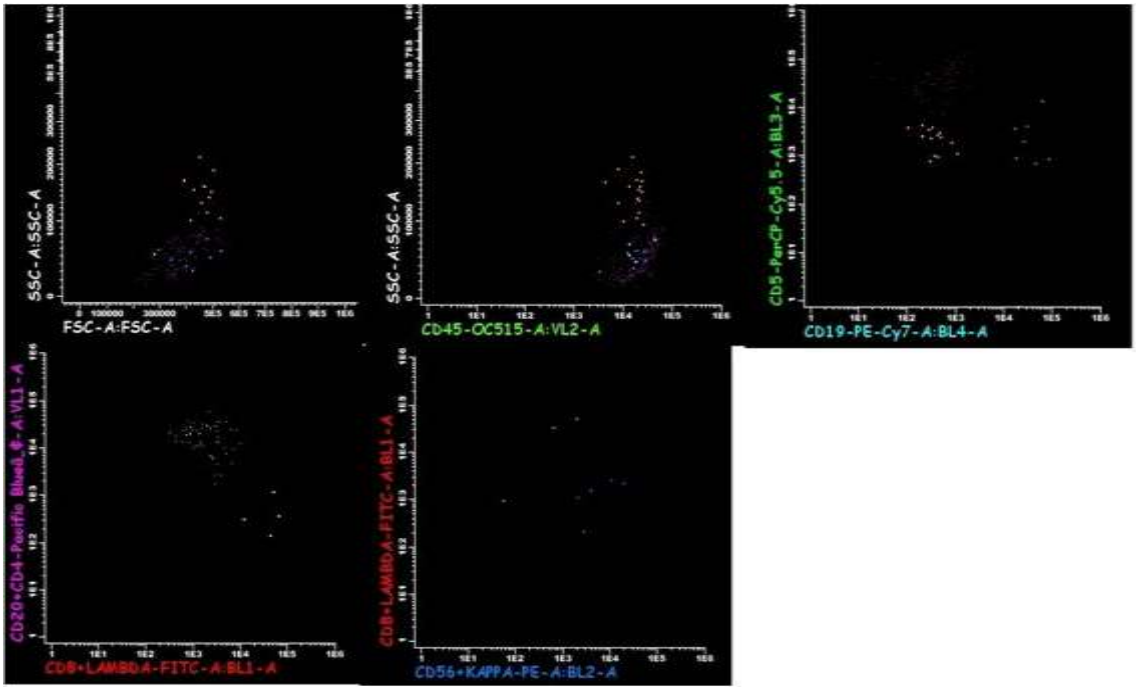

Figure 3b. Flow cytometric immunophenotyping of undiluted vitreous humor. Sample of patient affected from uveitis of unknown origin. There is no predominance for a cellular type from others. In blue: B lymphocytes, in pink: T-lymphocytes, in orange: macrophages and green: monocytes.

4.4. Molecular analysis techniques.

4.4.1. Polymerase chain reaction (PCR).

Polymerase chain reaction (PCR) is a fast and economical technique used to "amplify" and copy small segments of DNA. Requires considerable amounts of a DNA sample. Is employed in various laboratory and clinical techniques, including the evaluation of the anterior and posterior segment of the eye for infectious agents such as cytomegalovirus, varicella zoster virus, herpes virus, Epstein-Barr virus, toxoplasmosis, acanthamoeba, chlamydia, and others.[72] Although microbial culture remains the gold standard for idiopathic uveitis, the technique is limited by the low yield of the small volume of eye samples. Other studies have shown that aqueous PCR produces a diagnosis in one third of patients with posterior infectious uveitis with a sensitivity of 82% and specificity of 100%, equal to or better than vitreous biopsy.[73]

PCR can also be employed for the evaluation of genetic disorders or for the determination of clonal populations of cells. With the help of microdissection, In PVRL, PCR may be used to detect a clonally expanded lymphocyte population. Microdissection allows the selection of few or poorly preserved malignant or atypical lymphoid cells that would have been nondiagnostic for PVRL by routine cytological techniques. PCR can determine monoclonality by rearranging the genes of immunoglobulin heavy chains (IgH) and translocation t(14;18) of the B cell Lymphoma 2 gene (BCL-2), which inhibits cell apoptosis and predicts a more aggressive tumor course in B-cell lymphoma.[74-76] PCR has been found to be sensitive to PVRL at 80.6-100%,[77,78] and is being used to study

the genotypic classification of PVRL in order to identify prognostic factors: patients with translocation in the bcl-2 gene are significantly younger than patients who lacked translocation, suggesting that younger patients with translocation may need more aggressive treatment. Molecular analysis of B-cell receptor (BCR) and T-cell receptor (TCR) clonality as well as other p-markers has also been used.[79] Unlike cytology, molecular tests do not require expert interpretation and are therefore considered less subjective. Other advantages include the ease of handling the samples, the rapid response time and the relatively low number of cells needed compared to the number needed for cytology and flow cytometry. In addition, even tissue samples fixed with paraffin, where DNA quality is often fragmented and of low quality, can be evaluated using molecular testing methods.[80]

4.4.2. Analysis of DNA by exomic sequencing.

Analysis of DNA by exomic sequencing of large tumor samples and blood germ DNA from patients with PLCNS, allows the detection of somatic mutations in certain genes involved in key signaling pathways such as NFKB (nuclear factor κ-light-chain-enhancer of activated B cells), B-cell differentiation and cell cycle control, such as the mutated genes identified Myeloid Differentiation Primary Response 88: MYD88 (38%), CD79B (30%), PIM1 (22%) and TBL1XR1 (19%),[81] but the role of these markers in small samples such as those of vitreous humor has not been analysed. Later, more recent techniques such as dropplet-digital PCR (where PCR is subdivided into nanoliter partitions where confined target amplification can be enumerated independently) has shown 100% specificity for the detection of $MYD88^{L265P}$ in vitreous fluid and aqueous humour in patients with PVRL.[82]

4.4.3. MicroRNAs.

The role of microRNAs (miRNAs) has recently gained notable importance in the study of neoplastic diseases, like PVRL, as the expression of serum aberrant microRNAs can affect gene expression in a variety of cancer-related pathways. Specifically, serum miR-6513-3p may have the potential to discriminate uveitis from PVRL, and serve as an auxiliary diagnostic tool.[83]

4.4.4. Single cell analysis technologies (CSA).

They have been widely applied in different biomedical fields over the past decade and have great potential to guide the accurate diagnosis of PVRL. However, the number of cells typically available from vitreous biopsies used for the diagnosis of PVRL is limited and heterogeneous.

4.4.5. Interleukin analysis.

In patients with intermediate or posterior active uveitis, studies have shown an increase in IL-6 (T-cell cytokine) levels in the vitreous fluid, although this does not correlate with a specific type of uveitis,[84] suggesting that IL-6 is a common inflammatory mediator in several uveitis etiologies. IL-12, produced by monocytes, macrophages, B cells and mast cells, has also been found to be increased in aqueous humour and vitreous fluid in patients with low-grade intraocular inflammation and in uveitis in clinical remission for as long as 2 years.[85] But unfortunately, IL-12 levels are not diagnostic with inflammatory uveitis, as

they have also been found in infectious uveitis. On the other hand, clinical findings, systemic laboratory tests and response to treatment are commonly used and are sufficient to determine the cause of inflammation in uveitis.

PVRL is possibly the best example of eye disease in which intravitreous cytokines are most useful for diagnosis. An increase in the concentration of IL-10 in vitreous fluids has been found in patients with PVRL[76]; in contrast to the increase in the concentration of IL-6 characteristic of uveitis, so many authors have indicated that an IL-10/IL-6 greater than 1.0 is useful for the diagnosis of PVRL. IL-10 is linked to rapid disease progression and acts as a growth and differentiation factor for activated B lymphocytes, along with other mediators[47]. It is also anti-inflammatory and this can stifle immune defenses against tumor cells and produce the typical quiet eye of PVRL.[86] Cytokine analysis may be useful as an adjuvant test to corroborate the suspicion of PVRL and to determine if there is a significant response to treatment,[78,87] but it cannot be used only to make the diagnosis, as some studies have reported false-positive or false-negative results.[88] This ratio was associated with a sensitivity and specificity of 74.3 and 75.0%, respectively,[31] while a higher cut-off value of IL10 >400 pg/ml in vitreous humor was associated with sensitivity of 80% and specificity of 99%. **Figure 4**.

Simple Plex Kit Result Data Summary

Kit ID 128849
Analytes IL-10, IL-2, IL-17A, IL-4, IL-1ra, IFNg 3rd gen, TNF-a 2nd gen, IL-6 2nd gen
Run Date 04/22/2021 1:48 PM
Results Summary

	Sample Name	IL-10	IL-2	IL-17A	IL-4	IL-1ra	IFNg	TNF-a	IL-6 2
		Mean Conc (pg/ml)							
VITREO	59576105	15,7	2,02	286	*0.320	812	10	4,52	727

Figure 4. Ratio IL10/IL6 in a case of uveitis of unknown origin.

4.5. Cytogenetics.

The introduction of genetic prognosis has drastically changed the approach to the main intraocular tumors: uveal melanoma and retinoblastoma.

About 50% of patients with uveal melanoma die from metastasis, which occurs almost exclusively in patients whose tumor shows loss of chromosome 3 and gain of chromosome 8q.[89] Other chromosome abnormalities that are clinically relevant are loss in 1p, 6q, 8, and 9p, as well as gain in 1q, 6p, and 8q. Many cytogenetic and molecular tests have been investigated for uveal melanoma, each with its own advantages and disadvantages. Multiligant-dependent probe amplification (MLPA) is a variation of multiplex PCR validated for uveal melanoma and examines 38 loci on chromosomes 1p, 3, 6, and 8.[90]

More than 80% of uveal melanomas have mutations in the GNAQ gene or its GNA11 paralog encoding a G-protein coupled receptor that is involved in the RAF/MEK/ERK pathway.
GNAQ/GNA11 mutations are also found in benign precursor lesions such as congenital ocular melanocytosis and are believed to be initiating events in the pathogenesis of uveal melanoma.[91] The combination of chromosome 3 loss and

biallelic inactivation mutations in the BAP1 gene (which encodes BRCA1-associated protein 1) on chromosome 3p21 are strongly prognostic for aggressiveness and metastasis. Given the cost of BAP1 mutation analysis, immunohistochemical staining to evaluate BAP1 expression is a more economical practical alternative and gives faster results.[82] Negative staining for this protein confers probability of BAP1 mutation with a sensitivity of 88% and specificity of 97%.[92]

The **Gene Expression Profile (PGE)** analysis of specimens of uveal melanoma is also used for the prognosis of uveal melanoma. Two classes of melanomas with distinct metastatic potential have been identified: Class I melanomas resemble normal melanocytes and rarely metastasize (<5%); in contrast, Class II cells resemble primitive neuroectodermal stem cells and have a significant risk of metastasis (>90%). In addition to chromosome 3 monosomy, these cells contain inactivating mutations in the BAP1 gene. The patented GEP kit uses a set of 15 genes and has been shown to be an accurate prognostic predictor of uveal melanoma metastasis and is commercially available under the trade name DecisionDx-UM.[93] Genome-wide analysis can be performed through comparative genomic matrix hybridization (a-CGH), which is useful for detecting gains or losses from a large number of chromosome segments.[94] Differentially expressed miRNAs have been correlated with clinicopathological characteristics of UM with monosomy/disomy 3, so that they can be predictive of liver metastases and survival.[95]

In retinoblastoma cytogenetics will allow to offer tests for an early diagnosis or to know the prognosis and predisposition of individuals and their offspring to develop this pathology. The retinoblastoma gene (RBl) is a molecular marker of retinoblastoma tumors. This gene is located in chromosome 13q14.2 and encodes a nuclear phosphoprotein (pRB) of 110 KDa, which plays a major role in cell proliferation control through cell cycle–regulated phosphorylation/dephosphorylation cycles of this protein. The RBl gene is mainly affected by point mutations, which occur most frequently in exons 3, 8, 18 and 20.[96] Currently, many other genes have been identified with increased and decreased expression in retinoblastoma at all stages of the cell cycle and DNA remodeling.[97] Some of them were genes normally expressed in developing retinal photoreceptor cells, including retinal-specific transcription factors (NRL, CRX, NR2E3) and genes encoding retinal antigens (ROM, SAG, AIPL1, RPGRIP1, TULP1, and PDE6H). It has been suggested that genes expressed differentially in retinoblastoma belong mainly to pathways of response to DNA damage, including the BRCA1, BRCA2, ATM, ATR, E2F, and CHK1 genes.[98]

Chromosome abnormalities such as 1q and 6p gain and loss of 16q in retinoblastoma suggest that genes on these chromosomes contribute to the development of the disease.[99] A study of **gene expression profiles** has suggested that there are two distinct subtypes of retinoblastoma: Group 1 derived from a primitive and clinically invasive retinal progenitor cell type, and group 2, arising from more differentiated cells of cone photoreceptors and less invasive.[100]

Recently, a rare subset of unilateral retinoblastoma tumors has been described that lack mutations in the RB1 gene, but instead have amplification of the MYCN oncogene. These tumors are poorly differentiated and found in very young children. With these new findings, however, it is now recommended to test the number of copies of MYCN in addition to the analysis of the RB1 gene.[101]

4.6. Liquid biopsy.

Liquid biopsy is a test done on a liquid sample of a patient to look for circulating tumor cells or DNA pieces from them. A liquid biopsy may find cancer at an early stage, help in the treatment plan, determine its effectiveness, or find cancer recurrence. The ability to take several samples over time also helps understanding the molecular changes that are occurring in a certain tumor.

In uveal melanoma, there are several serum biomarkers that are useful for the detection and monitoring of systemic spread, but it does not tell us about the active or inactive status of the local disease. Levels of 5-S-cysteinaldopa (5SCD), a metabolite generated during the synthesis of pheomelanin, have been found to be significantly elevated in serum or urine, and also in vitreous, either by a direct discharge of the tumor in the vitreous or an alteration in the dynamics of intraocular fluids, in patients with UM and may reflect the progression of ocular melanoma.[102] Elevated vitreous levels of other proteins such as S100 β have also been found (although it is much more prevalent in skin melanoma).[103]

More recent studies on vitreous biopsy in UM showed elevated expression of other protein biomarkers such as HGFR, HGF and SCFR and lower expression of KLK7. These markers would be interesting to evaluate the potential of mestastasis in these patients and identify individually targeted treatments according to melanoma subtype and as adjuvant therapy: for example, the drug imatinib would inhibit SCFR, and HGFR/c-signalingMET is inhibited by cabozantinib,[104] however this field of research is still under development.

In retinoblastoma, due to the potential metastatic risk, attempts for liquid biopsy in the vitreous humor are not recommended, and prioritize other non-invasive access by blood or aqueous humor. The latter is being evaluated as a surrogate of tumor biopsy when tumor tissue is not available.[105]

5. Conclusions.

Vitreous sampling is a useful tool for the diagnosis of many pathologies affecting in particular the posterior chamber of the eye and the most important layers of the ocular wall such as the retina and choroid. There should be high clinical suspicion about the possible pathologies responsible for the clinical presentation before deciding on the most appropriate approach, taking into account the possible complications and associated undesirable effects.

In summary, in case of the suspicion of a non-philiated uveitis, the most appropriate approach would be to complement systemic tests by obtaining intraocular samples mainly for PCR, and microbiological tests. In PVRL vitrectomy linked to cytology, flow cytometry and the IL10/IL-6 ratio are able to detect all cases of PVRL analyzed with this combination of techniques.[106] If the quality of the cytology is ultimately poor,

then a second vitrectomy may be needed, but because the number of cells is likely to be low in a vitrectomized eye, a retinochoroid biopsy may be done at the time of vitrectomy surgery.[107] In uveal melanoma, when possible, fresh tumor tissue should be obtained in order to confirm the diagnosis and, even more, to be able to offer patients a chance to know their vital prognosis by means of cytologenetic tests. In pediatric retinoblastoma caution should be exercised in offering diagnostic and/or prognostic tests to these patients if sufficient experience is not available due to the associated high rate of spread.

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