Special Issue

Local and Systemic Inflammatory Biomarkers of Diabetic Retinopathy: An Integrative Approach

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Purpose. To review the usefulness of local and systemic inflammatory biomarkers of diabetic retinopathy (DR) to implement a more personalized treatment.

Methods. An integrated research (from ophthalmologist and diabetologist point of view) of most significant literature on serum, vitreous, and aqueous humor (AH) biochemical biomarkers related to inflammation at early and advanced stages of DR (including diabetic macular edema [DME] and proliferative DR) was performed. Moreover, novel imaging retinal biomarkers of local "inflammatory condition" were described.

Results. Multiple inflammatory cytokines and chemokines are increased in DR in both serum as well as in the eye (vitreous and AH). Nevertheless, local rather than systemic production of proinflammatory cytokines seems more relevant in the pathogenesis of both DR and DME. In the eye, retinal glia cells (macroglia and microglia) together with RPE are major sources of proinflammatory and angiogenic modulators. Retinal imaging allows for noninvasive clinical evaluation of retinal inflammatory response induced by diabetes mellitus.

Conclusions. Proinflammatory cytokines/chemokines play an essential role in the pathogenesis of DR. Therefore, circulating biomarkers and retinal imaging aimed at assessing inflammation have emerged as useful tools for monitoring the onset and progression of DR. In addition, "liquid biopsy" of AH seems a good option in patients with advanced stages of DR requiring intravitreous injections. This strategy may permit us to implement a more personalized treatment with better visual function outcome. Further evaluation and validation of circulating and local biomarkers, as well as multimodal imaging is needed to gain new insights into this issue.

Keywords: biomarker, inflammation, diabetic retinopathy, diabetic macular edema, aqueous humor

Diabetic retinopathy (DR) is the leading cause of visual impairment and preventable blindness[1] and represents a significant socioeconomic cost for health care systems worldwide.[2–4] DR prevalence in the diabetic population is approximately one-third, and 10% have vision-threatening states, such as diabetic macular edema (DME) or proliferative DR (PDR).[1] Because DR is the most common complication of diabetes and is expected to increase from 415 million in 2015 to 642 million by 2040, DR will become an even more serious problem in the future.[5] The potentially substantial worldwide public health burden of DR highlights the importance of searching for new approaches beyond current standards of diabetes care.

The current available treatments for DR (laser photocoagulation, intravitreal injections of corticosteroids or anti-VEGF agents, and vitreo-retinal surgery) are applicable only at advanced stages of the disease and are associated with significant adverse effects.[6–9] In early stages, the only therapeutic strategies that physicians can offer are a tight control of the risk factors for DR. The principal risk factors for developing DR are diabetes duration, glycemic control, and hypertension, but only the two latter can be druggables.[9]

Although there is no doubt regarding the relationship between the glycemic control and the appearing and progression of DR, the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group showed that HbA1c values explained up to 11% of the risk of DR, and that the unexplained 89% of variation in risk is due to elements of the diabetic milieu not captured by mean HbA1c value.[10,11] Therefore, new diagnostic tools are urgently needed for early detection and monitoring the effects of treatment on DR. In this regard, retinal imaging and circulating biomarkers could be useful in detecting early disease, in identifying those diabetic patients most prone to progressive worsening who ought to be followed more often, and who could obtain the most benefit from these therapies.

A biochemical biomarker is a molecule found in blood or other biological fluid that represents a sign of abnormal process of a condition or a disease. Ideally, a biomarker should be measured in accessible tissues. Biomarkers may help to identify subjects at risk of developing a disease, patients with subclinical disease, or those patients in whom the disease will progress. In addition, they may help to monitor the therapeutic response.[12–14] In the setting of DR, it is important to separate

the circulation biomarkers from the biomarkers obtained by sampling an eye-related biologic fluid, such as the vitreous or aqueous humor (AH).

As the retina constitutes a small proportion of total body weight, a circulating biomarker for DR should be highly specific to the retina rather than a marker of systemic vascular disease. In addition, renal function (i.e., creatinine levels or even better the glomerular filtration rate [GFR]) should be taken into account when using a biomarker that is significantly eliminated by glomerular filtration. On this basis, circulating biomarkers could be useful to detect early disease, to identify diabetic patients most prone to progressive worsening, in whom intensified therapy could be prioritized, and to monitor the effectiveness of new drugs for DR before advanced DR stages will be developed.

Local biomarkers reflect more directly the pathogenic events that are taking place in the retina. However, to obtain a sample of AH, and in particular vitreous fluid, is quite invasive but could be useful for selecting the best option of intravitreal treatment (see below). In recent years, the emerging technologies in imaging have opened up a new concept of "imaging biomarker," which permits us a better understanding of the main pathogenic process involved in DR in a particular patient. The combined information obtained by imaging and the assessment of local biomarkers is a promising area that fully connects with the precision medicine.

Most of reported biomarkers are based on the assessment of molecules involved in the pathogenesis of DR (i.e., advanced glycation endproducts [AGEs], molecules reflecting basal membrane turnover, oxidative stress, inflammation and angiogenesis). Because inflammation has a crucial role in the pathogenesis of DR[15,16] the present review will be focused on this issue.

Serum Biomarkers Related to Inflammation

Circulating Proinflammatory Cytokines

Several inflammatory molecules have been proposed as serum biomarkers of DR.[17] TNF-α has been the proinflammatory cytokine that has been more consistently related to DR. TNF-α is a cytokine that promotes the irreversible adhesion of leukocytes to the endothelium (leukostasis), increases the production of reactive oxygen species, and is implicated in the blood-retinal barrier breakdown.[18,19] Baseline circulating TNF-α was a predictor of DR incidence,[20] as well as of diabetic complications progression.[21] In addition, a strong correlation between plasma levels of TNF-α and PDR has been reported.[22,23] However, Klein et al.[24] reported that this correlation was mediated by the presence of kidney disease. Zorena et al.[25] found that the risk of nonproliferative DR was strongly dependent on TNF-α levels in the pediatric population. In addition, it has been observed that TNF-α level in tears is highly correlated with severity of DR.[26] Recently, Kocabora et al.[27] found increased levels of TNF-α in serum and AH from patients with DME in comparison with healthy controls.

IL-6 has been found elevated in vitreous fluid of patients with diabetes with DR and has been implicated in the pathogenesis of DR.[28,29] Higher levels of systemic IL-6 were detected in children with diabetes with DR than in those without DR.[30] Moreover, Shimizu et al.[31] found that serum IL-6 concentration correlates significantly with the severity of macular edema, and also could be a predictor of PDR. However, these results have not been confirmed in larger studies.[22,32,33]

The EURODIAB Prospective Complication Study hypothesized that a *z*-score composed of C-reactive protein, TNF-α, and IL-6 could be associated with the presence of vascular complications in patients with diabetes. The investigators found a positive correlation between these inflammatory factors and DR, diabetic nephropathy (DN), and cardiovascular disease.[34]

Retinol-Binding Protein 4

Retinol-binding protein 4 (RBP4) is an adipokine that is secreted by hepatocytes and adipose tissue and acts as a transport protein for vitamin A.[35] Circulating levels of RBP4 have been found related to body mass index (BMI), waist-to-hip ratio, triglyceride levels, systolic blood pressure, and decreased high-density lipoprotein cholesterol levels; all of them components of the metabolic syndrome. In addition, RBP4 levels have been associated with insulin resistance in subjects with obesity, impaired glucose tolerance, or type 2 diabetes mellitus (T2DM).[36] Furthermore, it has been postulated that elevated RBP4 contributes to insulin resistance by downregulating glucose transporter type 4.[37]

There is a close relationship between RBP4 and inflammation. In this regard, a correlation between RBP4 and inflammatory factors, such as C-reactive protein and IL-6, as well as a reduction of elevated serum RBP4 in obese children by lifestyle intervention, have been reported.[38] In addition, RBP4 induced inflammation in human retinal endothelial cells by stimulating the expression of proinflammatory molecules.[39] Moreover, in an animal model overexpressing RBP4, an early-onset microglia activation followed by a progressive retinal degeneration mediated by an increased expression of pro–IL-18 was observed.[40] Therefore, RBP4 could be involved in the inflammatory process of DR and could be considered a biomarker of early stages of DR. Takebayashi et al.[41] found elevated RBP4 levels in T2DM patients in comparison with nondiabetic subjects, and significant increased levels in patients with PDR versus no DR or nonproliferative DR. However, this relation was reduced after adjusting for urinary albumin excretion (UAE). Moreover, Li et al.[42] found that both UAE and serum RBP4 levels were significantly higher in PDR patients. Nevertheless, other studies have not found any association between RBP4 levels and DR.[40,41]

There are some factors that could influence RBP4 levels, such as BMI, some drugs (i.e., antidiabetic and hypolipidemic agents), vitamin A deficiency, and GFR.[35,41,43] Therefore, further research taking into account these confounding factors is needed.

In patients with T2DM, there is a low grade of systemic inflammation and both adipose tissue and macrophages are the main responsibility of the proinflammatory cytokine production. Plasma diffusion favored by the breakdown of the blood-retinal barrier could participate in the development and progression of DR but the local synthesis is the most important source of proinflammatory cytokines/chemokines. In other words, the proinflammatory cytokines produced by RPE and glial cells (macroglia and microglia) play a primary role in the pathogenesis of DR (Fig. 1).

Local Biomarkers Related to Inflammation

Proinflammatory Cytokines

Müller cells are the principal retinal macroglia cells and are considered to be "the communicator" cells between vessels and neurons, participating in regulation of neuronal nutrition, development, and metabolism.[44] Besides their crucial role in structural neuronal support and signaling, Müller cells regulate potassium and water homeostasis (by expression of aquaporins, and in particular aquaporin 4 [AQP4]), participate in the

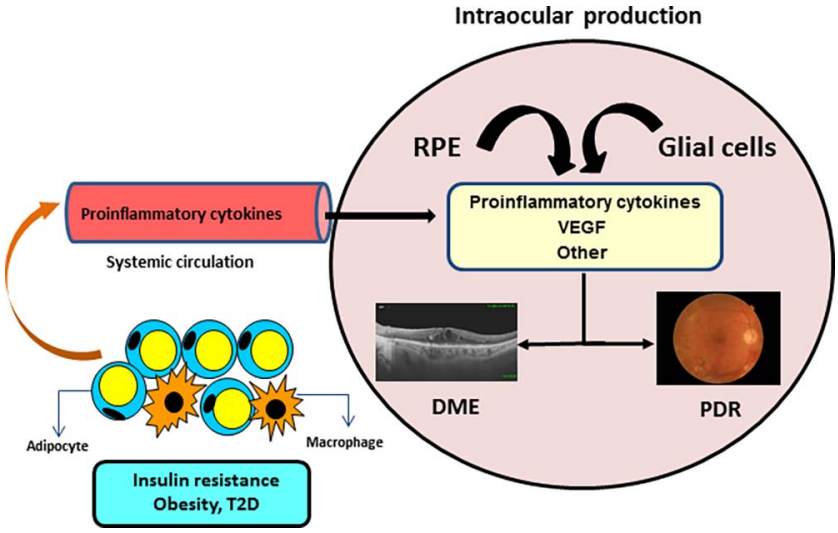

FIGURE 1. In situations such as insulin resistance, obesity, and T2DM, there is a low grade of systemic inflammation with an increase of serum levels of proinflammatory cytokines, which are mainly produced by adipose tissue and macrophages. These circulating cytokines could play a role in the pathogenesis of DR in T2DM. However, the main contributor to the developing of both DR and DME is the intraocular production of proinflammatory cytokines.

protection against oxidative stress, contribute to recycling of photopigments, maintain the integrity of the blood-retinal barrier, and participate in the local retinal inflammatory response with release of neuroinflammatory and vasoactive mediators, such as VEGF, pigment epithelium-derived factor (PEDF), matrix metalloproteinase, IL-1β, IL-6, TNF-α, TGF-β, monocyte chemotactic protein (MCP-1), β-catenin, nitric oxide (NO), cyclooxygenase (COX) 2, prostaglandin E2 (PGE2), inducible NO synthase (iNOS), AGE receptor (RAGE), calcium-binding protein B (S100B), glutamate, D-serin, adenosine triphosphate (ATP).[45–49] Müller cells react to hyperglycemia by facing a reactive gliosis, with an increase in glial fibrillary acidic protein (GFAP), nestin and vimentin, functional activation, and cellular proliferation.[44] An increase in GFAP has been documented in experimental studies and in diabetic donors.[47,48,50–53] In human ocular fluids, and specifically in the AH, GFAP increase has been documented in patients with diabetes with no clinical signs of DR and with nonproliferative DR compared with healthy controls.[54] Moreover, an increase in AQP4 (a channel protein that allows the flow of free water through the cell membrane, regulated by Müller cells) has been documented by histology in animal models in diabetes mellitus (DM).[55–58] Recently, an increase in AQP4 has been reported in human ocular fluids, specifically in the AH, even in patients with no clinical signs of DR.[54] Therefore, GFAP and AQP4 can be considered as AH biomarkers of Müller cell (activation). Retina macroglial cell activation in DR has also been confirmed by clinical studies using optical coherence tomography (OCT).[59]

Microglia cells are the major resident immunocompetent cells in the central nervous system and they share the phenotypic markers of monocytes and macrophages.[60,61] In DM, microglia cells undergo a shift in the activity phenotype (from so-called "surveying microglia" to "activated microglia")[62] and change their location in the retina, migrating from the inner to the outer retinal layers.[63–65] Many signals and modulators can trigger a transformation of microglial cells to the activated (alerted or reactive) states, including complement; antibodies; cytokines; chemokines; neurotrophic factors; surface structures; and DNA/RNA of viral, bacterial, or fungal origin, abnormal endogenous proteins, plasma components, proteins and peptides, neurotransmission-related compounds, ions, and so forth.[62] Recently, a general increase in retinal glia cells of proinflammatory cytokines was documented in the AH in diabetic patients without DR and with mild DR, compared with nondiabetic subjects.[66] In particular, in patients with diabetes with no clinical signs of DR, IFN-γ, IL-1α, IL-3, and MCP-2 were significantly increased compared with nondiabetic controls; in diabetic patients with mild DR, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, IL-10, IFN-γ–induced protein (IP-10), regulated and normal T-cell expressed and secreted (RANTES), and soluble TNF receptor (sTNF-R) II were significantly increased versus controls. In diabetic patients with mild DR, macrophage inflammatory protein (MIP-1b), GM-CSF, RANTES, and sTNF-RII were significantly increased versus diabetic patients with no clinical signs of DR.[66]

In more advanced stages of DR, such as the presence of DME or proliferative DR, increased concentration of following cytokines was reported in the AH: IL-6, VEGF, IL-12, IL-8, IP-10, MCP-1, platelet-derived growth factor (PDGF), and intercellular adhesion molecule (ICAM)-1,[66–73] whereas in the vitreous: IL-6, IL-8, TNF-α, IL-1β, VEGF, ICAM-1, MCP-1, and complement factor were increased.[74–81]

Although vitreous fluid may more reliably reflect the pathophysiological events that are taking place in the retina than AH, its sampling is too invasive to be incorporated into clinical practice with the exception of those patients' candidates to vitrectomy.

Hemopexin

Apart from proinflammatory cytokines, hemopexin has been found overexpressed in diabetic retina, and in vitro studies have shown that increases the permeability of the outer blood-retinal barrier.[82] It is worth mentioning that this effect was detected using a hemopexin dosage in the range detected in the vitreous fluid of patients with DME (50 μg/mL).[82] Hemopexin is the best characterized permeability factor in steroid-sensitive nephrotic syndrome and its infusion induces experimental proteinuria.[83] In addition, hemopexin could be involved in either causing or perpetuating enhanced glomerular permeability in minimal change nephrotic syndrome.[30] T-cell–associated cytokines, like TNF-α, are able to enhance

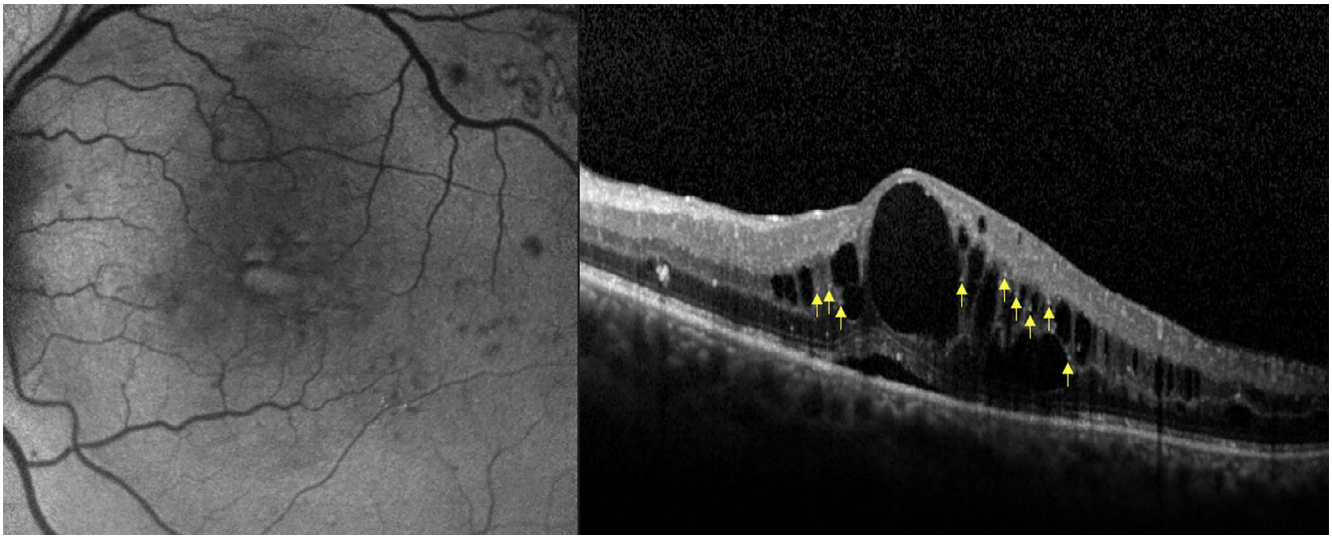

FIGURE 2. Fundus autofluorescence and linear OCT scan of the eye with cystoid DME with SND. *Yellow arrows* indicate the presence of hyperreflective retinal spots mostly located (in this case) in the inner nuclear layer considered to represent aggregates of activated microglial cells, thus inflammatory condition in the retina. These hyperreflective spots have specific characteristics: small in size (<30 μm), no back-shadowing, reflectivity similar to the nerve fiber layer.[65]

hemopexin production in mesangial cells in vitro, and this effect is prevented by corticosteroids.[83] Therefore, it could be postulated that the increase of hemopexin induced by diabetes plays a similar role in the retina, thus contributing to the vascular leakage (hyperpermeability) that is the main pathogenic factor of DME. In fact, dexamethasone significantly reduced the hyperpermeability induced by hemopexin.[84]

Noninvasive Imaging Retinal Biomarkers of Inflammation in DR and DME

Recently there has been an increasing interest in determination and validation of noninvasive imaging retinal parameters, as possible biomarkers of local retinal "inflammatory condition" in DR and DME (both prognostic and predictive of treatment response) by using different imaging modalities, but mostly spectral-domain (SD)-OCT and fundus autofluorescence. These imaging biomarkers include subfoveal neuroretinal detachment (SND) and hyperreflective retinal spots/foci (HRS) evaluated on SD-OCT, and foveal hyperautofluorescence (FAF) evaluated on fundus autofluorescence.[63,65,85–87]

DME associated with SND is a specific pattern of DME associated with higher concentration of inflammatory cytokines, specifically IL-6 in the vitreous, when compared with DME without SND.[85] HRS have been recently evaluated in different retinal and choroidal conditions, such as early stages of DR, DME, AMD, and macular edema due to retinal vein occlusion.[63,65,88–97] HRS with specific characteristics, such as dimension <30 μm, reflectivity similar to nerve fiber layer, absence of back-shadowing, and location in both inner and outer retina, were suggested to represent activated aggregates of microglial cells, thus can be considered an imaging biomarker of retinal inflammatory response (Fig. 2).[65] An increase in number of HRS was reported in preclinical and early clinical DR, as well as in DME.[63,65,86,89,95–97] A higher number of HRS was present in DME with SND versus DME without SND.[48] Also, an increased area of FAF was described in eyes with DME.[86] HRS, SND, and FAF all decrease after either anti-VEGF or steroid intravitreal treatment in DME, although major SND decrease occurred after intravitreal steroids.[86,89,95–97] As HRS correlate inversely with retinal sensitivity determined with microperimetry in DME, it was suggested that HRS may become a new OCT parameter for evaluation of functional efficacy of treatments in center-involving DME.[96]

Apart from these potential biomarkers, a significant increase in the thickness of the inner nuclear layer on SD-OCT (mostly formed by the nuclei of bipolar and Müller cells) has been reported in patients with nonproliferative DR, indicating that this finding may represent a clinical sign of Müller cell activation due to hypertrophy of these cells.[59]

Further evaluation and validation of multimodal imaging biomarkers is needed to gain new insights in DME. Prognostic and predictive biomarkers evaluating both morphology and function, which are minimally invasive, or even better noninvasive, may help in choosing more personalized treatment with better visual function outcome.

Therapeutic Implications

The identification of reliable biomarkers of the development and progression of DR will permit us to obtain different phenotypes based on the primary pathogenic mediators and will provide a valuable information that could modulate the therapeutic strategy (Table). In addition, both circulating biomarkers and new imaging techniques can give us useful information for monitoring the response to a specific treatment in an individualized manner. At present, one of the most important therapeutic implications derived from the measurment of molecular biomarkers relies on advanced stages of DR and DME (see below).

Personalized Treatment Using Intravitreal Injections Based on "Liquid Biopsy"

VEGF plays a crucial role in the pathogenesis of both DME and PDR. However, it should be noted that in the study by Aiello et al.,[98] 36% of diabetic patients with PDR had undetectable levels of VEGF in the vitreous fluid. This finding explains why intravitreal anti-VEGF treatments fail in a significant proportion of patients and reveals that VEGF-independent pathways play a primary role in these patients. Therefore, the development of therapeutic strategies for blocking other growth factors and/or

TABLE. Proposed Systemic and Local Inflammatory Biomarkers in DR

Type of Biomarkers	Circulating			Imaging		
	Diagnostic	Prognostic	Predictive of Treatment	Diagnostic	Prognostic	Predictive of Treatment
Proposed systemic inflammatory biomarkers	TNF-α, IL-6, RBP4	TNF-α, IL-6, RBP4				
Proposed ocular inflammatory biomarkers	TNF-α, IL-6, VEGF, IL-12, IL-8, IP-10, IL1β, MCP-1, complement factor, PDGF, ICAM-1, GM-CSF, IFN-γ, IL-10, IP-10, RANTES, sTNF-RII, matrix metalloproteinase, TGF- β, PEDF, β-catenin, NO, COX 2, PGE2, iNOS, Hemopexin, RAGE, S100B, glutamate, D-serin, ATP, GFAP, AQP4	TNF-α, IL-6, MIP-1b, GM-CSF, RANTES, sTNF-RII	IL-6, VEGF, IP-10, MCP-1	SND, HRS, FAF	SND, HRS, FAF	SND, HRS, FAF

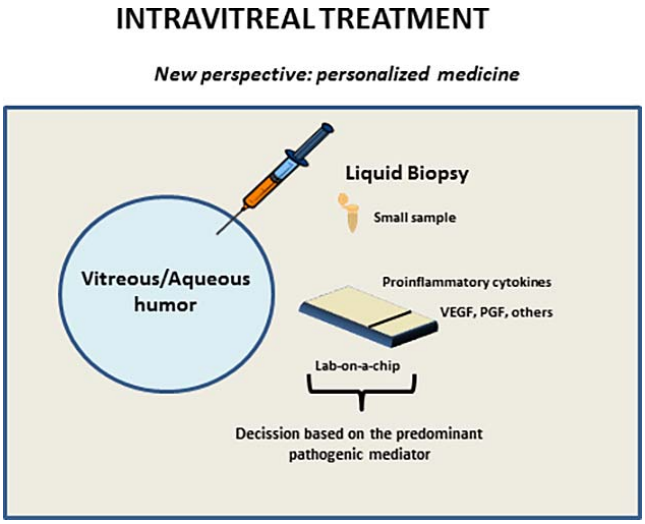

FIGURE 3. Strategy based on the use of liquid biopsy of AH (or eventually vitreous humor in patients needing vitreoretinal surgery) for selecting the most appropriate approach for the treatment of DME or DR.

proinflammatory cytokines/chemokines seems to be necessary. The use of AH during the first injection could be useful for examining the predominant pathogenic pathway in a personalized manner ("liquid biopsy"). This approach would permit us to select a more rationale and probably a more cost-effective treatment (Fig. 3).

CONCLUSIONS

The assessment of reliable biomarkers of DR or DME is a challenge to be met. Because inflammation plays an essential role in the pathogenesis of DR, serum biomarkers and retinal imaging aimed at assessing the presence of inflammation have emerged as useful tools for monitoring the appearance and progression of DR. However, further evaluation and validation of serum and multimodal imaging biomarkers is needed to gain new insights into this issue.

Prognostic and predictive biomarkers evaluating both morphology and function may help in choosing more personalized treatment with a better visual function outcome. In this regard, growing evidence suggests the reliability and usefulness of AH humor samples in characterizing the intraocular phenotypes. This approach based on "liquid biopsy" will permit us to optimize the treatment, thus resulting not only in a more efficient treatment but also in one less expensive for health care providers.

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Investigative Ophthalmology & Visual Science

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Investigative Ophthalmology & Visual Science