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Markers of hypoxia in aqueous humor as factors for assessing the severity of diabetic retinopathy

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Маркери гіпоксії у внутрішньоочній рідині як фактори оцінки тяжкості діабетичної ретинопатії

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Abstract

Purpose. *To establish the diagnostic value of lactate versus hypoxia-inducible factor (HIF)-1α concentration in the aqueous humor (AH) for determining the severity of diabetic retinopathy DR.*

Material and Methods*. Totally, 110 type 2 diabetics with DR (110 eyes) were involved in the study and divided into five groups from no apparent retinopathy (DR0) to proliferative DR (PDR) based on the 2003 international classification. The control group included 25 non-diabetics. Lactate concentrations (mg/mL) were determined in AH samples obtained during cataract surgery, and HIF-1α concentrations (pg/mL) were determined in this cohort of patients in our previous study.*

Results. *AH lactate level increased with disease progression ($p < 0.001$), with a median level ranging from 0.32 mg/mL in controls to 6.49 mg/mL in group 5 (PDR). A total accuracy was moderate (~60%) for both markers for the discrimination between all grades of the disease, but a high AH lactate concentration (>8.56 mg/mL) was found to be highly specific (98.9%) for confirming PDR. To assess the risk of DR progression, a total study sample was divided into two categories (mild-to-moderate DR vs severe DR) on the basis of AH HIF-1α concentration, and a threshold of >377 pg/mL provided for a total prediction accuracy of 71.9% and specificity 95.2%.*

Conclusion. *AH lactate and HIF-1α concentrations reflect a gradient of hypoxic load in DR. Determining AH lactate concentrations (a rule-in marker) is effective for severe conditions, whereas determining AH HIF-1α concentrations should be used for stratifying patients into risk groups to guide planning the intensity of supervision and treatment.*

Keywords: *diabetic retinopathy, type 2 diabetes mellitus, retina, lactate, hypoxia-inducible factor 1α, biomarkers, aqueous humor, disease progression.*

Резюме

Мета. *Оцінити діагностичну цінність вмісту лактату у внутрішньоочній рідині у співставленні з іншим маркером гіпоксії — гіпоксія-індуцибельним фактором-1α (HIF-1α), для визначення тяжкості діабетичної ретинопатії.*

Матеріал та методи. *Обстежено 110 пацієнтів (110 очей) із цукровим діабетом 2-го типу, розподілених*

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на п'ять груп відповідно до тяжкості ДР за міжнародною класифікацією (2003): від відсутності ретинопатії (ДР0) до проліферативної стадії (ПДР). Контрольну групу склали 25 пацієнтів без цукрового діабету. У внутрішньоочній рідині (ВОР), отриманій під час факоемульсифікації катаракти, визначали вміст лактату (мг/мл), а рівні HIF-1α (пг/мл) були визначені у нашому попередньому дослідженні цієї самої когорти пацієнтів.

Результати. *Рівні лактату у ВОР статистично значущо зростали паралельно з тяжкістю захворювання (p<0,001). Медіана лактату підвищилася від 0,32 мг/мл у контролі до 6,49 мг/мл при ПДР. Точність діагностики конкретних п'яти стадій була помірною (~60% для обох маркерів). Проте високі концентрації лактату (>8,56 мг/мл) виявилися високоспецифічним інструментом (98,9%) для підтвердження проліферативної стадії. Для оцінки ризику прогресування було застосовано поділ на дві групи (легка/помірна проти тяжкої ДР) на основі HIF-1α: поріг >377 пг/мл забезпечив загальну точність прогнозу 71,9% при специфічності 95,2%.*

Висновок. *Вміст лактату та HIF-1α у ВОР відображає градієнт гіпоксичного навантаження при ДР. Визначення лактату є ефективним для підтвердження тяжких станів («rule-in» маркер), тоді як HIF-1α доцільніше використовувати для стратифікації пацієнтів у групи ризику задля планування інтенсивності нагляду/лікування.*

Ключові слова: *діабетична ретинопатія; цукровий діабет 2-го типу; сітківка; лактат; гіпоксія-індуцибельний фактор-1α; біомаркери; внутрішньоочна рідина; прогресування захворювання.*

Introduction

Diabetic retinopathy (DR) remains a major cause of global vision loss and substantially affects the working-age population. It was a leading global cause of blindness in those aged 50 years and older in 2020 [1]. Despite advances in ophthalmology, the global burden of eye disease and visual disability is still high and has been steadily increasing in recent decades [1]. In type 2 diabetes treated with or without insulin, the incidence with 5 years of evolution is 20% while with 15 years of evolution it reaches 80% [2].

Around 537 million people were living with diabetes in 2021 and these figures are constantly growing, with a projection of 783 million in 2045, and with a further increase in the number of patients with DR [3]. It is technological progress (autonomous screening for the disease, optical coherence tomography angiography [OCTA] and novel biomarkers) and personalized management of patients that have been identified in prognostic reviews as key contributors to the efficacy of vision loss prevention due to DR until 2030 [4].

The International Clinical Diabetic Retinopathy Disease Severity Scale (ICDR) includes no apparent retinopathy, mild, moderate and severe non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR), whereas diabetic macular edema (DME) is assessed separately from the grade of DR [5]. However, structural fundus changes observed during dilated ophthalmoscopy insufficiently reflect the ischemic component of DR which manifests before the appearance of severe microvascular changes [6]. OCTA evidence of deep capillary non-perfusion at baseline in eyes with clinically referable NPDR can predict short-term DR complications with high accuracy [7], and low macular vessel area density significantly correlated with visual function in treatment-naïve diabetic eyes with edema [8]. Therefore, there is a rise in the need for identifying the biochemical markers capable of reflecting the degree of retinal hypoxia in DR.

Hypoxia-inducible factor (HIF)-1α is a key component of ocular tissue response to hypoxia; it is a transcription factor that activates the expression of proangiogenic genes and metabolism-regulating genes (e.g., vascular endothelial growth factor [VEGF]) and shifts the metabolism of glucose from oxidative phodphorylation to glycolysis to reduce oxygen consumption by cells [9, 10]. In DR, this is reflected by increased vascular permeability, formation of microaneurysms, progressive capillary non-perfusion and neoangiogenesis. Endothelial dysfunction and inflammation also play a major role and increase hypoxic distress [11]. Taken in total, these mechanisms substantiated the use of biomarkers of ocular tissue hypoxia for stratifying the severity of DR and predicting DR complications.

Lactate, along with HIF 1α, has attracted the attention of researchers of DR; it is a metabolite that reflects the intensity of glycolysis and cell/tissue redox status, and can be quantitatively measured [12]. Today, it is considered not only a marker of hypoxia, but also a signaling molecule with wide-ranging effects [12]. Metabolomic analysis of aqueous humor (AH) in patients with DR demonstrated a shift in the energy profile with an increase in the levels of intermediary products of glycolysis (e.g., L-lactate) [13, 14]. Additionally, concentrations of lactate in the AH are higher than those in the serum, which confirms the local origin of this metabolite and, consequently, that it is advisable to measure it in the AH as a marker of ocular tissue hypoxia [15]. Therefore, this provided preconditions for assessing the two complementary markers of hypoxia, HIF 1α (as a regulatory marker of hypoxia) and lactate (as a metabolic fingerprint of hypoxia) directly in the AH.

Terminal effectors of angiogenesis, VEGF and pigment epithelium-derived factor (PEDF) are still the most clinically validated biomarkers for DR [16-18]; however, a marker of early hypoxia (HIF-1α) and a marker of metabolic changes in hypoxia (lactate) may reflect the early pathogenetic components of DR. It is these characteristics that may potentially provide an early signal on the transition to severe phenotypes of the disease, which is critically important for screening tools. We have previously established the diagnostic value of HIF-1α [19]; however, the

search for more readily available metabolic indicators of hypoxia (like lactate) is still ongoing, and these indicators have not yet been directly compared with HIF-1α.

The purpose of this study was to establish the diagnostic value of lactate versus HIF-1α concentration in the AH for determining the severity of DR.

Material and Methods

This study followed ethical standards as outlined in the 1964 Helsinki declaration and its later amendments, the European Convention on Human Rights and Biomedicine, and relevant laws of Ukraine. The study was approved by the local bioethics committee of the Bogomolets National Medical University. This was a randomized cross-sectional prospective cohort study. Informed consent was obtained from all subjects.

The study included 110 type 2 diabetics with DR (110 eyes) who received surgery for age-related cataract. The worse eye was used to classify the severity of DR [19]. Patients were divided into groups based on the ICDR severity scale [5]: group 1, no apparent retinopathy (DR0; 15 eyes); group 2, mild NPDR (NPDR1, 40 eyes); group 3, moderate NPDR (NPDR2, 25 eyes); group 4, severe NPDR (NPDR3, 12 eyes); and group 5, PDR (18 eyes).

Patient age ranged from 50 to 76 years (median, 62.5; interquartile range [IQR], 51.3-69). The patient sample consisted of 92 males (83.6%) and 18 females (16.4%), and there was no difference between males and females in DR stage (p = 0.970). DME was present in 12 patients (10.9%). Of these 12 patients, 10 (83.3%) were patients of groups 4 and 5. The control group included 25 age- and gender-matched non-diabetics who were treated surgically for senile cataract.

Patients received visual acuity assessment with a premium-quality chart projector (CSO srl, Florence, Italy) and Takagi VT-5 View Tester (Takagi Seiko, Nagano, Japan); autorefractometry (Topcon KR-7000P, Topcon Europe BV, Capelle a/d IJssel, Netherlands); tonometry with a Non-Contact Air-Puff Tonometer (Huvitz HNT 7000, Huvitz, Dongan-gu, Anyang-si, Gyeonggi-do, South Korea); corneal pachymetry with the HNT-1P apparatus (Huvitz); slit-lamp biomicroscopy with the SL-9900 LED 5X slit lamp (CSO srl) and an indirect ophthalmoscope (Heine Omega 600, Optotechnik GmbH, Gilching, Germany); gonioscopy with a three-mirror Goldmann lens; ophthalmoscopy with a Volk Digital Wide Field Lens (Volk Optical, Mentor, OH, USA) and a three-mirror Goldmann lens; and OCT imaging with a HOCT-1F apparatus (Huvitz).

A 0.2-ml aqueous humor sample was obtained via an anterior chamber paracentesis during cataract surgery. Enzyme-linked immunosorbent assays from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA) were used to determine HIF-1α levels (pg/mL) in the same cohort of patients in our previous study [19]. Aqueous humor lactate levels (mg/mL) were determined enzymatically [20].

EZR v.1.54 (graphical user interface for R statistical software version 4.0.3, R Foundation for Statistical Computing, Vienna, Austria) [21] was used for statistical analysis. Medians and IQR were calculated due to the skewness of the data. The Kruskal-Wallis test was used for group comparisons, followed by Dunn's test for post hoc pairwise comparisons. Statistical significance was set at $P < 0.05$ for all analyses [22].

An open-source package for R and S+ was used to analyze and compare receiver operating characteristic (ROC) curves and determine diagnostic ranges for lactate and HIF-1α levels [23].

Results

Groups were comparable for age (p = 0.108). Median age was 56 years for controls and ranged from 55 to 67 years for groups of patients (Table 1). Diabetes duration increased with the severity of DR, from 5 years in group 1 (no apparent retinopathy) to 16.5 years in group 4 (severe NPDR) ($p < 0.001$).

AH lactate level increased with disease progression ($p < 0.001$), with a median level ranging from 0.32 mg/mL in controls and 0.64 mg/mL in group 1 (no apparent retinopa-

Table 1. Age, diabetes duration, and aqueous humor lactate concentrations (median, interquartile range) in the study groups and controls

Characteristic	Groups						p
	Controls	Group 1 (no apparent DR)	Group 2 (NPDR1)	Group 3 (NPDR2)	Group 4 (NPDR3)	Group 5 (PDR)	
Age, years	56 (52.1–65)	55 (51.3–64.8)	59.5 (46.5–69.5)	66 (51.5–73)	67 (58–74.5)	65.5 (61–69)	0.108
Diabetes duration, years	-	5^{cde} (3.3–7.5)	10^{e} (5–14.5)	14^{a} (10–18.5)	16.5^{a} (10–21)	15.5^{ab} (15–25)	<0.001
Lactate, mg/mL	0.32^{abcde} (0.25–0.4)	0.64^{0bcde} (0.58–0.76)	1.61^{0acde} (1.19–2.37)	3.4^{0abe} (2.61–4.60)	5.75^{0ab} (5.3–7.3)	6.49^{0abc} (5.81–7.2)	<0.001

Note: The Kruskal-Wallis test was used for group comparisons, followed by Dunn's test for post hoc pairwise comparisons. ^{0}, significant difference ($p < 0.05$) from controls; ^{a}, significant difference ($p < 0.05$) from group 1; ^{a}, significant difference ($p < 0.05$) from group 1; ^{b}, significant difference ($p < 0.05$) from group 2; ^{c}, significant difference ($p < 0.05$) from group 3; ^{d}, significant difference ($p < 0.05$) from group 4; ^{e}, significant difference ($p < 0.05$) from group 5

thy) to 6.49 mg/mL in group 5 (PDR). Statistical analysis confirmed significant differences between controls and all clinical groups and between most intermediate stages.

We have determined previously [19] that AH HIF 1α level (pg/mL; median [IQR]) increased more substantially with disease progression compared with increases in AH lactate level, from 43.65 (26.8-57) in controls and 65.3 (53.3-83.3) in DR0 to 165.40 (111-229.5) in NPDR1, 285.50 (203.9-327) in NPDR2, 405.10 (316-443.1) in NPDR3 and 461.20 (399.5-517) in PDR, $p < 0.001$.

Taking in account the relationship established previously between the AH lactate level and DR progression, we used a "one-vs-all" classification (a multiclass classification technique) and generated ROC curves separately for each stage of DR [22, 23] (Figs. 1) to select the best threshold.

The curves reflect a noticeable but incomplete distinction among classes, with the clearest distinction for the outermost phenotypes (DR0 and PDR) and some intersection of ranges between adjacent non-proliferative stages, which was previously reported [19] for AH HIF-1α levels.

Further interpretation was based on determining cut-off ranges for each class (Fig. 2; Table 2).

The following AH lactate ranges (mg/mL) were established: < 0.86 for DR0; 0.86–2.92 for NPDR1; 2.93–5.35 for NPDR2; 5.36–8.56 for NPDR3; > 8.56 for PDR. Corresponding sensitivity and specificity percentage values reflected different accuracy levels for recognizing separate DR stages: the sensitivity and specificity of 100% and 92.6, respectively, for DR0; 67.5% and 61.4%, respectively, for NPDR1; 52.0% and 87.1%, respectively, for NPDR2; 75.0% and 83.7%, respectively, for NPDR3; and 11.1% and 98.9%, respectively, for PDR. Additionally, a total accuracy of 60% (52-69%) was established for the multiclass classification. The following AH HIF 1α ranges (pg/mL) have been established previously [19]: <113.8 for DR0; 113.8–247.8 for NPDR1, 247.9-408.4 for NPDR2, 408.5-509.3 for NPDR3, and >509.3 for PDR. Additionally, corresponding sensitivity and specificity percentage for recognizing separate DR stages were 100% and 52.6%; 57.5% and 60.0%; 60.0% and 58.8%; 50.0% and 60.2%; and 33.3% and 64.1%, respectively. Moreover, a total accuracy of 59% (49-68%) was established for the multiclass classification.

Because our attempt to divide the total sample of patients into five narrow groups resulted in a moderate recognition accuracy due to natural continuity of the pathological process, we decided to use a more clinically advisable approach, binary stratification with separation of mild-to-moderate DR groups (NPDR1+NPDR2) and severe DR groups (NPDR3+PDR). HIF-1α was selected as a key biomarker for this model because it is key regulating factor of hypoxia response which initiates pathological changes before the development of massive metabolic consequences reflected by lactate [9]. Consequently, HIF-1α-based stratification is more pathogenetically justified for predicting a transition to severe DR (Fig. 3).

The following HIF-1α thresholds (pg/mL) were obtained: <94.60 for controls, 94.70-113.85 for DR0, 113.85-377.05 for mild-to-moderate DR, and >377.05 for severe DR. Additionally, corresponding sensitivity and specificity percentage for recognizing mild/moderate DR were 73.8% and 87.1%, respectively; and for recognizing severe DR, 70.0% and 95.2%, respectively. Additionally, the integral accuracy was 71.9% (CI 63.5-79.2%) (Table 3). Therefore, the extension of classes improved the appropriateness of using HIF 1α for practical patient stratification for the risk of DR progression.

Hence, our findings indicated that lactate and HIF-1α had comparable predictive values for the risk of DR pro-

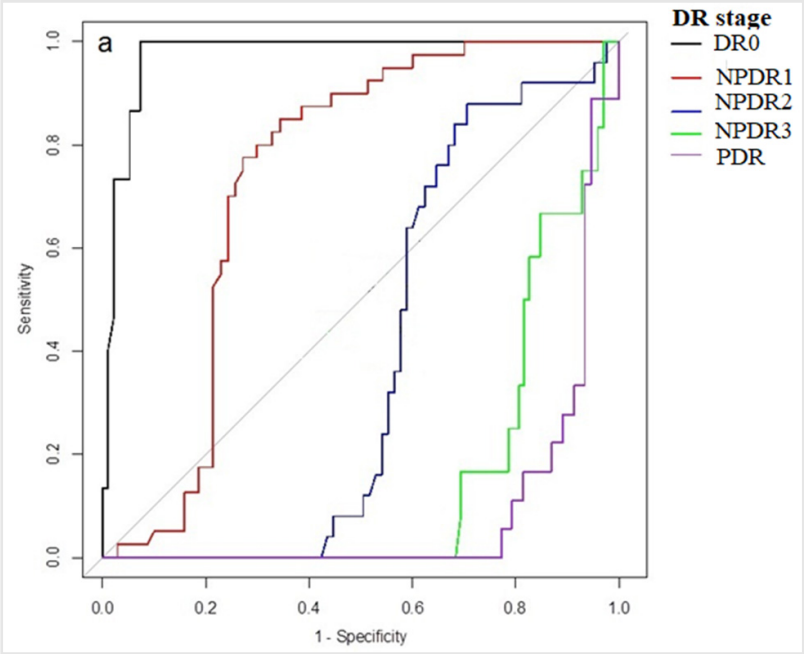

Fig. 1. Receiver operating characteristic (ROC) curves for multiclass classification models generated separately for each class on the basis of aqueous humor lactate concentrations.

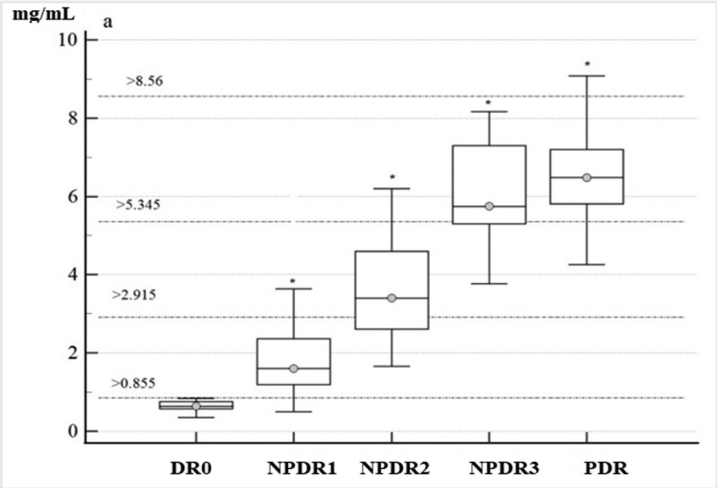

Fig. 2. Cut-off ranges of aqueous humor lactate concentrations.

Note. * - a significant difference ($p < 0.05$) from the DR0 group. The plot presents cut-off values of aqueous humor lactate concentrations (mg/mL) for predicting the stage of DR.

Table 2. Analytical characteristics of prediction depending on the aqueous humor lactate concentration

Characteristic	DR stages				
	DR0	NPDR1	NPDR2	NPDR3	PDR
Lactate concentration threshold, mg/mL	<0.86	0.86-2.92	2.93-5.35	5.36-8.56	>8.56
Sensitivity, %	100	67.5	52.0	75.0	11.1
Specificity, %	92.6	61.4	87.1	83.7	98.9
Accuracy, 60% (52%-69%)					

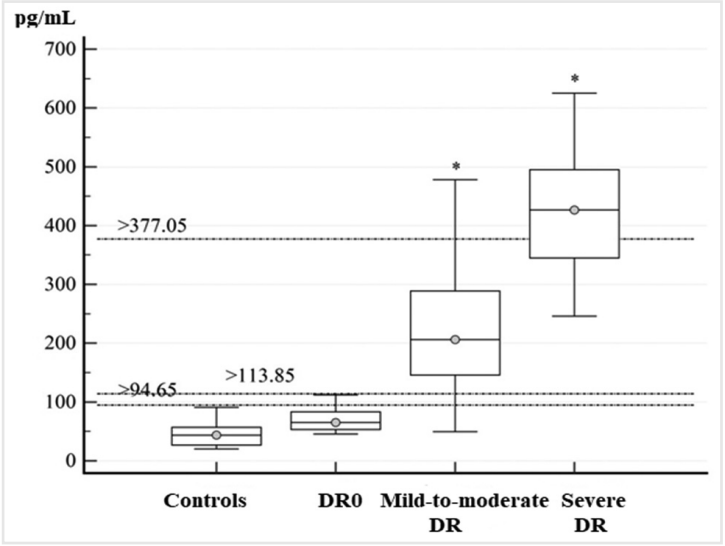

Fig. 3. Cut-off ranges of aqueous humor HIF-1α concentrations.

Note. * - a significant difference ($p < 0.05$) from the DR0 group. The plot presents cut-off values of aqueous humor HIF-1α concentrations (pg/mL) for predicting mild-to-moderate DR (NPDR1+NPDR2) versus severe DR (NPDR3+PDR)

Table 3. Analytical characteristics of prediction depending on the aqueous humor HIF-1α concentration

Characteristic	DR categories			
	Controls	DR0	Mild-to-moderate DR	Severe DR
HIF-1α concentration threshold, pg/mL	<94.6	94.7-113.9	114-377	>377
Sensitivity, %	100	20.0	73.8	70.0
Specificity, %	82.7	97.5	87.1	95.2
Accuracy, 71.9% (63.5%-79.2%)				

gression: they increased synchronously with an increase in the severity of DR and had comparable accuracy metrics. Due to a high statistical similarity of the results, it has been considered futile to duplicate binary stratification for both characteristics. The final model for predicting the risk of mild to moderate DR vs severe DR was built on the basis of HIF-1α as a primary component of the pathogenesis of DR. Additionally, AH lactate concentration can be more easily measured than HIF-1α, and its primary advantage was high- specificity verification of PDR.

Discussion

In this study, we compared the diagnostic value of AH lactate measurements versus previously established AH HIF-1α measurements [19]. We found that, similarly to the AH HIF-1α concentration, the AH lactate concentration increases with an increase in the severity of DR from no apparent DR (DR0) to PDR, with statistically significant differences among the groups. The accuracy of the markers was moderate (~59–60%) after an attempt to discriminate among all five clinical stages of DR: the clearest distinction was observed for the outermost phenotypes of DR, and there was some intersection of ranges between adjacent non-proliferative stages of DR. However, a transition to the distribution of patients into two risk groups (mild-to-moderate DR vs severe DR) enabled substantially improved prediction accuracy (to 71.9% on the basis of HIF-1α) to meet the clinical needs for separating patients requiring more intensive observation (Tables 1 to 3; Figs. 1 to 3).

Our findings are in agreement with current understanding of the hypoxia/HIF 1α/angiogenesis axis and metabolic re-arrangement in the retina: HIF 1α activates VEGF pathways, induces a shift in energy metabolism towards anaerobic glycolysis, increases vascular permeability and promotes neoangiogenesis, which characterizes the essential features of DR progression [9, 10, 24-26].

Our findings are also in agreement with (1) those of studies noting the role of HIF 1α VEGF/VEGFR2 signaling in neoangiogenesis under conditions of hyperglycemia and retinal ischemia [25, 26] and (2) clinical observations of high levels of hypoxia-associated factors and proangiogenic factors in ocular tissues affected by ischemic injury [27-29].

OCTA studues demonstrated deep capillary nonperfusion at the level of the deep capillary plexus, which is associated with the risk of DR complications even in moderate NPDR and severe DR complications in ICDR [7, 8, 30, 31]. Our findings complemented these data, proposing biochemical thresholds of AH lactate/ HIF 1α concentrations as indicators of hypoxic stress in the clinical setting.

The increase in the AH lactate concentration with an increase in the grade of DR is pathophysiologically justified: HIF 1α induces an anaerobic glycolytic shift and decreased cellular oxygen metabolism, which is reflected in the accumulation of glycolysis metabolites e.g. L-lactate [9, 10, 32, 33]. Metabolomic studies in patients with DR and/or DME confirmed a shift in the energy profile with an increase in the AH levels of intermediary products of glycolysis [13, 15, 34, 35], and technical easiness of enzymatic determination of lactate makes it a promising candidate marker for routine examination [20].

A type of classification errors in multiclass analysis is a substantial practical aspect. The outermost phenotypes had a high specificity (e.g., an AH lactate range > 8.56 mg/mL for PDR) but a low sensitivity (~11.1%). A more balanced reduction in both characteristics was seen in intermediate DR grades (Table 2). Such a distribution was natural because ocular biomarkers reflect gradual pathological changes, whereas the ICDR severity scale provides for subdivision of this continuous process into separate categories. The transition to the extended binary approach (mild-to-moderate DR vs severe DR) provided a better balance between sensitivity and specificity for both AG HIF-1α concentration thresholds, which improved the practical value of the method for stratification of the risk of DR progression.

The clinical value of HIF-1α as an early indicator of hypoxic stress has been confirmed by numerous in vivo and ex vivo studies, which demonstrated that the activation of the HIF-1α/VEGF axis regulates vascular permeability and promotes neoangiogenesis, whereas the inhibition of this axis reduces pathological vascular growth and retinal edema, the mechanisms underlying the efficacy of current anti-VEGF therapy for DR and DME [9, 10, 36]. Therefore, findings of this study have a practical value, pointing to the possibility of using HIF-1α as a molecular reference point in personalized management of patients with DR. Recent clinical reviews and real data have confirmed a key role of anti-VEGF medications in therapy for DME and PDR and highlight the advantages of early interventions in severe NPDR [17, 37-39].

In this context, our finding of threshold levels of AH HIF-1α for integrated DR severity groups (mild-to-moderate DR, 114–377 pg/mL; severe DR, >377 pg/mL) has a significant clinical potential for managing patients with DR. These characteristics may be used for operative risk stratification to identify patients requiring frequent eye examinations, early referral to subspecialties and timely therapy escalation (e.g., administration of anti-VEGF medications or laser treatment in patients with OCTA evidence of ischemic changes). Such an approach enables personalizing patient management, focusing on the groups of patients with increased risk of disease progression, and may be integrated into clinical algorithms of early intervention to prevent the development of PDR [17, 37].

However, such a stratification strategy should be considered along with systemic predictors of DR progression. It has been demonstrated that diabetes duration, average HbA1c level, longitudinal variation of the latter and even the rate of glycemia reduction over time have a substantial impact on the risk of retinopathy and its complications [40-44]. Therefore, the integration of ocular biomarkers of hypoxia (HIF-1α and lactate) with systemic metabolic

control characteristics provides a framework for complex prediction of the risk of DR progression which combines local pathogenetic mechanisms with systemic metabolic determinants of the disease.

Our findings regarding AH lactate concentration confirmed that it is a promising additional marker of DR severity. A high specificity of maximum values of this characteristic in PDR indicated its potential usefulness as a rule-in marker (which is helpful for confirming the presence of a severe disease in suspects for fast disease progression or in cases when comprehensive fundus examination is not possible). In this context, lactate may be considered a metabolic fingerprint of hypoxia which complements HIF-1α that is more appropriate for binary risk stratification in the clinical setting.

Our observations are in agreement with findings of recent studies that demonstrated high AH lactate concentrations in patients with PDR [45] and studies using metabolomic analysis of AH and vitreous [13, 15, 34, 35, 46]. These works demonstrated steady increases in the concentrations of glycolytic metabolites (e.g., lactate), with an increase in the severity of DR, which confirms the role of lactate as a reliable indicator of local hypoxic and metabolic stress.

Therefore, HIF-1α and lactate reflect two interrelated layers of the pathogenesis of DR, the former as a transcriptional regulator of the hypoxic response, and the latter as metabolic marker of glycolysis intensity. Their use in combination with each other may improve the accurateness of patient stratification for the risk of DR progression and provide new opportunities for personalized monitoring and early interventions.

Advantages and limitations: The advantage is parallel assessment of two hypoxia markers in AH with rearrangement of class-specific thresholds and check of practical binarization to improve clinical appropriateness of the approach. Limitations of this study include a single-center and cross-sectional design, the use of cataract patients as controls (although this is a common practice, the presence of cataract may affect the composition of aqueous humor [47]), possible effects of pre-analytical factors on measurements (time to freezing, storage conditions, and variability between assay kits), effects of co-founders (concomitant hypertension, nephropathy, diabetes duration, and glycemic metrics) and small subgroups of the mildest and most severe grades of DR. The last limitation could have caused increased confidence intervals for metrics, which will result in the need for validation of thresholds in independent cohorts from various laboratories.

Conclusion

We found that, in eyes of type 2 diabetics, the AH lactate concentration steadily increased with an increase in the grade of DR ($p < 0.001$) in a way similar to that for the AH HIF-1α concentration reported by us previously. This confirms a close relationship between the accumulation of glycolytic metabolites and clinical disease progression. The following diagnostic thresholds for AH lactate concentrations (mg/mL) were established for the differentiation between the grades of the disease: <0.86 (no apparent retinopathy), 0.86–2.92 (mild NPDR), 2.93–5.35 (moderate NPDR), 5.36–8.56 (severe NPDR) and > 8.56 (PDR). These values correlated with corresponding thresholds for AH HIF-1α concentrations determined for this cohort of patients. A total accuracy was moderate (~60%) for the attempt to use thresholds of AH concentrations of both markers for the discrimination between all grades of the disease. No apparent retinopathy and PDR were most reliably identified, but there was some intersection of ranges between adjacent non-proliferative grades of DR. Dividing patients into two risk groups (mild-to-moderate DR and severe DR) is most effective for practical predictions. The use of threshold values of AH HIF-1α concentrations (114–377 pg/mL for mild-to-moderate and >377 pg/mL for severe DR) enabled improving the accuracy of prognosis to 71.9%, which is sufficient for selecting a clinical strategy. Determining AH HIF-1α and lactate concentrations may have different clinical applications: a high AH lactate concentration (>8.56 mg/mL) is a specific test (98.9%) for confirming PDR, whereas determining AH HIF-1α concentrations should be used for early screening, stratifying patients into risk groups and to guide planning the intensity of supervision and treatment. The thresholds and approaches proposed require external validation in multicenter longitudinal studies taking into account pre-analytical factors and potential confounders. The integration of ocular biomarkers of hypoxia (HIF-1α and lactate) with systemic metabolic control characteristics and OCTA metrics of retinal ischemia may improve the prognostic accuracy and clinical usefulness of models for predicting the course of DR.

Author Contributions

All authors reviewed the results and approved the final version of the manuscript.

Sources of support

None.

Conflict of interest

The authors declare no conflict of interest that could influence their views on the subject matter or materials described and discussed in this manuscript.

Disclaimer

This manuscript reflects the views of the authors and may not reflect the views of their institution or sponsor.

Data Availability Declaration

All the data obtained or examined during this study has been incorporated into this published article.

Abbreviations

AH, aqueous humor; DM, diabetes mellitus; DME, diabetic macular edema; DR, diabetic retinopathy; HIF 1α, hypoxia-inducible factor 1α; NPDR, nonproliferative

diabetic retinopathy; OCTA, optical coherence tomography angiography; PDR, proliferative diabetic retinopathy; PEDF, pigment epithelium-derived factor; VEGF, vascular endothelial growth factor.

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