

Quantification of Intraocular Interferon- γ and IgG in Cataract and Diabetes

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Received May 24, 2000; revised June 20, 2000; accepted July 31, 2000

We applied the machine learning procedure to the analysis of serum and aqueous humor albumin, IgG and interferon- γ patterns. The data were analyzed with respect to the cataract, type of diabetes, retinopathy and blood-aqueous humor barrier damage. Intraocular production of IgG was detected in diabetic patients, since the IgG index values were increased in some diabetic groups (especially those with type I diabetes and retinopathy). Comparison of numerical methods and clonal IgG detection suggested that intraocular IgG patterns in diabetes are predominately polyclonal. Interferon- γ (IFN- γ) was pathological in serum and aqueous humor of patients with type I diabetes and to a lesser extent in the type II diabetes group. Machine learning system based on C5.0 classifier extracted 98.5% accurate rules for discrimination of the senile cataract group, diabetes group and diabetic retinopathy group. Our results confirm that the combination of immunochemical and numerical methods with a proper statistical analysis may contribute to the diagnosis of the pathologic ocular immune response and the

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related retinal or vascular disorders in diabetes. Significant savings in laboratory material and efforts may be obtained by means of the machine learning based optimization of the tests.

Key words: diabetes, retinopathy, cataract, machine learning, C5.0, interferon- γ , aqueous humor, serum, IgG, prognosis, barrier.

INTRODUCTION

Immunochemical information of different immunochemical tests strongly depends on the validity of the procedure, sample preparation and data analysis. Improvement of sensitivity, specificity and accuracy of enzyme-immunoassays, and the procedure of freezing and storage of body fluid samples at low temperatures have directed more attention to the data mining methods in biomedical analyses of proteins. New computer softwares enable easy and accurate application of different statistical tools that extract the links between the measured tests and complex biological systems influencing their results.

The aim of this study is to perform an immunochemical analysis of proteins in two body fluids and combine it with the new machine learning procedure based on artificial intelligence. The specific problem that we address is to classify serum and aqueous humor protein patterns obtained by means of the immunochemical tests into groups of control, diabetic and retinopathy samples in cataract, paying heed to the blood-aqueous humor barrier function.

In uveitis, diabetes and complicated cataracts, the protein pattern of aqueous humor is often changed because of the barrier leakage, accumulation of inflammatory cells and local intraocular cytokine and immunoglobulin production.¹⁻⁸ Intraocular protein changes can be evaluated by means of immunochemical or electrophoretic aqueous humor analyses and calculation of relative concentration aqueous/serum ratios, *i.e.* indexes.¹⁻⁸

Leakage of the blood-aqueous barrier and changes in the aqueous humor protein patterns have been observed in some cases of diabetic retinopathy.⁷ Diabetes mellitus of type I is thought to be an autoimmune reaction to the pancreas β -cells, where the local and systemic immunological reaction depends on the IL-2 and interferon- γ (IFN- γ).⁹⁻¹¹ Similarly to type I diabetes, the autoantibodies in the type II disease are of the IgG class, against insulin receptors.¹¹⁻¹²

Preretinal membranes of the diabetics are characterized by different pathologic changes, including aggregation of inflammatory cells and increased discharge of IL-2, IL-4 and IFN- γ .¹³⁻¹⁵ Local and systemic presence of the antiretinal antibodies of the IgG class has also been established.¹⁴⁻¹⁶ The

mentioned laboratory and clinical findings are more prominent in the type I diabetes.^{17,18}

Therefore, in this study we investigated the serum and aqueous humor patterns of IFN- γ and IgG in type I and II diabetes with and without retinopathy. The results of the immunochemical tests were compared to the senile cataract controls since they do not exhibit any local or systemic pathologic immune response, with respect to the analyzed protein fractions.¹⁻⁸ In addition to the standard statistical procedure of immunochemical parameter analysis, we applied the new C5.0 machine learning system to define the links between different tests and groups.¹⁹ The method of decision tree and rules estimation for different parameters (tests) has been recently reported to be useful procedure in immunochemical data analysis.²⁰

MATERIALS AND METHODS

Immunochemical Parameters Determination

Albumin and IgG in serum were evaluated on Partigen[®] plates for the standard single radial immunodiffusion technique (Behring, Marburg, Germany). Aqueous humor concentrations of albumin and IgG were determined with LC-Partigen[®] plates for low protein concentration measurements (Behring, Marburg, Germany).^{1,2,4,6-8}

Intraocular IgG synthesis was evaluated by means of the IgG index, calculated with the following formula:^{2-4,6-8}

$$\text{IgG AH index} = (\text{AH/S})_{\text{IgG}} \times (\text{S/AH})_{\text{albumin}}$$

Oligoclonal IgG were detected in diluted serum (1:500) and unconcentrated aqueous humor by isoelectric focusing of proteins in ultrathin polyacrylamide gel (0.4 mm), followed by direct immunofixation with monospecific IgG antisera and silver nitrate staining.^{4,6-8} Samples with aqueous humor oligoclonal IgG without matched serum bands were considered positive.^{4,6-8}

IFN- γ in aqueous humor and serum was analyzed using the quantitative enzyme immunoassay test kit Immunotech[®] (Marseille, France). The assay is specific for both natural IFN- γ and recombinant human IFN- γ , and demonstrates no cross-reactivity to other interferons. The procedure was performed according to the manufacturer's instructions. Briefly, in the first incubation, IFN- γ from 50 μ L of pipetted serum or aqueous samples was bound with primary monoclonal anti-IFN- γ Ab of the wells into Ab/Ag immunocomplex. Then, 0.050 mL of the secondary antibody was added into each well, followed by 100 μ L/well of streptavidine bound to horse-radish peroxidase. After 30 minutes of this secondary reaction in which the secondary antibody bound to primary complex and HRP-streptavidine binds to biotine, 100 μ L/well of tetramethylbenzidine was added to activate peroxidase. The intensity of colour, proportional to IFN- γ concentration, was measured spectrophotometrically at 450 nm.

Sample Collection

Aqueous humor (AH) samples were collected at the time of cataract surgery according to the procedure described by Zirm¹ and Grabner *et al.*,² taking account of the recommendations of Tripathi *et al.*²¹ During the collection every precaution was taken to avoid contact with corneal endothelium, iris or lens.⁴ 100–200 μ L of each aqueous sample was collected. The material was stored at -70 °C and analyzed later. Serum samples were obtained by standard venepuncture and stored at -70 °C. While conducting the investigation, the principles of the Helsinki Declaration were observed and informed consent was obtained from the subjects.

The control group consisted of 23 patients with senile uncomplicated cataract without diabetes, retinopathy and other systemic diseases that could influence their immunological status. Fifteen of the patients were males (M) and eight were females (F). The mean age of the group was 67.7 years and the mean cataract duration was 2.2 years.

Type I diabetes group had 22 patients (11 M, 11 F; 9 without, 13 with retinopathy), and the type II diabetes group had 23 patients with diabetic cataract (11 M, 12 F; 11 without, 12 with retinopathy). The mean age (type I 69.4 years, type II 71.2) and cataract duration (type I 2.3 years, type II 2.6) were not statistically different from the control group. Patients with retinopathy had suffered from diabetes longer than the ones without retinopathy ($p < 0.05$).

C5.0 Machine Learning Program

The C5.0 program is a commercially available successor to the landmark C4.5 decision tree program.¹⁹ C5.0 decision tree induction is essentially the same as in C4.5, while the tests show some differences but negligible improvements. However, the C5.0 rule generation has been greatly speeded up compared to C4.5.¹⁹

C5.0 and C4.5 define the possible decision tree by means of a hill-climbing search based on the statistical property measure called information gain.^{19,20,22} Information gain measure defines how well a given attribute separates the training examples according to their target classification, and selects candidate attributes at each step of the tree.^{19,20,22} Consequently, this measure is the expected reduction in Shannon's entropy caused by partitioning the examples according to the attribute in classifying the training data.^{19,20,22}

The elements of the decision tree are either leaves or decision nodes.^{19,20,22} The leaf shows a class and the decision node specifies the test to be implemented on an attribute value, with one branch and subtree for each possible result of the test.^{19,20,22} The starting node is the root node and a tree is used to predict a case by starting at the root and moving through the tree until the leaf is encountered.^{20,22} For any tree, all paths lead to a leaf corresponding to a decision rule that is a logical conjunction of various tests.^{19,22} If there are multiple paths for a given class, then the paths represent logical disjunctions.^{19,20,22} All paths are mutually exclusive. For any new case, one and only one path in the tree will always have to be satisfied.

Statistical Analysis

Software STATISTICA[®], version 5.0, was used for data analysis. The values of the IgG index, serum and aqueous humor IgG and albumin were compared by means of two-sided t-test with the correction according Cochran & Cox. IFN- γ values and oligoclonal IgG bands in serum and aqueous human were compared by means of the Fisher exact probability test.

RESULTS

Protein Patterns in Control Samples

IgG index in senile cataract controls was 0.49 ± 0.11 , *i.e.* within the normal range typical of aqueous humor IgG measurements. Total albumin and IgG values in serum and aqueous humor were also within the normal range (Table I). Oligoclonal IgG bands were negative in all samples. Aqueous humor levels of IFN- γ in senile cataract controls were below the detection limit of the test and serum IFN- γ levels were detectable in only four cases (15.6%, Table II).

Albumin and IgG in Diabetes and Retinopathy

In the group of patients with type I diabetes without retinopathy a statistically significant difference ($p < 0.05$) in aqueous humor albumin was found compared to the senile cataract controls (Table I). Differences in IgG indexes among groups were also observed. Group of the type I diabetes patients with retinopathy differed from the control group, type I diabetes without retinopathy and type II diabetes with retinopathy (Table I). In type II diabetes group, the differences were significant only between its retinopathy group and type I retinopathy.

Aqueous humor oligoclonal IgG were found in only 5–10% of the cases in different diabetic groups; however, the values were not significantly different when compared to the senile cataract control group that had no aqueous IgG bands ($p > 0.05$).

IFN- γ in Diabetes and Retinopathy

Pathological aqueous IFN- γ was found in several groups with diabetic complications and in none of the senile cataract controls. However, statistical difference from the senile cataract controls was observed only in type I diabetes without retinopathy (2 pathological IFN- γ results out of 9, $p < 0.05$, Table II).

TABLE I

Albumin and IgG in serum and aqueous humor of patients suffering from senile cataract, cataract complicated with diabetes without retinopathy (-R), and cataract complicated with diabetes and diabetic retinopathy (+R)

Concentrations g L ⁻¹	Albumin Serum	Albumin AH	IgG serum	IgG AH	IgG index
Senile cataract					
X±SD	39.9±5.1*	0.167±0.065	12.3±2.7	0.024±0.015	0.490±0.110 [†]
range	27.5–46.4	0.061–0.290	8.1–19.9	0.000–0.051	0.264–0.679
Type I diabetes -R					
X±SD	41.9±2.1*	0.275±0.233	12.5±4.7	0.031±0.021	0.453±0.195 [§]
range	38.3–45.4	0.072–0.758	7.4–22.3	0.000–0.070	0.242–0.873
Type I diabetes +R					
X±SD	42.1±2.2	0.180±0.091	13.4±3.2	0.034±0.013	0.678±0.210 ^{§†‡}
range	39.3–47.2	0.056–0.370	8.6–20.9	0.000–0.049	0.377–1.032
Type II diabetes -R					
X±SD	41.9±2.2	0.194±0.128	11.9±2.1	0.023±0.017	0.525±0.123
range	38.4–44.3	0.043–0.398	9.8–17.3	0.000–0.053	0.347–0.713
Type II diabetes +R					
X±SD	41.9±2.7	0.233±0.149	12.8±2.5	0.031±0.020	0.450±0.131 [‡]
range	36.7–45.8	0.066–0.611	8.4–17.8	0.000–0.069	0.233–0.668

* $p < 0.03$; $§p < 0.05$; $†p < 0.01$; $‡p < 0.01$.

Serum IFN- γ values in type I diabetes without retinopathy were significantly elevated (5 out of 9, $p < 0.05$). Consequently, the whole type I diabetes group significantly differed from the cataract controls ($p < 0.05$, Table II).

Decision Tree and Rule Analysis

C5.0 machine learning system extracted 3 decision trees with the corresponding rules for accurate prediction of the groups of senile cataract controls, diabetes without retinopathy and diabetes with retinopathy. Table III presents the most accurate tree and rules algorithm (85.3%). Boosting procedure with 3 trials enables the overall precision of 98.5% for the decision rules and 91.2% for the decision tree (Table IIIb).

TABLE II

Serum and aqueous humor values of interferon- γ in patients suffering from senile cataract, cataract complicated with diabetes without retinopathy (-R), and cataract complicated with diabetes and diabetic retinopathy (+R)

Concentrations in IU mL ⁻¹	IFN- γ Serum	IFN- γ AH
Senile cataract		
X \pm SD	0.210 \pm 0.470*	0.000**
range	0.000-1.490	0.000
Type I diabetes -R		
X \pm SD	1.346 \pm 1.526*	0.164 \pm 0.394**
range	0.000-3.690	0.000-1.180
No. \uparrow (>2SD)	5	2
Type I diabetes +R		
X \pm SD	0.621 \pm 1.307	0.005 \pm 0.017
range	0.000-4.250	0.000-0.006
No. \uparrow (>2SD)	3	1
Type II diabetes -R		
X \pm SD	0.402 \pm 0.931	0.000
range	0.000-2.790	0.000
No. \uparrow (>2SD)	2	0
Type II diabetes +R		
X \pm SD	0.241 \pm 0.834	0.002 \pm 0.006
range	0.000-2.890	0.000-0.020
No. \uparrow (>2SD)	1	1

* $p < 0.05$; ** $p < 0.05$.

DISCUSSION

Systemic and intraocular aberrations of protein patterns reflect the changes of cellular and humoral immune response in both types of diabetes.⁹⁻¹⁸ Our results of IFN- γ and IgG in serum and aqueous humor confirm the latter (Table I and Table II).

Albumin is a protein that is not synthesized intraocularly.¹⁻⁸ Therefore, it often serves as a marker of protein transfer through blood-ocular barriers.¹⁻⁸ We found no differences in the aqueous humor albumin concentration between groups. Similar applies to aqueous/serum albumin concentration ratio, which suggested that protein transfer through the blood-aqueous barrier was not significantly impaired in diabetes and retinopathy.^{1-4,6,8}

TABLE III (a-c)

C5.0 decision tree and rule analysis of immunochemical parameters in patients suffering from senile cataract (C), cataract complicated with diabetes without retinopathy (D), and cataract complicated with diabetes and diabetic retinopathy (R)

a. Options:

Generating rules
 Rule utility ordering (1/5's)
 Boost using 3 trials
 Fuzzy thresholds

Class specified by attribute 'diagnosis'
 C = Senile cataract control;
 D = Diabetes without retinopathy;
 R = Diabetes with retinopathy.
 Read 68 cases (10 attributes)

--- Trial 0 --- Decision tree:

```

albuminS <= 36 (36.35): C (6)
albuminS >= 36.7 (36.35):
:...albuminAH >= 0.37 (0.334):
  ...albuminS <= 39.3 (40.7): R (2)
  : albuminS >= 42.1 (40.7): D (7/1)
  albuminAH <= 0.298 (0.334):
  ...IgGAH >= 0.034 (0.033):
    ...IgGindex >= 0.919 (0.812): R (3)
    : IgGindex <= 0.705 (0.812):
    :   ...IgGAH >= 0.044 (0.043): C (2)
    :   : IgGAH <= 0.042 (0.043):
    :   :   ...IgGindex <= 0.504 (0.514): C (4/1)
    :   :   : IgGindex >= 0.524 (0.514): R (6)
    :   IgGAH <= 0.032 (0.033):
    ...IFNgamaS >= 2.19 (2.105): R (3)
    IFNgamaS <= 2.02 (2.105):
    ...IFNgamaS >= 1.56 (1.525): D (3)
    IFNgamaS <= 1.49 (1.525):
    ...IFNgamaS >= 1.32 (1.25): C (2)
    IFNgamaS <= 1.18 (1.25):
    ...albuminS >= 44.9 (44.6):
    : ...albuminS <= 45 (45.35): C (2)
    : albuminS >= 45.7 (45.35): R (3/1)
    albuminS <= 42.7 (44.6):
    ...IgGAH >= 0.023 (0.022): D (7/2)
    : IgGAH <= 0.021 (0.022):
    : ...IgGindex = N/A: N (5/2)
    :   IgGindex <= 0.353 (0.402): R (4/1)
    :   : IgGindex >= 0.451 (0.402):
    :   :   ...IgGindex >= 0.713 (0.661): D (2/1)
    :   :   : IgGindex <= 0.609 (0.661):
    :   :   :   ...IgGindex <= 0.451 (0.488): D (3/1)
    :   :   :   : IgGindex >= 0.494 (0.488): C (4)
    
```

b. Evaluation on training data (68 cases):

Trial	Decision Tree			Rules	
	Size	Errors		No	Errors
0	18	10 (14.7%)		18	10 (14.7%)
1	10	29 (42.6%)		10	24 (35.3%)
2	15	19 (27.9%)		14	17 (25.0%)
boost		6 (8.8%)		1 (1.5%)	<<

(a)	(b)	(c)	<-classified as
23			(a): class C
1	19		(b): class D
		25	(c): class R

Time: 0.1 secs

TABLE III (continued)

c. Extracted rules 0/1–9:	Extracted rules 0/10–18:
Rule 0/1: (7, lift 2.4) albuminS > 36 IgGAH > 0.032 IgGAH <= 0.042 IgGindex > 0.508 -> class R [0.889]	Rule 0/10: (4/1, lift 1.8) IFNgamaS <= 1.49 albuminS > 36 IgGAH <= 0.022 IgGindex <= 0.402 -> class R [0.667]
Rule 0/2: (6, lift 2.6) albuminS <= 36 -> class C [0.875]	Rule 0/11: (2, lift 2.2) IFNgamaS > 1.18 IFNgamaS <= 1.49 -> class C [0.750]
Rule 0/3: (7/1, lift 2.6) albuminS > 40.5 albuminAH > 0.298 -> class D [0.778]	Rule 0/12: (3/1, lift 1.6) albuminS > 45 IgGAH <= 0.032 -> class R [0.600]
Rule 0/4: (7/2, lift 2.3) IFNgamaS <= 1.18 albuminS > 36 albuminS <= 44.3 IgGAH > 0.022 IgGAH <= 0.032 -> class D [0.667]	Rule 0/13: (3, lift 2.2) IgGAH > 0.032 IgGindex > 0.786 -> class R [0.800]
Rule 0/5: (5, lift 2.5) albuminS <= 44.3 IgGAH <= 0.022 IgGindex > 0.482 IgGindex <= 0.645 -> class C [0.857]	Rule 0/14: (2, lift 2.0) albuminS <= 40.5 albuminAH > 0.298 -> class R [0.750]
Rule 0/6: (3, lift 2.2) IFNgamaS > 2.02 albuminAH <= 0.298 -> class R [0.800]	Rule 0/15: (2, lift 2.2) albuminS > 44.3 albuminS <= 45 -> class C [0.750]
Rule 0/7: (3, lift 2.7) IFNgamaS > 1.49 IFNgamaS <= 2.02 albuminAH <= 0.298 -> class D [0.800]	Rule 0/16: (3, lift 2.4) albuminAH <= 0.298 IgGAH > 0.042 IgGindex <= 0.786 -> class C [0.800]
Rule 0/8: (5/2, lift 1.9) IFNgamaS <= 1.18 albuminS <= 44.3 IgGindex = N/A -> class D [0.571]	Rule 0/17: (4/1, lift 2.3) IFNgamaS <= 2.02 albuminS > 36 IgGAH <= 0.032 IgGindex > 0.402 IgGindex <= 0.482 -> class D [0.667]
Rule 0/9: (5/1, lift 2.1) albuminAH <= 0.298 IgGAH > 0.032 IgGindex <= 0.508 -> class C [0.714]	Rule 0/18: (3/1, lift 2.0) IgGAH <= 0.032 IgGindex > 0.645 -> class D [0.600]
	Default class: R

Total IgG in serum and aqueous humor were not significantly elevated for any of the groups, although some patients had elevated rates. The latter might be caused by intraocular IgG production, enhanced IgG filtration (leakage) due to the barrier damage, or by their combination. Since the relative concentration ratio (index) of transferred albumin and IgG on both sides of the barrier defines the rate of aqueous humor IgG synthesis much better than total aqueous IgG, we calculated and compared IgG indexes of all groups.^{2-4,6,8}

IgG indexes were elevated in type I diabetes with and without retinopathy. In type II diabetes, indexes were just above the borderline (Table II). More pathology was found in the patients with type I diabetes and in retinopathy. This confirmed the findings of other authors that the immune response tends to be more pronounced in type I diabetes.^{17,18}

The breakdown of blood aqueous-barrier in retinopathy may influence the passage of systemic antibodies.^{1-4,6,8} However, we found only a few cases with aqueous oligoclonal IgG (1 in type I and 2 in type II diabetes), while much more IgG pathology was reflected by positive IgG indexes. This negative oligoclonal findings and positive IgG index values suggest a more pronounced polyclonal intraocular IgG synthesis in diabetic patients.⁶ The detected protein pattern is consistent with our previous results for type II diabetes.⁷

IFN- γ is a cytokine strongly involved in the breakdown of the ACAID system.²³⁻²⁵ It was not detected in the aqueous humor of the control patients with senile cataract. This seems to suggest that IFN- γ is not directly involved in senile cataract formation. Normal values of IgG indexes, low total aqueous IgG values and the absence of aqueous oligoclonal IgG seem to exclude involvement of the IgG immune response in this type of cataract.¹⁻⁸

Significant deviations of systemic IFN- γ were found in diabetes, especially in the type I disease.⁹⁻¹¹ We measured heightened levels of this cytokine in the aqueous humors and serums of type I disease, in contrast to the senile cataract controls. This supports several experimental and clinical investigations linking IFN- γ (and related Th1 lymphocytes) to type I diabetes.⁹⁻¹¹

Machine learning program C5.0 extracted the decision rules that enable a very accurate classification of 98.5% of subjects (Table IIIb) into the groups of patients with senile cataract, diabetes without retinopathy and diabetes with retinopathy. One case misclassified to the control group had a mild type II diabetes, which indicates that this insignificant error in the classification occurred due to the less pronounced course of the disease. The decision tree result was somewhat less precise, but still acceptable, 91.2% of correct classifications.

The machine learning procedure presented in Table III does not discriminate between different types of diabetes, which may be due to the insufficient selectivity and small number of immunochemical parameters measured. The complexity of the decision tree and rules is also influenced by the parameter specification.¹⁹ However, the decision rules may predict the onset of retinal vascular complications, *i.e.* retinopathy, with almost 100% accuracy from only three serum and aqueous humor parameters (albumin, IgG and IFN- γ). In this context, predictive values of albumin in serum and aqueous

ous humor may be related to the leakage of albumin through damaged vessels. It is worth mentioning that C5.0 creates a supporting program that performs the classification for each new case. Following addition of the new case to the database, corrected trees and rules of enhanced precision are generated.

Diabetic retinopathy is a severe and common complication of diabetes. Extraction of algorithms for the accurate prediction of its onset, in different types of the disease and from the serum parameters only, may be of great prognostic and therapeutic value. Analyses of IgG subclasses, complement components and other cytokines by means of machine learning²⁰ or neural network²⁶ based methods might provide a better understanding and prediction of the ocular immune-mediated processes in diabetes and retinopathy.

The procedure of data mining applied in this study may be useful adjunct to the standard immunochemical tests. Moreover, in the analysis of the chemical information obtained from the parameters of the complex biological systems, artificial intelligence based analysis might be a prerequisite for the extraction of implicit, previously unknown and potentially useful algorithms describing the results. Significant savings in the laboratory material and efforts may be obtained by means of the machine learning based optimization of the tests.

Acknowledgement. – The financial support of the Ministry of Science and Technology of the Republic of Croatia is highly appreciated (research grant No. 00981108).

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SAŽETAK

Kvantifikacija intraokularnog interferona- γ i IgG kod katarakte i dijabetesa

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U radu je analizirana nova metoda interpretacije imunokemijskih testova zasnovana na umjetnoj inteligenciji. Odnosi albumina, IgG i interferona- γ dobiveni imunokemijskim mjerenjima seruma i očne vodice, analizirani su strojnim klasifikatorom C5.0 s obzirom na utjecaj i tip dijabetesa, oštećenje mrežnice (retinopatiju) i propusnost krvno-očne barijere. Utvrđeno je da ispitanici s dijabetesom tipa I imaju patološke vrijednosti interferona- γ u očnoj vodici i serumu, te da je intraokularna sinteza IgG u dijabetesu primarno poliklonska. Program C5.0 za strojno učenje dao je algoritam pravila odlučivanja koji je uz 98.5% točnosti razlučio skupinu kontrolnih ispitanika sa staračkom kataraktom od skupine s dijabetesom i dijabetičkom retinopatijom. Programi strojnog učenja mogu biti korisna dopuna analizi i interpretaciji imunokemijskih pokazatelja standardnim statističkim metodama.