Comparison of Inflammatory Status and Biochemical Changes in Patients with Open Angle Glaucoma and Type II Diabetes

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Comparison of inflammatory status and biochemical changes in patients with open angle glaucoma and type II diabetes represents a novel approach for a better understanding of possible correlations between these pathologies. The aim of this study was to perform such analysis and report evidences of common pathogenic pathways. We elaborated a protocol for a prospective cohort study in a tertiary ophthalmology center; patients with open angle glaucoma and diabetes were selected between October 2012-October 2014. Study included two research groups: 74 open angle glaucoma eyes (control group) and 44 eyes with open angle glaucoma + diabetes (study group). All patients were clinically evaluated by experienced ophthalmologists, and biochemical profile was assessed individually. The two study groups proved similar on age and sex ratio distribution; visual field parameters were significantly different between groups. We detected significant differences in biochemical profile between groups, that matched the difference in the functional status in glaucoma vs diabetic glaucoma patients. Therefore our study proves by comparison that changes in the biochemical profile are visible in glaucoma versus glaucoma and diabetes patients. As expected the biochemical parameters are influenced by diabetes in terms of increased inflammation, immune response and active phase reactants, but also important changes were detected in glaucoma patients. Based on this result authors could explain common pathogenic theories in glaucoma and diabetes using simple and cost effective biochemical methods.

Key words: open angle glaucoma, diabetes, biochemical profile

Glaucoma and diabetes represent very frequent pathologies and their prevalence is increasing to alarming levels; therefore the two become an epidemiological problem and a matter of public health [1]. Among all forms of glaucoma, primary open angle glaucoma (POAG) represents the most frequent type [2]. The main characteristic is a progressive optic neuropathy leading to blindness due to optic nerve damage and loss of ganglion cells. Diabetes was recently finally acknowledged as risk factor in POAG, both in determining the disease and in glaucoma progression [1]. What connects the two clinical entities are some common pathogenic pathways, basically biochemical changes related to autoimmunity, inflammation, oxidative stress and neurodegeneration. Loss of biochemical homeostasis attributed to ischemia and vascular dysregulation produce progressive disease in both cases [3]. Up to now many studies proved through complicated biochemical analysis (immunoproteomics, metabolomics) that similar molecules (immunomediators - cytokines, chemokines, oxidative stress mediators – nitric oxide or enzymes - malonyldialdehyde, superoxide dismutase, catalase etc) are responsible for neurodegeneration and inflammation in diabetes and in experimental glaucoma [4, 5].

In human subjects the results of these studies remained controversial. What is generally accepted is that in glaucoma there is a continuous glial cell activation in the retina and optic nerve head [6, 7]. This particular state was defined as *para-inflammation* and represents the basis for neuroinflammation and neurodegeneration in POAG [4, 8, 9]. The intensity is lower than a classic inflammatory process, but the mediators are similar. While the physiological purpose of para inflammation is to restore tissue homeostasis and functionality, it may become chronic or turn into over destructive process if persists for longer time. In diabetes there were also described similar inflammatory pathways and autoimmune processes. The hallmark of autoimmune diseases generally involves the presence of self-reactive T cells, autoantibodies and inflammation [4]. In diabetes the autoimmune compound is widely accepted, while in glaucoma this aspect is fraught with difficulty because not one laboratory test fully supports such a diagnosis. Typically, multiple expensive laboratory tests are needed and none is specific. Still, there are some parameters (C reactive protein, ESR, fibrinogen, immunoglobulines) that might be useful to assess disease activity [4, 10]. These proteins are mainly produced by the liver in response to stress and can also be called acute phase reactants. Pro-inflammatory cytokines such as IL-1, IL-6, and TNF-alpha stimulate synthesis of some acute phase reactants that include CRP, fibrinogen and haptoglobin. The inflammatory markers are not diagnostic of inflammation, but reflect abnormalities that are seen in autoimmune diseases, infections, malignancies and other illnesses. Therefore their quantification can provide tools for diagnosis and management in patients with autoimmune diseases and might offer prognosis, or indicate severity of organ involvement/ damage [4].

The purpose of this study was to compare biochemical profiles in glaucoma patients versus glaucoma and diabetes patients, having in mind that diabetes has specific and well established biochemical changes, therefore can be used as landmark for inflammatory status assessment. Moreover the combination of two chronic diseases could augment the severity of each one of them, individually.

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Since in common clinical care ophthalmologists do not have access to high sensitivity detection methods or specialized laboratories as those attached to powerful research centers, our study tried to validate a biochemical model of optic progressive neuropathy (glaucoma) combined or not with diabetes on less complex basis. Therefore, without immunoproteomics analysis techniques, just based on typical active phase reactants and inflammatory markers, we tried to establish and compare individual biochemical profile of glaucoma/ glaucoma+diabetes patients. Previous studies of Wax (2008, 2009) and Hammam (2008) represented the start up point for our work hypothesis, stating that glaucoma belongs to a subset of monoclonal gammopathies (immunoglobulinic alterations) and abnormal T cells activity [11-13]. Also C reactive proteins, normally increased in inflammatory processes, may reach higher levels in glaucoma patients by promoting innate immune response [14]. If proved efficient, our results may represent a simple and cost effective method to label glaucoma as inflammatory disease. This study was approved by the Ethical Board of Grigore T. Popa University of Medicine and Pharmacy – Iasi and each patient was informed and signed an informed consent.

Experimental part

POAG was defined in the presence of open anterior chamber angle on goniscopy, glaucomatous optic disc damage on clinical examination (focal or diffuse neuroretinal rim thinning, localized notching, or nerve fiber layer defect) and corresponding visual field (VF) defects. Glaucoma severity was graded according to Hodapp criteria [15].

Clinical opthalmological parameters were recorded: visual acuity, intraocular pressure, central corneal thickness, visual field parameters (mean deviation, pattern standard deviation), optical cohorence tomography parameters (retinal nerve fiber layer, ganglion cell layer thickness, neural rim area, optic disc area).

Diabetes was defined if fasting plasmatic glucose level was above 126 mg/dL or if previous diagnosis was already made. Arterial blood pressure (systolic and diastolic) was recorded in both groups using a conventional sphingomanometer. Mathematical formula was then used to calculate the ocular perfusion pressure (OPP) [16] .

Biochemical analysis included measurements for plasma glucose level, glycosilated hemoglobin, C reactive protein, erythrocite sedimentare rate, fibrinogen, serum lactate and immunogram.

In order not to have confounders for functional alterations in glaucoma patients, we selected the cases with early and moderate forms of POAG, having maximum a mild form of diabetic retinopathy. Eyes with significant lens opacities, ocular comorbidities, refractive errors >5D spherical and >3D cylinder were excluded.

C reactive proteins, serum lactate and immunoglobulines were measured using Architect c400 automated analyzer**,** to make clinical chemistry determinations using colorimetric, enzymatic and immuno turbidimetric methods (table 1). Plasma glucose was measured by Gluc2 HK, Cobas® analyzer (enzymatic reference method with hexokinase).

Enzymatic method

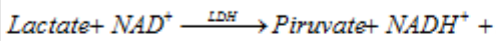
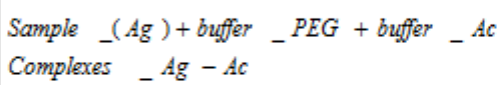
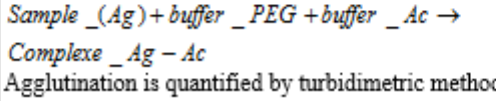
Enzymesare organic biocatalyzers that modify the speed of biochemical reactions, without consuming themselves. Most important characteristic is specificity. Under the action of specific enzymes, the substrate disintegrates and forms different compounds. The speed of these compounds formation or of substance disintegration, measured at different moments in time with the appropriate wave lengths is proportional with the activity of the enzyme, therefore with its concentration, expressed in U/L. For serum lactate which was quantified through this method, normal values were considered between 4.5-19.8mg/dL [17-21].

Turbidimetric method is based on quantification of agglutination that appears in antibody-antigen reactions, specific to each parameter. The quantity of light that is transmitted through a solution, without deviation represents the principle of determination. The higher the particle concentration is, the less transmitted light passes through. Results are expressed as mass concentration (mg/dl or g/l), considering Beer Lambert law [17-21].

Erythrocyre sedimentation rate (ESR) is measured from venous blood, harvested in special ESR vaccutainerscoated with 3.8% sodium citrate. The ESR is the measure of the quantity of red blood cells (RBC) that precipitate in a tube in a defined time and is based upon serum protein

Table 1

PRINCIPLE OF CHEMICAL REACTIONS (MODIFIED AFTER TIETZ, 2006, [20])

Parameter	Biochemical analysis method	Principle of method	Wave lenths(nm)
Serum lactate	Standard enzymatic method;	NAD is reduced to NADH; rate of NADH production, measured my photometric methods, is proportional to serum lactate activity 	340/404
CRP	Standard turbidimetric method	 Agglutination is detected as a change in absorption capacity, rate of this change being proportional with the quantity of C reactive protein in the analyzed sample	572
Immunoglobulines (A, M, G)	Immunoturbidimetric method	 Agglutination is quantified by turbidimetric methods.	700 700 340/700

concentrations and RBC interactions with these proteins. Inflammation causes an increase in the ESR. Multiple factors influence the ESR and include patient's age, gender, RBC morphology, hemoglobin concentration, and serum levels of immunoglobulin [17]. The sample must be handled appropriately and processed within a few hours to assure test accuracy. While the ESR is not a diagnostic test, it can be used to monitor disease activity and treatment response and signal that inflammatory or infectious stress is present. Normal value ranges between 2-10 mm/1h.

CRP, an innate immune protein, helps opsonize pathogens for phagocytosis and activates the complement system. CRP production is under the control of IL-1, IL-6, and TNF-alpha. Changes in serum CRP concentration change more quickly than ESR and therefore CRP maybe a better reflection of current inflammation. Unlike the ESR, CRP is a fairly stable serum protein whose measurement is not time-sensitive and is not affected by other serumcomponents. The magnitude of inflammation directly relates to the concentration of CRP. Levels < 0.2 mg/dl are considered normal, while those >1.0 mg/dL are suggestive of inflammation and/or infection. More recently, the use of high sensitivity CRP has been utilized. This test may better quantify lower levels of inflammation and has been important in evaluating cardiac disease and other inflammatory states [22, 23].

Measuring total quantitative immunoglobulin (Ig) levels is a key component to any immunologic evaluation. Ig levels reflect B cell function (humoral production and T cell interaction) and serum Ig levels aid in disease detection [11]. Simple qualitative measurements of serum immunoglobulins reflect an individual's ability to mount a humoral immune response [17-21]. Normal IgG level ranged between 700-1600 mg%.

Fibrinogen, a hemostatic coagulation factor produced in response to tissue injury. Its synthesis is controlled at the transcription level and is increased in the presence of inflammation and stress that is mediated by IL-6. For determination, principle of method resides in thrombin (enzyme) capacity to convert the soluble plasma protein fibrinogen into its insoluble polymer, fibrin. The clotting time for diluted plasma is inversely proportional to the fibrinogen concentration of the plasma [18-22]. We used undiluted blood samples collected in sterile conditions from venous puncture; the samples were carefully mixed with sodium citrate solution (0.11mol/L) in proportion 9:1, avoiding the formation of foam. Immediately the samples were centrifuged for 10-15 min at 1500-2500 x g; supernatant plasma was removed. To the separated plasma we added the amount of dry thrombin that adhered to the tip of several applicator sticks or 1-2 drops (0.1mL) of reconstituted Dade (Siemens®) Thrombin reagent (100 units/mL) per 1 mL of sample. Mixing and incubation was set at 37°C for 5-10 min (table 2). Reference values were between 180-350 mg/dL.

Results and discussions

We included in our cohort a total of 118 eyes from 118 patients: 44 eyes in the study group (diabetes + glaucoma) and 74 eyes in the control group (open angle glaucoma).Majority of cases included primary open angle glaucoma (56.41%), followed by normal tension glaucoma (41.03%) and pseudoexfoliative glaucoma(2.56% cases). As a particular aspect, in the study group there were more cases of normal tension glaucoma (60.9%) whereas in the control group there were more hypertensive glaucoma cases (56.4% primary open angle glaucoma). From this point of view the groups might have diferent characteristics and behaviour regarding the susceptibility to systemic homeostatic changes. Yet the general aspect of the populations was comparable from the demographic and clinical point of view. This constitutes a good premise for our study since homogenous and comparable subjects were included in our analysis. Descriptive statistics was presented in table 3.

From the functional point of view, visual field Hodapp classification (described somewhere else by EGS) [15], found in both groups mostly early forms of glaucoma (77.27% - 34 eyes in the study groups vs. 83.78% - 62 eyes in the control group). Moderate forms were confirmed in 22.63% cases (10 eyes) in the study group and in 16.21%

Pipette into pre-warmed coagulation tubes as follows		
	Patient Plasma	Control Plasma
Plasma sample (diluted 1:10)	0.2 mL	0.2 mL
Control plasma (diluted 1:10)	-	-
	Incubate in waterbath at +37°C, 1-2 min or in a heat block at +37°C for 2-4 minutes (no longer than 5 minutes)	
Dade (Siemens®) Thrombin reagent (stored at +15 to +25°C)	0.1 mL	0.1 mL
	Start stopwatch simultaneously with addition of Dade (Siemens®) Thrombin Reagent	

Table 2
FIBRINOGEN DETERMINATION PROTOCOL

Table 3
DESCRIPTIVE STATISTICS BETWEEN STUDY GROUP (DIABETES AND OPEN ANGLE GLAUCOMA) AND CONTROL GROUP (OPENA ANGLE GLAUCOMA)

Parameter	OAG+diabetes	OAG	P<0.05 Student Fisher test
Mean age (years)	62.69+/-1.8	64.31+/-1.66	nss
Sex ratio (M:F)	1 : 2.9	1 : 1.5	-
Mean VA	0.85+/-0.02	0.81+/-0.02	nss
Sph. Eq.	0.34+/-0.24	0.32+/-0.19	nss
Mean IOP	15.93+/-0.66	17.25+-/0.53	nss
Nr of medications	1.43+/-0.9	1.62+/-1.13	nss
CCT	550.35+/-4.63	540.36+/- 3.30	nss
C/D(v) ratio - clinical	0.75+/-0.12	0.69/0.51+/-0.11	nss
Disc area – clinical	2.042	2.039	nss

Abbreviations: VA – visual acuity, Sph. Eq. – spherical equivalent, IOP – intraocular pressure, CCT – central corneal thickness, C/D – cup disc ratio, nss – not statistically significant

Table 4
VISUAL FIELD PARAMETERS COMPARED BETWEEN GROUPS

	OAG+DM	OAG	p (<0.05)
MD (db)	-4.8+/5.55	-3.15+/3.35	**0.048**
PSD (db)	4.30+/-3.29	3.05+/2.23	**0.029**

Abbreviations: MD – mean deviation,
PSD – pattern standard deviation

Parameter	Normal range	OAG+DM	OAG	p<0.05 (t Student test)
CRP	0.01-0.5 mg/dl	0.50 +/- 0.91	0.20 +/- 0.01	**p=0.000**
ESR/1h	2-10 mm/1h	17.95 +/- 2.28	11.75 +/- 0.95	**p=0.016**
Fibrinogen	180-350 mg/dl	371+/- 8.72	327.62 +/- 0.54	**0.000**
Seric lactate	4.5-19.8 mg/dl	23.97 +/- 1.23	19.37+/- 0.54	**p=0.01**
Glicemia	74-106 mg/ dl	136.85 +/- 7.74	97.41 +/- 1.25	-
HbA1c	4.80-5.90%	6.89 +/- 0.25	-	-
IgG	700-1600 mg%	1650 +/- 82.67	1529 +/- 45.93	**0.032**

Table 5
BIOCHEMICAL PROFILE - MEAN VALUES AND COMPARISONS BETWEEN GROUPS

(12 eyes) in the control. Means for visual field parameters were compared in table 4. Both MD and PSD values showed increased glaucoma damage in the group where diabetes was present.

Morphological changes in our study were assessed by optical coherence tomography (OCT). Parameters like cup-disc-ratio, neural rim area, disc area, ganglion cells complex thickness and retinal nerve fiber layer were compared my means. No statistically significant difference was obtained between groups ($p>0.05$) for any OCT parameter, fact that assured very comparable populations before we started to evaluate the biochemical changes between groups.

Glicemic control, inflammatory status and humoral immune response was checked for each patient, then means of all tested biochemical parameters were compared by t Student test. Results are presented in table 5.

Correcting the analysis after sex and age, the statistical difference remained significant between groups ($p<0.05$). In glaucoma patients the absolute values reached upper borderline of normal range, which was described by previous glaucoma studies as borderline inflammatory statuts or *para-inflammation.* When diabetes counted as a general disease, all biochemical markers raised as mean levels compared to glaucoma matching subjects.

Since at the beginning of the study we determined how many patients had early, respectively moderate perimetric defects we analyzed if this functional glaucoma change can be related to the biochemical profile in our patients. So, comparing all eyes with early perimetric defects (96 eyes) versus eyes with moderate defects (22 eyes), statistical analysis (Levene test for equality of variances) showed that ESRand IgG levels were more increased if the functional damage was more important, (sig.2 tailed =0.02, respectively 0.01).

Correlations between all parameters were calculated with Pearson test. We will provide only the information relevant for the biochemical status in glaucoma or diabetic glaucoma patients.

In the glaucoma group, CRP was positively correlated with the level of serum lactate ($r=0.288$, $p=0.01$).ESR correlated with fibrinogen mean level ($r=0.303$, $p=0.01$) and IgG level ($r=0.236$, $p=0.04$). From all inflammatory markers, for fibrinogen we detected most correlations with functional changes in glaucoma patients. Therefore, we detected significant correlations with visual acuity ($r=0.295$, $p=0.01$), mean MD ($r=-0.337$, $p=0.006$), PSD ($r=0.286$, $p=0.02$) and ESR ($r=0.303$, $p=0.01$). Even though we analyzed a group of patients without diabetes, in control eyes we established a correlation between plasma glucose level and intraocular pressure (IOP). Immunoglobulines (IgG) were only correlated with visual acuity level, in a negative direction ($r=-0.283$, $p=0.02$).

In the study group no correlation was found for the CRP level with any parameter. ESR was correlated with IOP ($r=0.479$, $p=0.002$), with specific glaucoma damage of the visual field –PSD ($r=0.411$, $p=0.008$) and fibrinogen mean level ($r=0.468$, $p=0.002$). Neural rim area correlated in these patients with fibrinogen level ($r=-0.408$, $p=0.009$). In diabetic glaucoma patients plasma glucose was also correlated with neural rim area ($r=-0.372$, $p=0.018$), with HbA1c ($r=0.663$, $p=0.000$) and serum lactate ($r=0.417$, $p=0.006$).HbA1c was supplementary found correlated with functional perimetric global defect – MD ($r=-0.360$, $p=0.023$), OCT neural rim area ($r=-0.421$, $p=0.007$) and IgG ($r=0.316$, $p=0.042$). IgG was the only biochemical parameter that was correlated with a visual field specific defect (PSD, for $r=0.380$, $p=0.016$) in this group.

Vascular perfusion in the optic nerve head is impaired both in glaucoma and diabetes based on endothelial dysfunction [24, 25]. In this case, if blood flow is reduced ischemia, consecutive metabolic and biochemical changes occur. According to Flammer [27] an ocular perfusion pressure below 50 mmHg is considered dangerous for the optic nerve. Therefore we calculated the OPP and correlated this parameter with all the biochemical measurements in the two groups. Systolic blood pressure, diastolic blood pressure and OPP had no statistical differences between groups (table 6), yet in both cases OPP was lower than the afore mentioned limit (50 mmHg) creating the metabolic premises for ischemic biochemical changes in both groups (e.g. increased serum lactate). In glaucoma cases systolic blood pressure was correlated positively with CRP levels ($r=0.288$, $p=0.016$) and with fibrinogen ($r=0.241$, $p=0.045$). Diastolic blood pressure was negatively correlated with lactate level ($r=-0.244$, $p=0.039$). All these biochemical correlations with vascular parameters increase the likelihood that glaucoma lesions are connected to the inflammation response, degree of hypoxia and systemic risk factors such as high blood pressure. In the study group, our results that vascular changes (systolic and diastolic blood pressure) arenegatively correlated at significant levels ($r=-0.399$, $p=0.028$ and $r=-0.310$, $p=0.046$) with the metabolic diabetes control (HbA1c). Same trend was found for systolic pressure and plasma glucose ($r=-0.316$, $p=0.042$) or serum lactate ($r=-0.305$, $p=0.044$), suggesting that low systemic perfusion affects the blood supply in the optic nerve head and generates homeostatic biochemical changes. Yet no correlation was found in either groups for OPP and biochemical changes.

Glaucoma is a major cause of blindness and the influence of diabetes in open angle glaucoma has been a

Parameter (mean)	OAG group	OAG+diabetes group	p<0.05(T student test)
Systolic blood pressure (mmHg)	149 .02 +/- 20.12	135.81 +/- 13.31	p = 0.48
Diastolic blood pressure (mmHg)	72.10 +/- 15.25	72.09 +/- 8.67	p = 0.12
OPP (mmHg)	44.27 +/- 9.02	45.46 +/- 8.47	p=0.09

Table 6
PERFUSION PARAMETERS IN GLAUCOMA *vs.* DIABETES+GLAUCOMA GROUP

subject for debate from pathogenic point of view. Studies report that diabetic eyes are at higher risk of injury from exterrnal stressors, such as elevated IOP [27]. Alternatively, diabetes may cause ganglion cell loss, which becomes additive to glaucoma neural damage.Abnormal vascular regulation, inflammation and aberrant immunity lead in both cases to neurodegeneration [28, 29].Up to now many studies validated this *connecting* glaucoma-diabetes theory based on complicated biochemical analysis [1]. Our study intended to prove that using ordinary inflammatory markers one can individualize a different biochemical profile in glaucoma vs diabetic glaucoma patients and establish relevant differences.

Based on statistical results, we showed that biochemical profile is modified in open angle glaucoma patients. If diabetes is over imposed these changes become more prominent. Yet, authors admit that the study group contains in more than 60% a special category of glaucoma – normal tension glaucoma, which is particular vulnerable to systemic changes compared to hypertensive forms of glaucoma (POAG) [26]. Since majority of our patients were hypertensives who received lowering blood pressure medication, a connection between low blood pressure and low OPP might be mentioned [30]As such, any attempt to lower the systemic blood pressure influences in a higher degree the perfusion in the optic nerve head and changes the biochemical profile. In our study we could not find statistically significant differences between OPP in the two groups, but both mean values entered the *dangerous* ischemic zone.

Acute phase reactants (CRP and ESR) were increased in glaucoma group, pleading for a certain low grade inflammation [11, 22, 23]. Endothelial dysfunction due to hypoxia lead to increased lactate levels in both groups. In the study group structural changes in OCT exam (neural rim area) were correlated with biochemical changes in the glycemic status. Some inflammatory markers correlate with IOP level as some other studies prove similar findings based on connections between the choroid and systemic vascular bed.

Authors acknowledge that larger studies are needed and a confirmation by more specific tests could fully validate out working hypothesis.

In another paper were studied the biomarkers of inflamation in patients with type 2 diabets mellitus and hepatic steatosis [31].

Conclusions

Our study proves by comparison that changes in the biochemical profile are relevant in glaucoma vs diabetic glaucoma patients. As expected the biochemical parameters are influenced by diabetes in terms of increased inflammation, immune response and active phase reactants, but also similar changes were detected in glaucoma patients. Based on this result authors could explain common pathogenic theories in glaucoma and diabetes using simple and cost effective biochemical methods.

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