Nat. Volatiles & Essent. Oils, 2021; 8(6): 2232-2244

Estimated Some Immunological Aspects In Iraqi Patients With Primary Open Angle Glaucoma

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Abstract

Glaucoma can be defined as a group of optic neuropathies marked by a gradual degradation of retinal ganglion cells as a result of certain immunological factors. The objective of this study is the evaluation the role of some cytokines (TGF-β1, TGF-β2, and TNFα), CD4+, CD8+ markers and FoxP3 in pathogenesis of Glaucoma. Blood samples from patients with Primary Open Angle Glaucoma (POAG) were taken at Baghdad's Ibn Al-Haitham Teaching Eye Hospital. Also, the samples have been taken from 75 unrelated Iraqi POAG patients (49 males and 26 females) and 75 unrelated healthy people (50 males and 25 females) within the age of 40 –70 years through the period from April to September 2021. For detecting CD8, CD4, D4/CD8 ratio, FOXP3, TNF-α, TGF-β2, TGF-β1, ELISA and flowcytometry approaches are utilized. The results show the age groups of 50-59 and 60-69 years scored highest percentage in patients than controls ($p<0.05$)**.** There are low levels of CD4, FoxP3 and Ratio and high levels of CD8, TGF-β1, TNF-α, and TGF-β2 in patients than controls with significant different ($p<0.05$). The TGF-β1, TNFα, and TGF-β2 were scored highest sensitivity (96%, 97% and 92%) respectively than others parameters with significant different ($p<0.05$). Conclusions: Glaucoma is increased with age progression. TNF-α, TGF-β1 and TGF-β2 parameters can be used as predictors biomarkers for Glaucoma diagnosis.

Keywords; Glaucoma, T regulatory, TGF-β1 and TGF-β2, FOXP3, CD4/CD8, TNF-α.

Introduction

Glaucoma can be defined as one of the neurodegenerative conditions affecting the eyes and is linked to increased intraocular pressure (IOP). If left untreated, patients' visual field might be lost over time, and they can eventually lose their sight entirely. It is the world's second biggest blindness cause (Bezuidenhout and Schellack, 2015). Adults and individuals over the age of 60 are particularly susceptible to this condition. Optic nerve deterioration takes place in the Optic Disc (OD) region referred to as Optic Nerve Head (ONH). Glaucoma cannot be cured, and the damage it causes is largely irreparable. The only choices offered to patients are strategies to reduce the disease's course. The effectiveness of all treatments depends on a timely diagnosis of the condition (Abdullah et al., 2021). Pathogenesis, time of onset, etiology, and anatomy of the anterior chamber angle are all used to classify glaucoma (Bowling, 2016).

Glaucoma is expected to impact about 80 million individuals by 2020, and 111.80 million individual by 2040, with a greater number of people living in African and Asia (Tham et al., 2014). Glaucoma is expected to be present in between (3 and 5)% of white population, also between (5 and 7)% of black population in South

Africa (Bezuidenhout and Schellack, 2015). POAG is linked to age, and individuals of African descent have a high risk to develop it compared to non-Africans (Broman and Quigley, 2006). Glaucoma has a significant economic impact because it impairs both the nation's and individual's productivity. Almost all population is ignorant of their glaucoma state, making glaucoma care a problem for eye health providers (Abdullah et al., 2021). PCG-causing variations have been discovered in 10 Arab nations, with the majority of them being found in CYB1P1 gene. Furthermore, genotype-phenotype associations in Arab patients with PCG appear to be unique (Jemmeih et al., 2021).

The eye is known to be an immune-privileged site. In the eyes, the active local immunosuppression is specified via the blood-aqueous and blood-retina barriers, the pigment epithelial cells' distinctive characteristic, and local synthesis of immunosuppressive cytokines and neuro-peptides, among other things (Stein-Streilein, 2008). Inflammation, such as that which takes place in response to pathological or surgical, carries the risk of vision impairment. Because immune responses are such an important part of the repair response, the eye has evolved its own methods for delivering immune responses to injury in avascular areas (Menko and Stepp, 2021).

The retina's activated glial cells in experimental glaucoma models express MHC-II proteins and release pro-inflammatory chemokines and cytokines such as TNF-α (Baudouin et al., 2020). Aqueous TGF-β has a high diagnostic performance in identifying glaucoma sub-types and can be prospective glaucoma biomarkers (Igarashi et al., 2021). The activation regarding Fas-FasL signaling causes immune cells which enter the eye as a response to injury to infection to undergo apoptosis without producing tissue damage or inflammation (Jiang et al., 2020).

The introduction of antigenic material into the eye causes immune deviation or inhibition of T cell-mediated immunity, resulting in peripheral immune tolerance to antigens, an approach known as Anterior Chamber Associated Immune Deviation (Moalem et al., 1999). Glaucoma patients had a lower number of Treg, and their CD4+ T cells had a higher stimulation response, which included enhanced proliferation and pro-inflammatory cytokine secretion. CD3+CD8+ lymphocyte frequency values were shown to be higher in patients with primary open-angle glaucoma (POAG) and normal tension glaucoma (NTG), with CD8+HLA-DR+ lymphocytes being more prominent in NTG. Increased expression regarding the soluble IL2 receptor, a marker of the T cell activation, accompanied this (Jiang et al., 2020). Latest research also suggests that systemic adaptive immune responses play a key role in the glaucoma's pathogenesis. In the glaucoma patient's sera, complex patterns related to retinal proteins as well as autoantibodies against retinal specific antigens were discovered (Beutgen et al., 2019). The purpose of this work is to determine the role of certain cytokines, FoxP3 Treg and CD markers in the glaucoma's pathogenesis.

Materials and methods

Samples collection

The collection of blood samples and practical work for the presented work lasted from April to September 2021. Also, the samples have been taken from a total of 75 unrelated Iraqi patients with POAG (26 females and 49 males) and a total of 75 unrelated healthy individuals (25 females and 50 males) serving as controls in this work. All controls and patients are of the same ethnicity, have no ocular or systemic disease, and are between

the ages of 40 and 70. Patients experiencing POAG have been recruited from Baghdad's Ibn Al-Haitham Teaching Eye Hospital. In addition, the patients clinically diagnosed via the Glaucoma Department's consulting medical team. The following are the diagnostic criteria used for diagnosing POAG: Patients' age, a high IOP of more than 21 mmHg, a high cup-to-disk (c/d) ratio, and an expanded cornea of more than 11 mm. A venous blood sample of 5 milliliters was obtained and divided into 2 parts. The first part (3 ml) has been placed in gel tubes and allowed to sit for 15 mins at room temperature before being centrifuged at 2000 rpm for a period of 15 mins to obtain sera, which were after that stored at a temperature of -20 Celsius till utilized for cytokine (TGF-β1, TNF-α, and TGF-β2) measurement via ELISA method. The second part (1 ml) was placed in EDTA tubes for flow cytometry analysis of CD markers, CD4/CD8 Ratio, and FOXP3treg.

Detection of study parameters

A- Detection of CD markers, FOXP3 Tregs and CD4/CD8 Ratio by flow cytometry technique.

In this study, Immunophenotyping CD4+, CD8+, CD4/CD8 ratio and foxp3+ were investigated by using fully equipped desktop four–color flowcytometry (BD FACS, USA).

1- Procedure

A- Antibody labeling:

One hundred microliters (100µl) of whole blood or isolated leukocytes were mixed thoroughly with 10 µl of conjugated antibodies (BD FACS, USA) in a test tube, then incubated for 15 minutes in the dark at room temperature.

B- Leukocyte's fixation:

From reagent A (BD FACS, USA), 100 µl were mixed and subjected to incubations for 10mins at room temperature in the dark.

C- Erythrocyte Lysis:

From reagent B (BD FACS, USA), 2.5 ml were added and shake gently and subjected to incubations for 20mins at room temperature in the dark. The sample then analyzed by flow cytometer.

- CD8-APC H7(mouse IgG), (catalog no. E-AB-F1110S, Elabscience, USA)
- CD4-FITC (mouse IgG), (catalog no. E-AB-F1231C, Elabscience, USA)
- FOXP3-PE (mouse IgG), (catalog no. IC8970P, R&D, USA
- Flow Cytometry FOXP3 staining buffer (120ml), (R&D, USA)
- Flow cytometry Staining Buffer (50ml), (BD FACS., USA)

B- Detection of TNF-α, TGF-β1 and TGF-β2 by ELISA technique

Serum levels of cytokines (TGF-β1, TNF-α, and TGF-β2) were quantified by sandwich ELISA (Humareader HS, Germany) technique using the commercially available kit (KOMA BIOTECH. Korea). The concentrations of the various cytokines detected were in pg/ml. measure observance at 450 nm (as the manufacturer's protocols).

Statistical analysis

The Chi-square (X2) test was used for comparing percentages in the present work's data. (Mean ± St. Error) was used to characterize numerical data. For comparing two numeric variables, the Student T test is utilized. The F test was also employed to assess the association between study parameters and age groups in this investigation. The test was run using a significance level of no more than (p=0.05). Present data was analyzed using (Graph pad prism v.6 and SPSS v.22) programs.

Results

Demographic characters of POAG patients show that the rate of open angle glaucoma increased with the age of the study sample and the age group of 60-69 is about half of the POAG patients 53.3% (p = 0.001). The males are more affected with POAG as compared with females (65.3%, p=0.008). The family history is a risk factor for POAG (P= 0.001). Myopia and Diabetes are also associated with the development of the disease (64.0%, p= 0.015 and 62.7%, p=0.02, respectively) as well as Blood Group O+ (39.0%, p= 0.001). Therefore, POAG was significantly associated with age, gender, family history, myopia, diabetes, and blood groups but there is no significant association with the history of hypertension (p= 0.21). Table 1 shows the association of these demographic data with the POAG.

Table 1: Demographic characteristics of POAG patients

		Number	Percent	P-value
Age (Years)	40-49	7	9.3%	0.001***
	50-59	18	24.0%	
	60-69	40	53.3%***	
	>69	10	13.3%	
Gender	Males	49	65.3%**	0.008**
	Females	26	34.7%	
Family history	Yes	57	76.0%***	0.001***
	No	18	24.0%	
Myopia	Yes	48	64.0%*	0.015*
	No	27	36.0%	
Diabetes m.	Yes	47	62.7%*	0.02*
	No	28	37.3%	
Hypertension	Yes	43	57.3%	0.21(N.S)
	No	32	42.7%	
Blood groups	A+	19	25%	0.001***
	A-	1	1%	
	B+	16	21%	
	O+	29	39%***	
	O-	3	4%	
	AB+	7	9%	

Chi-square test. * Significant different, **and * Highly significant , N.S = Non significant(P>0.05). POAG: primary open angle glaucoma.**

The results in table 2 show that among age groups of the patients, the age group of 60-69 years scored the highest percentage in patients (53.3%, p=0.001). As compared with the control, the age group of >69 years scored significantly higher (13.3% p=0.02). Based on gender, the males have scored the highest percentage in patients (65.3%, p=0.008) than females (table 2).

Table 2: Age and gender of POAG Patients and Control statistical analysis.

			Groups		Total	P-value
			Patients	Controls		
Age	40-49	N	7	15	22	0.088
		%	9.3%	20.0%	14.7%	
	50-59	N	18	30	48	0.083
		%	24.0%	40.0%	32.0%	
	60-69	N	40	28	68	0.145
		%	53.3%	37.3%	45.3%	
	>69	N	10	2	12	0.02*
		%	13.3%	2.7%	8.0%	
P value			0.001***	0.001***	0.001***	
Gender	Males	N	49	50	99	0.96
		%	65.3%	66.7%	66.0%	
	Females	n	26	25	51	0.93
		%	34.7%	33.3%	34.0%	
P value			0.008**	0.004**	0.001***	

Chi-square test. * Significant different, **and * Highly significant. POAG: primary open-angle glaucoma.**

The males are at a significantly having high risk to acquire POAG than females (p=0.008). In the age group of 50-59 years, the males are significantly having a high risk to acquire POAG as compared with the same age group of females (p=0.018). Female age group 60-69 years are significantly having a high risk to acquire POAG than another age group of females, p=0.001 (Table 3).

Table 3: Male and female age groups of POAG patients

Age	Male		Female		Total		P-value
	N	%	n	%	N	%	
40-49	7	14%	0	0%	7	9%	1.00
50-59	14	29%	4	15%	18	24%	0.018*
60-69	18	37%	22	85%	40	53%	0.572
>70	10	20%	0	0%	10	13%	1.00

Total	49	100%	26	100%	75	100%	0.008**
P-value	0.137		0.001***		0.001***		

Chi-square test. * Significant different, **and * Highly significant. POAG: primary open-angle glaucoma.**

The results of the conducted study show that CD4% of the control group was significantly higher than CD4% of POAG patients (56.86±0.89, p=0.03). While CD8% shows the no-significant difference between patients and control. FoxP3Treg%was is highly significant in control (85.53±1.43, p=0.001) as compared with POAG patients (68.74±3.00). The CD4/DC8 ratio was significantly high in control (1.87±0.08, p=0.02). Table 4 illustrates these results.

Table 4: Some immunological parameters in POAG patients and controls

Groups		N	Mean ±Std. Error Mean	P-value
CD4%	Patients	75	53.83±1.12	0.03*
	Controls	75	56.86±0.89	
CD8%	Patients	75	36.22±1.24	0.06 (N.S)
	Controls	75	32.45±1.03	
FoxP3Treg%	Patients	75	68.74±3.00	0.001***
	Controls	75	85.53±1.43	
CD4/CD8 Ratio	Patients	75	1.61±0.08	0.02*
	Controls	75	1.87 ±0.08	

***Significant difference, ***highly significant as compared to control group(P<0.05), N.S= Nonsignificant (student t-test). POAG: primary open-angle glaucoma.**

The results in table 5 show that the immunological parameters, CD4, CD8, FoxP3Tregs, and CD4/CD8 ratio, of POAG patients in all age groups, are the same, and no significant difference among all these groups in these immunological parameters.

Table 5: Some immunological parameters of POAG patients according to the age groups

Age groups		N	Mean± Std. Error	P-value
CD4%	40-49	7	54.77±3.69	0.56 (N.S)
	50-59	18	53.81±1.64	
	60-69	40	52.74±1.79	
	>69	10	57.60±2.01	
CD8%	40-49	7	35.41±3.51	0.53 (N.S)
	50-59	18	36.94±1.85	
	60-69	40	36.48±1.80	

	>69	10	31.17±4.32	
FoxP3 Tregs%	40-49	7	71.97±9.83	0.77 (N.S)
	50-59	18	69.73±6.13	
	60-69	40	66.14±4.22	
	>69	10	75.12±8.13	
CD4/CD8 Ratio	40-49	7	1.76±0.29	0.61 (N.S)
	50-59	18	1.44±0.11	
	60-69	40	1.62±0.13	
	>69	10	1.78±0.20	

F test. N.S= Non significant different (P>0.05). POAG: primary open angle glaucoma.

The CD4% in female POAG patients was significantly higher than in males (57.20±1.82, p=0.02). On the other hand, there is no significant association between males and females in the rest immunological parameters, CD8%, FoxP3Tregs%, and CD4/CD8 ratio (table 6).

Table 6: Some immunological parameters of POAG patients according to the gender

Gender		N	Mean± Std. Error	P-value
CD4%	Males	49	52.05±1.36	0.02*
	Females	26	57.20±1.82	
CD8%	Males	49	36.40±1.58	0.48(N.S)
	Females	26	34.62±1.99	
FoxP3Tregs %	Males	49	68.61±3.79	0.95(N.S)
	Females	26	68.98±4.99	
CD4/CD8 Ratio	Males	49	1.51±0.09	0.14(N.S)
	Females	26	1.80±0.17	

Student t test *Significant different (P<0.05) , N.S = Non significant (P> 0.05). POAG: primary open angle glaucoma.

The three cytokines of this study, TNF-α, TGF-β1, and TGF-β2, are measured in the serum of POAG patients and control. The results are illustrated in table 7 and all three cytokines are significantly higher in POAG patients as compared with control (132.85±5.18, 367.32±19.54 and 313.32±15.28, p=0.001) respectively

Table 7: The level of TNF-α, TGF-β1, and TGF-β2 in POAG patients and control.

Groups		N	Mean± Std. Error (Pg/ml)	P-value
TNF-α	Patients	75	132.85±5.18	0.001***
	Controls	75	71.52±2.07	
TGF-β1	Patients	75	367.32±19.54	0.001***
	Controls	75	147.45±4.82	
TGF-β2	Patients	75	313.32±15.28	0.001***
	Controls	75	150.28±5.22	

***** Highly significant difference as compared to control (student t-test). POAG: primary open angle glaucoma.**

The results in table 8 show that the TNF-α, TGF-β1, and TGF-β2 of POAG patients in all age groups are the same, and no significant difference among all age groups in these cytokines.

Table 8: The level of TNF-α, TGF-β1, and TGF-β2 in POAG patients and control according to the age groups.

Age groups		N	Mean ± Std. Error (pg/ml)	P-value
TNF-α	40-49	7	108.72±18.78	0.33(N.S)
	50-59	18	145.13±11.67	
	60-69	40	131.97±6.73	
	>69	10	131.19±12.79	
TGF-β1	40-49	7	353.23±26.91	0.09(N.S)
	50-59	18	374.79±34.01	
	60-69	40	336.54±26.10	
	>69	10	486.89±73.58	
TGF-β2	40-49	7	242.98±27.16	0.43(N.S)
	50-59	18	309.93±27.65	
	60-69	40	317.91±22.12	
	>69	10	350.30±49.19	

N.S = Non-significant difference (P>0.05) by using the F test. POAG: primary open angle glaucoma.

Similarly, there was no significant difference in the concentrations of TNF-α, TGF-β1, and TGF-β2 between male and female POAG patients (Table 9), which means there is no correlation between gender and cytokines levels of POAG patients.

Table 9: The level of TNF-α, TGF-β1, and TGF-β2 in POAG patients and control according to gender.

Gender		N	Mean± Std. Error (pg/ml)	P-value
TNF-α	Males	49	128.97±5.86	0.34(N.S)
	Females	26	140.17±10.06	
TGF-β1	Males	49	352.35±20.81	0.32(N.S)
	Females	26	395.54±40.52	
TGF-β2	Males	49	315.41±20.37	0.84(N.S)
	Females	26	309.37±22.17	

N.S = Non-significant difference (P>0.05) by using student t-test. POAG: primary open angle glaucoma

Discussion

The occurrence of Glaucoma is higher in individuals over 60 years old in the presented work. The high age-specific prevalence related to glaucoma in old adults indicates that the glaucoma's severity starts at an earlier age, which might be due to a lack of treatment and early diagnosis. (Kelly et al., 2020). Male gender is determined to be one of the significant risk factors in the presented work, which is consistent with prior results (Zhang et al., 2021). The actual reasons are unknown. Males have been found to have a deeper anterior chamber depth (ACD) and a longer axial length in multiple investigations (Hashemi et al., 2012). Anatomical differences can be one of the causes for the gender disparity in patients experiencing POAG.

A family history of glaucoma is generally accepted as a risk factor for the disease. A history of glaucoma in the family is widely recognized as a risk factor for the condition. POAG is more common in some families than others, which could be due to environmental or genetic causes, or a combination of both. It is obvious that genetic factors have a role in at least some of the PAOG's inheritance (Cook and Foster, 2012).

Myopia was a significant risk factor for Glaucoma pathogenicity and many related studies suggest the correlation of Myopia with POAG (Nitta et al., 2017). Myopia affected the distribution of the Retinal nerve fiber layer (RNFL) thicknesses around the optic disc, mean, inferior, and superior quadrants of the RNFL are thinner than that of the normal control eyes (Fang et al., 2018). Diabetes history was found to have a substantial link with POAG in the presented work. Many suggestions were offered about the biological relation between glaucoma and diabetes. Diabetes might disrupt vascular autoregulation, raise the risk of neuronal damage, and perhaps compromise the aqueous humor outflow system, resulting in POAG (Chen and Zhao, 2017).

The recent study has indicated that there is no association between hypertension and POAG and these findings may be due to the prevalence of glaucoma in males than females in our survey and the prevalence of hypertension is higher in men than women over age 45 until menopause (Santosa et al., 2020), when woman ovary stops producing the hormones estrogen and progesterone. Increased androgen levels have

been shown to increase blood pressure in women with polycystic ovary syndrome and in animal models, the loss of estrogens at menopause, (Reckelhoff, 2001).

Also, primary open angle glaucoma in this study was found to be common in blood group/Rh O+ve and less common in A-ve with the absence of blood group/Rh B-ve and AB-ve. Blood group O in Iraqi people was more prevalent than the others (Hattem et al.,2009, Jaff, 2010). Blood groups are a genetic marker in glaucoma (Brooks et al.,1988)

A study of blood samples from patient groups with glaucoma has detected some abnormalities in T-cell subsets (Guo et al., 2018). In this study, the increase in CD8+ T cells could be explained via the diminished apoptosis of CD8+ which are less vulnerable to (FasL) mediated apoptosis in POAG (Fahmy et al., 2010). The drop in CD4+Tcells observed in our results suggests that the glaucoma-activated immunity might not be adequately counter-balanced via immune suppression . More research is needed to see if shifting T-cell homeostasis contributes to glaucoma neurodegeneration and/or if T-cell subset imbalance can be used as a bio-marker for autoimmune vulnerability (Yang et al., 2019).

A low CD4/CD8 ratio reflects β-cell destruction and may predict diabetes diagnoses in first-degree relatives of type 1 diabetic probands. In a population study of solid neoplasms, an inverted CD4/CD8 ratio is associated with metastatic disease as compared with cancer patients without metastasis (Alegria et al., 2017).

The CD4+Foxp3+ regulatory T (Treg) cells have an important role in the maintenance of immune tolerance and the preventing autoimmunity. Deficiencies in Treg cell development or function lead to uncontrolled immune responses accordingly and can lead to inflammatory disorders and autoimmunity. Manipulating Treg cells is a strategy to treat autoimmune diseases (Dominguez-Villar and Hafler, 2018).

There were no noticeable differences among the CD4+ T cells, CD8+ T cells, FoxP3 Tregs, and CD4+/CD8+ ratio by age groups, and may that due to approximate ages between study groups. The percentage of CD8+ T cells, FoxP3 Tregs, and CD4/CD8 ratio were similar between males and females group but the percentage of CD4+ T cells was high in females than males. Sex differences in immune responses are affected by hormonal, genetic and environmental factors (Klein et al.,2016).

The results of this study show high levels of TNF-α in POAG patients than in the control group. Tumor necrosis factor-α (TNF-α) plays an important role in neuroprotection and neurodegeneration, there is a balance between the two actions (Agarwal and Agarwal, 2012). Exposure to risk factors might initiate a shift in balance to favor the progression of the neurodegenerative process. Therefore, cytokines, such as TNF-α, play a critical role in the pathogenesis of POAG primarily through induction of apoptosis in retinal ganglion cells and therefore optic nerve degeneration (Simionescu et al., 2015).

Moreover, The levels of TGF-β2 and TGF-β1 were found to be considerably greater in patients compared to controls. Excess ECM was suggested to cause an increase in aqueous outflow resistance in trabecular meshwork TM related to glaucomatous eyes, and TGF-β1 cytokine affects extracellular matrix ECM metabolism. (Igarashi et al., 2021). POAG eyes have higher amounts of TGF-β2, a potent fibrotic agent, in comparison with normal eyes, but SOAG eyes have lower levels of aqueous TGF-β2 (Inatani et al., 2001).

According to recent results when comparing cytokines parameters (TNF-α, TGF-β1, and TGF-β2) with age and gender, there is no correlation between gender, age, and cytokines levels of POAG patients, and that may be due to factors inciuding sex differences in immune responses changing throughout life and being influenced by age, , sex hormones, reproductive status and environmental factors.

In conclusion**,** glaucoma is raised with age progression and it is a high incidence in males than females. Low levels of CD4, FoxP3treg, and CD4/CD8 ratio and high levels of CD8, TNF-α, TGF-β1, and TGF-β2 in patients than controls with significant differences. TNF-α, TGF-β1, and TGF-β2 parameters can be used as predictors biomarkers for glaucoma diagnosis.

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