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ROLE OF SERUM NEURON SPECIFIC ENOLASE IN DIAGNOSIS TYPE 2 DIABETIC PATIENTS WITH RETINOPATHY

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Abstract— In this study the aim of the work is to investigate the relation of neuron-specific enolase (NSE) and high sensitive C-reactive protein (Hs-CRP) in type2 diabetic patients with retinopathy if we use the funds exam to classify diabetic patients with retinopathy to non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) or if they are used as one group,80 subjects divided to healthy controls (n=20), Type2 diabetic patients without retinopathy (n=30) and type2 diabetic patients with retinopathy (n=30) divided to non- proliferative diabetic retinopathy (n=15) and proliferative diabetic retinopathy (n=15). In this cross-sectional study, diabetic retinopathy status was assessed by fundus examination. When we compared the levels of NSE or hs-CRP levels between the studied groups showed statistically highly significant elevation ($P < 0.0001$ separately) and when combined the NPDR and PDR as one group showed also highly significant elevation ($P < 0.0001$), ROC curve used for determination the AUC at the best cut off. So, NSE and hs-CRP can be used as potential biomarkers for the detection of type2 diabetic patients with retinopathy.

Keywords— Non-proliferative**,** Proliferative**,** Fundus examination**,** Retinopathy**,** Diabetes mellitus**.**

I. INTRODUCTION

Diabetes mellitus is rightly recognized as a global public health concern and is characterized by chronic hyperglycemia due to abnormal insulin secretion or insulin receptor or post-receptor events affecting metabolism of carbohydrate, protein, and fats. Chronic hyperglycemia is approximate cause of several diabetic complication such as nephropathy, retinopathy, cardiovascular disease, and peripheral neuropathy. Morever, chronic hyperglycemia evokes oxidation stress [1]**.** A large number of individuals with type2 diabetes (T2D) will be asymptomatic and thus unaware of their disease for an extended period of time before diabetes is clinically detected. As such, complications may be present at the time of diagnosis [2].Therefore, in persons with T2D, an initial dilated and comprehensive eye examination by an ophthalmologist or optometrist should be performed shortly after initial diagnosis. Subsequent examinations should be repeated annually by an optometrist. Less-frequent exams (i.e., every 2–3 years) may be considered following one or more normal eye exams [3]. Diabetic retinopathy (DR) affects 4.2 million Americans over the age of 40 years, 655,000 of whom have sight-threatening retinopathy [4]. Diabetic retinopathy is a microvascular complication of diabetes and is the leading cause of new cases of legal blindness in the United States [5].Development of diabetic retinopathy in patients with type 2 diabetes was found to be related to both severity of hyperglycemia and presence of hypertension in the U.K. Prospective Diabetes Study (UKPDS), and most patients with type 1 diabetes develop evidence of retinopathy within 20 years of diagnosis. Retinopathy may begin to develop as early as 7 years before the diagnosis of diabetes in patients with type 2 diabetes [6]. Worldwide, in 2010 it was estimated that DR affected 93 million persons, and 28 million were affected by vision-threatening diabetic retinopathy (VTDR). Diabetic retinopathy may become the leading cause of visual impairment globally [7]. It is a progressive disease associated with a decline in best-corrected visual acuity. The Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) noted that 3.6 % of type 1 diabetes mellitus (DM) and 1.6 % of type 2 DM patients were legally blind [8]. The role of screening eye evaluation in the early detection of DR occurrence and progression has been clearly documented [9]. Nevertheless nowadays almost 50 % of people with diabetes did not perform routinely dilated fundus examination and still 50 % of diabetics went blind without any treatment [10].The recommendations of the American Academy of Ophthalmology (AAO), the American Diabetes Association (ADA), and the American Optometric Association (AOA) suggested an annual dilated fundus examination for all diabetic patients by an eye-care professional. Fundus photography has been routinely performed since 1976 in

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multicenter clinical trials to record the occurrence and the progression of the DR and to classify the different degrees of the disease using a standardized method [11]. A higher inflammatory status, expressed by circulating levels of high sensitivity C-reactive protein (hs-CRP), is associated with diabetes [12]. Enolase (2-phospho-D-glycerate hydrolase) is a "metal-activated metalloenzyme" that catalyzes the dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate in the glycolytic pathway to generates ATP, Although α- enolase plays a main role in glycolysis additional cellular functions of this enzyme have been discovered [13].

II. Material and Method

The research protocol was approved by the Research Ethics Committee of the Departments of Chemistry, Cairo University, and National Institute of Diabetes and Endocinology. All procedures Performed were in accordance with recommendation of the Declaration of Helsinki, 1964 and its later amendments or comparable ethical standards. Informed written consents were obtained from all participants prior to enrollment in the study.

Our study cases divided to healthy controls (n=20), type 2 diabetic patients without retinopathy (n=30) and type 2 diabetic patients with retinopathy (n=30) was classified to non-proliferative diabetic retinopathy (n=15) and proliferative diabetic retinopathy (n=15) by using the fundus photograph made by Ophthalmologists. Right arm blood pressure was measured .In addition, the BMI was calculated as : BMI=body weight (Kg)/height $(m)^2$. Other variables (e.g. age ,drug use, duration of the diabetes) were collected by interviewer questionnaire.

A. Blood neuron specific enolase and other variables

Fasting blood samples were measured for NSE and hs-CRP concentration using an electrochemiluminescence immunoassay automatic analyzer (Stat fax,USA) according to the manufacturer's instructions. Hemolysis was a voided as far as possible during the procedure. Other blood variables such as FBG, GOT, GPT, Urea, Creat, Cholesterol, Triglyceride, HDL, LDL were measured using an automatic analyzer the Dimension®RxL Max® Integrated Chemistry System (DADE BEHRING instruments Inc, USA).HbA1c was measured using. *BIO-RAD® D-10* Hemoglobin testing system (United States, BIO-RAD laboratories, Inc., Hercules, CA94547 France, Bio-Rad, Mames-la-Coquette); Used for determination of HbA1c.(values of HbA1c are first reported as mmol/mol,,followed by %).

III. Statistical analysis

All statistical analyses were done by a statistical software package "SPSS 16.0 for Microsoft Windows, SPSS Inc.) and considered statistically significant at a two-sided $P < 0.05$. Numerical data were expressed as mean ± SD. Qualitative data were expressed as frequency (number=n) and proportion (%). Comparisons of quantitative data between multiple groups were done using the one way ANOVA for data with normal distribution and post hoc LSD test was used for comparison between each two groups. The chi-square (x^2) tests were used for qualitative variables. The correlation was evaluated by Pearson correlation coefficient. Multiple regression analysis was used to calculate the scores of combination markers.

IV. Result

We collected 80 subjects divided to healthy subjects (n=20) and type 2 diabetic patients without retinopathy (n=30) and type 2 diabetic patients with retinopathy (n=30),diabetic retinopathy is identified by high level of FBG , HbA1c and by the using of fundus examination is made by Ophthalmologists to classified diabetic retinopathy to non-proliferative diabetic retinopathy (n=15) and proliferative diabetic retinopathy (n=15) **(table 1)**and study show that no significant in some lab investigation as show in **(table 2)**.

ELISA work is done to determine serum NSE and hs-CRP levels **(Fig. 1)**and **(table 3)** showed highly significant value if we use the fundus photograph to classify the diabetic cases to NPDR and PDR or when are used as one group. **(Fig. 2)**From person correlation coefficient there was a positively highly significant correlation between the two biomarkers NSE and hs-CRP**.**

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	Control		T2DM			NPDR			PDR	
	Mean±SE	P^{CT}	Mean±SE	P^{CN}	P^{TN}	Mean±SE	P^{CP}	P^{TP}	Mean±SE	P^{NP}
Age (Year)	43.9±1.54	n.s	43.43±1.29	h.s	h.s	52.6±1.87	h.s	h.s	52.73±1.23	n.s
BMI (Kg/m2)	26.74±0.74	s	29.96±0.91	n.s	n.s	28.67±0.96	s	n.s	29.83±1.33	n.s
SBP (mmHg)	120±2.29	n.s	115.5±1.65	h.s	h.s	131.33±2.73	h.s	h.s	134±3.87	n.s
DBP (mmHg)	76±2.22	n.s	75.47±1.62	s	s	82.67±2.28	h.s	h.s	84.67±2.73	n.s
FBG (mg/dl)	89.8±2.37	h.s	212.23±14.41	h.s	n.s	203.93±15.70	h.s	n.s	223.6±29.88	n.s
HbA1c (%)	5.7±0.07	h.s	8.92±0.38	h.s	n.s	9.94±0.38	h.s	n.s	9.93±0.65	n.s
Urea (mg/dl)	29.15±1.77	s	24.4±1.24	n.s	s	30.13±2.27	n.s	h.s	32.87±2.45	n.s
Creatinine (mg/dl)	1.08±0.02	h.s	0.91±0.03	n.s	s	1.06±0.06	n.s	n.s	1.01±0.05	n.s
ALT (U/L)	22.3±1.59	h.s	32.3±3.18	n.s	n.s	25.6±2.01	n.s	n.s	28.87±2.39	n.s

Table 1:Show P-values of normal lab investigation (PCT) P-value of Control vs. T2DM,(PCP) P-value of Control vs. PDR , (PCN)P-value ofControl vs. NPDR , (PTP)P-value ofT2DM vs. PDR , (PTN) P-value of T2DM vs. NPDR , (PNP)P-value of NPDR vs. PDR.

	Control	T2DM	NPDR	PDR	
	Mean±SE	Mean±SE	Mean±SE	Mean±SE	P
Cholesterol (mg/dl)	193.8±6.62	200.17±6.14	220.87±11.55	219.47±15.44	n.s
Triglyceride (mg/dl)	112.4±11.18	141.27±15.41	151.33±18.46	129.73±16.18	n.s
HDL (mg/dl)	39.5±1.93	38.4±1.61	40.6±2.59	40.47±2.75	n.s
LDL (mg/dl)	128.8±5.65	130.5±4.48	143.13±11.76	136.93±8.86	n.s
AST (U/L)	24.3±1.72	29.1±3.08	26.2±2.61	29.2±2.22	n.s

Table 2: Show non-significant P-values of normal lab investigation.

	Control		T2DM			NPDR			PDR		PDR+NPDR		
	Mean±SE	P^{C}_{T}	Mean±SE	P^{CN}	P^{T}_{N}	Mean±SE	P^{C}_{P}	P^{TP}	Mean±SE	P^{N}_{P}	Mean±SE	$P^{C(P+N)}$	$P^{T(P+N)}$
NSE ng/ml	6.94±0.42	h.s	16.19±0.78	h.s	h.s	24.62±1.39	h.s	h.s	20.4±1.15	h.s	22.51±0.97	h.s	h.s
Hs-CRP mg/l	1.38±0.23	h.s	6.36±0.23	h.s	h.s	4.53±0.45	h.s	h.s	9.96±0.68	h.s	7.24±0.64	h.s	h.s

Table 3: Show highly significant values of NSE and hs-CRP levels.

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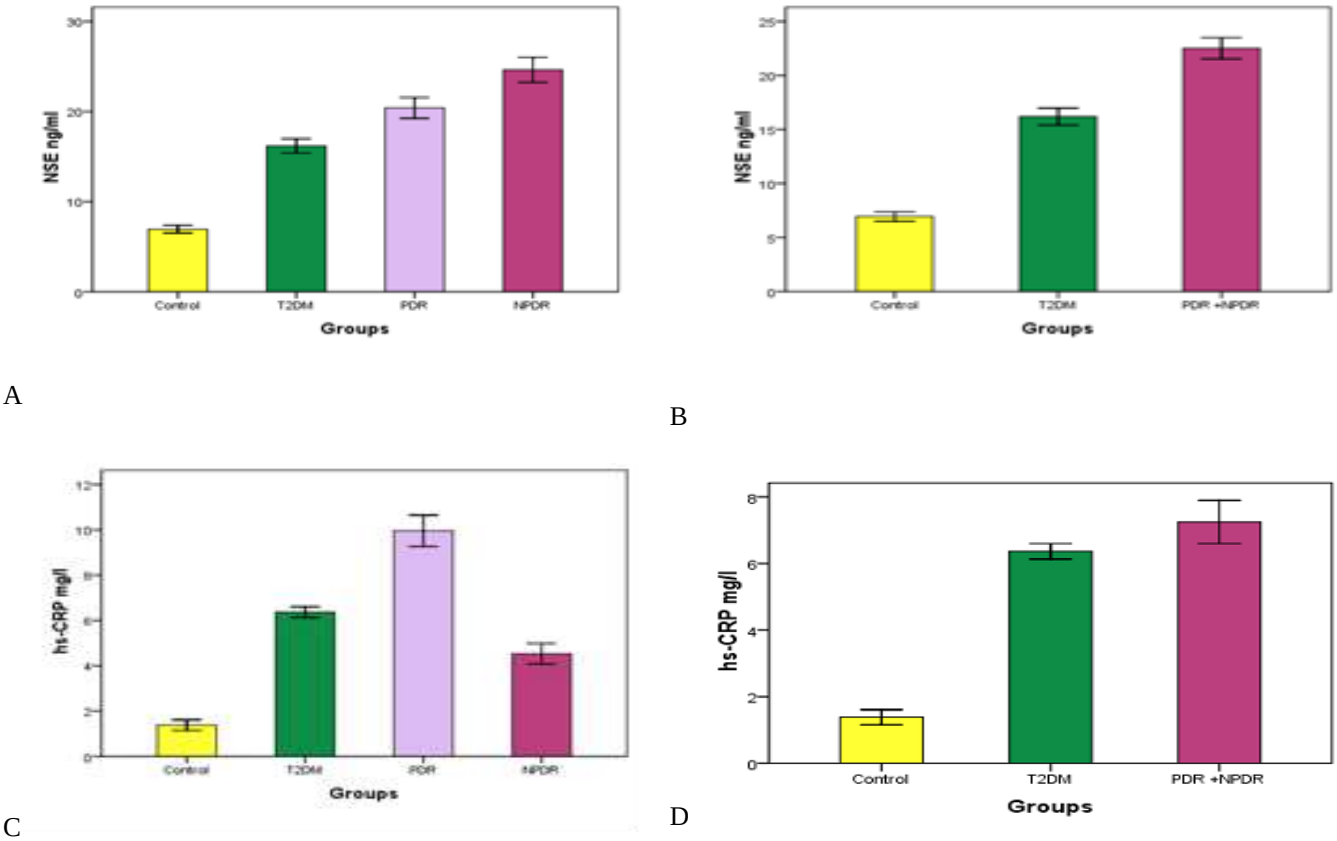

Fig. 1 : Show the mean ± SE of NSE if classify the diabetic retinopathy cases to NPDR and PDR by fundus photograph (A) or when are used as one group (B) and the mean ± SE of hs-CRP if classify the diabetic retinopathy cases to NPDR and PDR by fundus photograph (C) or when are used as one group (D).

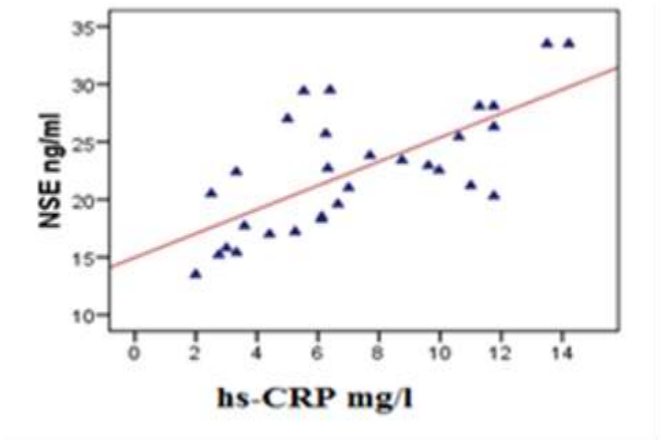

Fig. 2 : Show the positively highly significant correlation between NSE and Hs-CRP.

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Comparisons of AUCs for ROC curves of hs-CRP and NSE indifferent studied groups

In control vs. T2DM groups: the AUCs of hs-CRP and NSE were (1; 0.988 respectively). The pairwise comparisons of these AUCs unveiled no statistically significant difference in (Hs-CRP vs. NSE) (P =0.343)**.** In control vs. PDR groups: the AUCs of hs-CRP and NSE were (1; 1 respectively). The pairwise comparisons of these AUCs unveiled no statistically significant difference in (Hs-CRP vs. NSE) (P = 1).In control vs. NPDR groups: the AUCs of hs-CRP and NSE were (0.947; 1 respectively). The pairwise comparisons of these AUCs unveiled no statistically significant difference in (Hs-CRP vs. NSE) (P =0.139).In T2DM vs. PDR groups: the AUCs of hs-CRP and NSE were (0.871; 0.739 respectively). The pairwise comparisons of these AUCs unveiled no statistically significant difference in (Hs-CRP vs. NSE) (P =0.212).In T2DM vs. NPDR groups: the AUCs of hs-CRP and NSE were (0.780; 0.888 respectively). The pairwise comparisons of these AUCs unveiled no statistically significant difference in (Hs-CRP vs. NSE) (P =0.208).In PDR vs. NPDR groups: the AUCs of hs-CRP and NSE were (0.956; 0.731 respectively). The pairwise comparisons of these AUCs unveiled statistically significant difference in (Hs-CRP vs. NSE) (P =0.016) **(Fig 3)**
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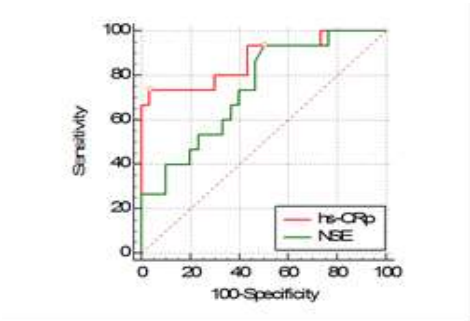
A.



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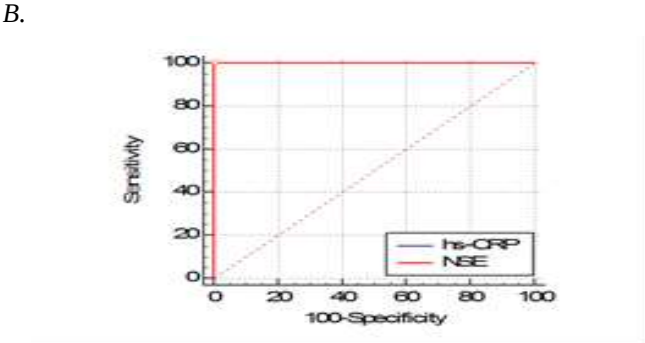

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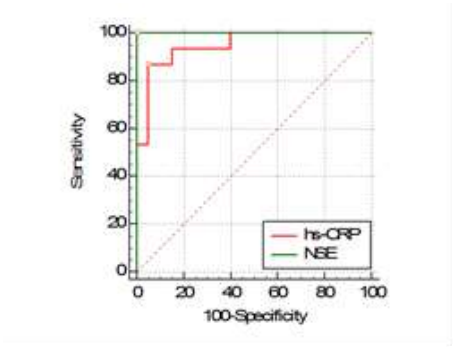

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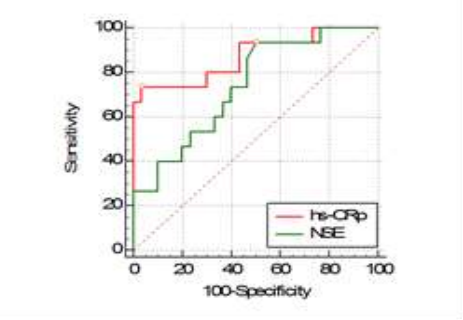

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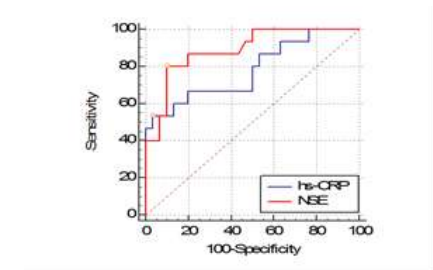

F.

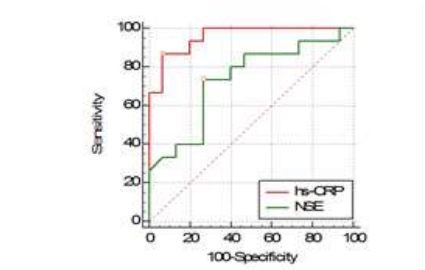

Fig. 3 : Show the pairwise comparisons of AUCs for ROC curves of hs-CRP and NSE in the studied groups,(A) Comparisons of AUCs for ROC curves of hs-CRP and NSE in control and T2DM groups,(B) Comparisons of AUCs for ROC curves of hs-CRP and NSE in control and PDR,(C) Comparisons of AUCs for ROC curves of hs-CRP and NSE in control and NPDR,(D)Comparisons of AUCs for ROC curves of hs-CRP and NSE in T2DM and PDR,(E) Comparisons of AUCs for ROC curves of hs-CRP and NSE in T2DM and

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NPDR,(F) Comparisons of AUCs for ROC curves of hs-CRP and NSE in PDR and NPDR groups

Comparisons of AUCs for ROC curves of hs-CRP and NSE in different studied groups

In control vs. (PDR+NPDR) groups: the AUCs of hs-CRP and NSE were (0.973; 1 respectively). The pairwise comparisons of these AUCs unveiled no statistically significant difference in (Hs-CRP vs. NSE) (P =0.150) . In T2DM vs. (PDR+NPDR) groups: the AUCs of hs-CRP and NSE were (0.546; 0.813 respectively). The pairwise comparisons of these AUCs unveiled statistically significant difference in (Hs-CRP vs. NSE) (P =0.014) **(Fig 4)**.

G.

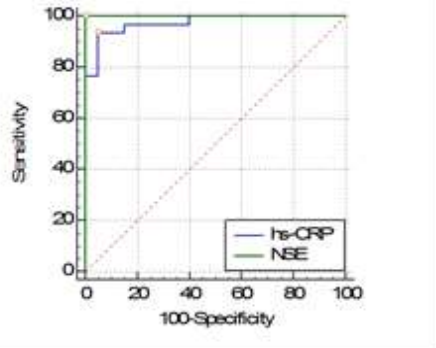

Fig. 4 : : Show the pairwise comparisons of AUCs for ROC curves of hs-CRP and NSE in the studied groups, (G) Comparisons of AUCs for ROC curves of hs-CRP and NSE in control and NPDR+PDR,(H) Comparisons of AUCs for ROC curves of hs-CRP and NSE in T2DM and NPDR+PDR.

V. DISCUSSION

Our study results of FBG and HbA1c showed the highly significant elevation (P < 0.0001 separately) may be due to; HbA1c give an indication of chronic glycaemia rather than being a test of glycaemia at a single point in time. It gives an integrated index of glycaemia over the entire 120-day life span of the red blood cell, but within this period of 120 days, recent glycaemia has the largest influence on the HbA1c value, with 50% of HbA1c formed in the month prior to sampling and 25% in the month before that **[**14]. HbA1c consists of a mixture of hemoglobin molecules that are glycated at different side chain amino positions. Assuming that the reaction rate is proportional to the hemoglobin concentration and that the accessibility of the side chain amino groups of hemoglobin for glucose and the lifetime of the red blood cells are constant, Only the glucose concentration should have an influence on the concentration of HbA1c in terms of percentage **[**15].HbA1c is not affected by prandial status and has no diurnal rhythm ,allowing measurement at any time of day. It is a relatively convenient test, not requiring the patient to fast and only using a single blood sample **[**16].Accordingly, HbA1c would be the perfect proxy for the blood glucose concentration over the average life span of red blood cells. HbA1c may be in the normal range in subjects with diabetes mellitus and among subjects with mild fasting hyperglycemia. Therefore, they claim that HbA1c alone is not a sufficiently reliable tool for recognizing particularly the early stages of diabetes and recommend that plasma glucose determination (FBG) should be used if a history or symptoms indicate a high risk for the presence of diabetes and HbA1cis < 6.5% **[**17]. Our experimental results of FBS and HbA1c levels are on par with studies done by Zelia Maria da silva correa and his coworkers**[18**]&Ishrat Kareem and his coworkers **[19**]Hyperglycemia-induced vascular injury leads to increased glucose flux through the polyol pathway, resulting in cellular damage, thereby resulting in the various microvascular and macrovascular complications **[**20]. HbA1c is also shown to have a special affinity for oxygen, causes tissue anoxia and plays a role in causation of micro and macro angiopathy.

Our study results showed that the level of neuron specific enolase highly significant elevated P < 0.0001 when we classified the diabetic patients with retinopathy (n=30) to non-proliferative diabetic retinopathy (n=15) and proliferative diabetic retinopathy (n=15) by using fundus examination when we compared between control, T2DM, NPDR and PDR groups. The level of neuron specific enolase increase in non-proliferative diabetic retinopathy group than proliferative diabetic retinopathy group, and when we collected the two group of diabetic retinopathy (n=30) and were used as one group showed highly significant elevation also P < 0.0001 when we compared between control, T2DM and NPDR+PDR groups. Slightly elevation of neuron specific enolase in serum of diabetic patients which not detect any central nervous system disordered as retinopathy. Pericyte loss induced by hyperglycemia. Which contributes to blood brain barrier disruption **[**21]and similarly, with in the retina, hyperglycemia induced intramural pericyte death and thickening of the basement membrane lead to in competence of the vascular walls and increased permeability **[**22], making elevate NSE in serum due to leaking it to serum in diabetic retinopathy in contrast to those without. However, the extent of elevation in NSE tended to decrease over the severity of retinopathy **[**23].

High sensitive C-reactive protein is an acute phase protein produce by liver cells in response to various inflammatory stimuli; it is found in the blood **[**24]. High sensitive C-reactive protein is a member of the pentraxin family of oligomeric proteins which is believed to play a fundamental role in natural host defense and innate immunity **[**24].

As a member of the class of acute –phase reactants, plasma hs-CRP level rises dramatically during a cute inflammatory processes **[**25]. Our study results showed that the level of high sensitive C-reactive protein highly significant elevated P < 0.0001 when we classified the patients with retinopathy

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(n=30) to non-proliferative diabetic retinopathy (n=15) and proliferative diabetic retinopathy (n=15) by using fundus examination, when we compared between control ,T2DM ,NPDR and PDR groups, and when we collected the two group of diabetic retinopathy (n=30) and were used as one group showed highly significant elevation also P < 0.0001 when we compared between control, T2DM and NPDR+PDR groups. Studies on western population have shown low grad systemic inflammation to be one of the mechanisms by which known risk factors such as obesity, smoking and hypertension promote the development of diabetes mellitus [26]. High sensitive C-reactive protein is strongly associated with diabetes mellitus and insulin resistance ,elevated in diabetic subjects compared with non-diabetic subjects [27].

Person correlation between serum biomarkers levels and other parameters in patient groups, was used to determine significant association between the parameters, by using the person correlation coefficient we found there is a positive highly significant correlation between the High sensitive C-reactive protein and neuron specific enolase levels (r = 0.692 , P < 0.0001). High sensitive C-reactive protein is involved in endothelial dysfunction and angiogenesis [28] which have been proposed to play an important role in the pathogenesis of DR [29].It is now well accepted that hs-CRP is a strong predictor of future cardiovascular events [30]. However, less is known about its relationship with microvascular complications of diabetes. In this regard, some, though not all, studies (especially prospective studies) have reported that circulating hs-CRP is associated with diabetic DR [31], NSE level increase in patients with diabetic retinopathy than without[32] ,so neuron specific enolase and high sensitive C-reactive protein can be used as biomarkers for diabetic retinopathy.

Receiver operating characteristics (ROC) analysis use to determine the sensitivity and specificity at the best cutoff value and make comparison between the two markers in the four studied groups [33]. High sensitive C-reactive protein Roc curves showed sensitivity and specificity (100,100 respectively) with P-value< 0.0001 and AUC 1 at cutoff >3.6 when compared the control group with T2DM group and sensitivity and specificity (100,100 respectively) with P-value< 0.0001 and AUC 1 at cutoff >3.6 as compared control groups and PDR, but when compared control group with NPDR sensitivity and specificity (86.7, 95 respectively) with P-value <0.0001 and AUC 0.947 at cutoff >2.65. When we compared T2DM with PDR sensitivity and specificity (73.33, 96.67 respectively) with p-value < 0.0001 and AUC 0.871 at cut off > 8.64 ,Compare T2DM with NPDR the sensitivity and specificity (53.33 , 96.67 respectively) with p-value 0.0004 and AUC 0.78 at cut off > 0.219 and when compared PDR with NPDR the sensitivity and specificity (86.7, 93.3 respectively) with p-value < 0.0001 at cut off > 0.158. The hs-CRP level increase in the T2DM according to the **Li et al.,(2004**) study which suggested that hs-CRP increase in DM due to inflammation as **Correale et al., (2008**) said in his study. **Nowak et al., (2010)** reported that hs-CRP have a relation with DR . Neuron specific enolase Roc curves showed sensitivity and specificity (100, 95 respectively) with p-value < 0.0001 and AUC 0.988 at cutoff > 9.59 when compared control group with T2DM group, sensitivity and specificity (100,100 respectively) with p-value < 0.0001 and AUC 1 at cutoff >11.67 when compared control group with PDR, and when compared control group with NPDR sensitivity and specificity (100,100 respectively) with P-value < 0.0001 and AUC 1 at cut off >11.67. When compared T2DM with PDR the sensitivity and specificity (93.3,50 respectively) with p-value 0.0019 and AUC 0.739 at cutoff > 14.9 , as compared T2DM with NPDR the sensitivity and specificity (80,90 respectively) with p-value < 0.0001 and AUC 0.888 at cutoff > 20.91 ,and when compared PDR with NPDR the sensitivity and specificity (73.33,73.33 respectively) with P-value 0.0155 and AUC 0.731 at cutoff > 22.4.To differ from the old studies we did comparison of AUCs for ROC curves of hs-CRP and NSE in different studies groups. When we compared the control vs. T2DM we found that AUC of hs-CRP more than AUC of NSE, the AUCs of hs-CRP and NSE were (1;0.988 respectively), and when we compared control with NPDR AUC of NSE more than hs-CRP, the AUCs of hs-CRP and NSE were (0.947;1 respectively), but when we compared between control vs. PDR we found AUC of NSE equal AUC of hs-CRP, the AUCs of hs-CRP and NSE were (1;1 respectively).

When we collected NPDR and PDR as one group we compared it with control group ,we found that AUC of NSE more than AUC of hs-CRP, the AUCs of hs-CRP and NSE were (0.973;1 respectively), and when it compared with T2DM, we found statistically significant difference AUC of NSE more than hs-CRP, that due to the increase in the NSE level in diabetic patients than healthy and in diabetic retinopathy than without retinopathy However, the extent of elevations in NSE tended to decrease over the severity of retinopathy and macular edema. Initially, the glycolytic enzymes are compensatively up regulated to increase survival of the neurons [34], because even early diabetic retinopathy involves a retinal neurodegenerative change [35].

VI. CONCLUSION

From the present study, it concluded that NSE and hs-CRP are the most important powerful new tools for diagnosis and surveillance of retinopathy in type 2 diabetic patients. That attributed to the following reasons ; hs-CRP can be used as biomarker for diabetic retinopathy due to the inflammation in diabetic cases.Neuron specific enolase can be used as biomarker in diabetic retinopathy because it is increase to

survive the nurons when it be under stress as (hypoxia, ischemia) ,if we classify it to nonproliferative and proliferative retinopathy or not classify. The elevation of NSE tend to decrease over the severity of diabetic retinopathy.

VII. ACKNOWLEDGMENT

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