

Relationship between hyperglycemia, insulin resistance and serum apoptosis marker m30- antigen in patients with type 2 diabetes mellitus

İhsan Boyacı^{1*}, Türkan Yiğitbaşı², Handan Ankaralı³

¹ Dept of Internal Medicine, Istanbul Medipol University, Fatih, Istanbul, TR

² Dept of Medical Biochemistry, Istanbul Medipol University, Beykoz, Istanbul, TR

³ Dept of Biostatistics and Medical Informatics, Istanbul Medeniyet University, Üsküdar, Istanbul, TR

* Corresponding Author: İhsan Boyacı E-mail: iboyaci@medipol.edu.tr

ABSTRACT

Objective: The amount of evidence suggests that the apoptosis marker M30-antigen (an antibody that recognizes the cytokeratin-18 fragment) has an association with hyperglycemia.

Material and Methods: In this study, serum M30 levels of 145 patients diagnosed with prediabetes (n = 28) and type 2 diabetes, which was divided into four groups according to their HbA1c levels, were measured. The health control group (n = 24) was composed of healthy individuals. Serum concentrations of M30 antigen were measured using an enzyme-linked immunosorbent assay system and expressed as mean ± SD. HbA1c concentration was determined by boronate affinity technology according to NGSP standards.

Results: M30 levels were comparable in the healthy control group (64,39±3,9 U/L) and prediabetes group (82,07±13,7). Type 2 diabetes Groups A, B, C, and D had levels of 109,38±16 U/L, 117,46±14,3 U/L, 173,69±48,1 U/L, and 163,40±37,3 U/L, respectively. The analysis of the data has shown that serum levels of M30 in the control and prediabetes groups were significantly lower than Type 2 diabetes Groups C and D (p=0.043). When all groups were taken into consideration, a significant relationship was found between HbA1c and serum M30 levels (r=0.231, p=0.002).

Conclusion: Apparently, the increase in glycemia is followed by a rise in the serum levels of the, suggesting that apoptosis occurs as a secondary effect immediately after hyperglycemia.

Keywords: type 2 diabetes mellitus, prediabetes, hyperglycemia, apoptosis, M30-antigen, insulin resistance

INTRODUCTION

Diabetes Mellitus (DM) is a long-lasting metabolic disease that prevents the body from using carbohydrates, fats, and proteins due to insulin deficiency or defects under the influence of insulin. Furthermore, diabetes is associated with a heavy-economic burden on the healthcare systems since it requires perpetual medical care. Diabetes is an endemic disease worldwide due to the changes in the lifestyles and increasing length of life. It is estimated that more than 700 million people will be affected by this disease by 2045 (1). Since DM appears in earlier ages in the last several decades, a detailed evaluation of the body response to hyperglycemia is required (2). In type 2 diabetes mellitus (T2D), lipotoxicity, increased production of reactive oxygen species (ROT) and insulin resistance cause damage in pancreatic β-cells. Many factors contribute to the development of T2D, including breakdown of insulin secretion, relative insulin deficiency, and IR (3). It has been shown that 25%-50% of necrosis in β-cells occurs through intrinsic and extrinsic apoptotic pathways in T2D (4).

Apoptosis, also called programmed cell death, is a physiological mechanism to maintain body homeostasis. Apoptosis is pathological when the rate of apoptosis is altered. During apoptosis, apoptosis enzymes, such as caspases and cysteine proteinases, cleave many intracellular proteins (4).

Research Article

Received 28-03-2020

Accepted 11-04-2021

Available Online: 13-04-2021

Published 30-04-2021

Distributed under
Creative Commons CC-BY-NC 4.0

OPEN ACCESS



The cleavage of these proteins releases proteolytic fragments such as cytokeratin-18 fragment (CK-18) into the circulation. CK-18 is a type 1 intermediate filament protein, which is the main component of single-layer and glandular epithelial cells (5).

An antibody against the CK-18 neo epitope has been developed (5, 6). This antibody, called M30- antigen, recognizes CK-18 fragments after the induction of apoptosis by Caspases. This fragment is not present in normal and necrotic cells. The use of different kinds of CK-18 in the serums of patients (apoptosis antigen M30 and necrotic antigen M65) reveals that they can be used to examine in vivo the way of various death of cells (7).

Serum cytokeratin levels are used as tumor markers. Its clinical benefits were demonstrated in lung and breast cancers. They were also partly observed in gastric and oral cancer (8). It is also claimed that they can be useful in nonalcoholic fatty liver disease (NAFLD), differentiating early fibrosis from severe fibrosis and in the distinction between nonalcoholic steatohepatitis (NASH) and simple steatosis (9). Many studies have reported that serum CK-18 levels in patients with NAFLD were significantly higher than nondiabetic controls (10).

It has been shown that pancreatic β -cells are affected by apoptosis in both type 1 and T2D(2,11). It has been suggested that hyperglycemia causes apoptosis in insulin-deficient β -cells, decreasing the production of pancreatic insulin in long term(4). By augmenting oxidative and nitrosative stress, and activating pro-apoptotic Bcl-2 subgroup proteins and caspase cascade, hyperglycemia affects many steps in the apoptotic signaling (2). Unraveling the mechanism behind the apoptosis in hyperglycemia result in a better understanding of diabetic complications, and pave the way for new treatment strategies.

It is unclear whether apoptosis is associated with the hyperglycemia, and subsequently diabetic complications in T2D. This study investigates the possible relationship between these two processes in T2D to examine whether hyperglycemia causes apoptosis or apoptosis causes hyperglycemia.

MATERIAL and METHODS

The study was conducted between January and March 2016 with 169 individuals consisting of prediabetes (PD) (n=28), overt T2D (n=117) and healthy control (HC) (n=24) which were enrolled from the Istanbul Medipol University, Medical Faculty, Department of Internal Medicine outpatient clinics. Subjects between the ages of 40 and 70 were included in the study by monitoring their fasting blood glucose (FBG), hemoglobin A1c (HbA1c) and 75g oral glucose tolerance test (OGTT). Eighty-one of the participants were female and eighty-eight were male. The value of HbA1c was stated as a percentage according to the National Glycohemoglobin Standardization Program (NGSP). Healthy volunteers with the history of any diseases were included in the HC group. The inclusion criteria for the healthy volunteers were; HbA1c levels lower than 5.7%, and normal OGTT level. The people, whose HbA1c values were $5.7\% \leq \text{HbA1c} \leq 6.4\%$, and who had impaired fasting glucose (IFG) [FBG levels 100 mg/dL to 125 mg/dL], and had impaired glucose tolerance (IGT) [2-h values in the OGTT of 140 mg/dL to 199 mg/dL], was

recruited to the PD group. The subjects were grouped according to their HbA1c levels as; group A (Gr A) ($\text{HbA1c} \leq 6.4\%$), group B (Gr B) ($6.5\% \leq \text{HbA1c} \leq 7.9\%$), group C (Gr C) ($8.0\% \leq \text{HbA1c} \leq 9.9\%$), and group D (Gr D) ($10.0\% \leq \text{HbA1c}$). The patients for Gr A were individuals who have taken one or more than one antidiabetic agents with $\text{HbA1c} \leq 6.4\%$ levels. The T2D and PD diagnosis criteria were based on that of American Diabetes Association (ADA)(12). The medical treatment received by the patients had not interfered during the study.

Patients with diabetes were also excluded from the study if they were diagnosed with cancer or were undergoing cancer treatment, were severely malnourished, along with those receiving immunosuppressive treatment. Moreover, patients with cirrhosis, anemia and hemolysis or who were pregnant were excluded from the study because of the interference with the HbA1c levels.

All participants provided written informed consent. The protocol of the study was reviewed and verified by the Ethical Committee of Faculty of Medicine, Istanbul Medipol University.

Screening: The following variables were recorded for each subject; age, gender, and background. A medical history of the patients, including the duration of diabetes, and the treatments were recorded at the outpatient clinic. A detailed physical examination was conducted and the symptoms were examined during the examination, their anthropometric measurements were recorded. The subjects were classified according to the degree of obesity measured by their body mass index (BMI). The standard waist-hip ratio (WHR) for males was set at <1 cm, and for females, it was set at <0.8 cm.

Blood Analysis: Blood and spot urine samples of all study subjects were collected after at least 10 h of fasting overnight. Blood specimens were collected in 8.5 mL vacutainers (Becton Dickinson) tubes for biochemical parameters. HbA1c samples were carved up/divided into 2 mL tubes including ethylene diamine tetra-acetic acid (EDTA). Depending on the results on the HbA1c, FBG, and OGTT tests, patients were classified into study groups. To assess the M30-antigen levels, the samples were centrifuged (Hence - Xiang Yi 500 model) and the supernatant was collected. The samples were then kept at -80°C until further analysis. Two hours after the breakfast, blood samples were collected again for postprandial glucose (PPG) levels. Boronate affinity technology determined (Quo-Lab®, EKF Diagnostics plc, Cardiff, UK) the HbA1c concentration according to standard NGSP. Plasma glucose was measured by the glucose oxidase method for diagnostic criteria. Standard 75-g glucose OGTT determined IFG and IGT. ADVIA Centaur XP (Siemens) analyzed C-peptide fasting and Cobas e411 measured insulin fasting level. To detect the Homeostatic Model of Assessment-Insulin Resistance (HOMA-IR) the following formula was used $\text{fasting insulin level } (\mu\text{U/ml}) \times \text{fasting glucose level } (\text{mg/dl}) / 405$. FBG, PPG, triglyceride (TG), total cholesterol (Col), high-density lipoprotein cholesterol (HDL-c), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, and uric acid levels were measured by VITROS® 350 Chemical Systems (Ortho Clinical Diagnostics). Low-density lipoprotein cholesterol (LDL-C) concentration was calculated using the Friedewald

equation ($\text{LDL-C} = [\text{Tcol}] - [\text{HDL-c}] - [\text{TG}] / 5$). C-reactive protein (CRP) and microalbumin, that in spot urine, were tested with the use of i-chroma reader device (Boditech).

ELISA: The level of CK-18 M30 neo-epitope was measured through an immunoassay according to manufacturer's instructions using M30-Apoptosense ELISA kit (Peviva AB, Sweden). The M30 neo-epitope (K18Asp396-NE) is a sensitive and integrative indicator-specific for epithelial cell death involving caspase-3, -7 or -9-activation. M30-antigen concentrations were stated as unit per liter ($1 \text{ U/L} = 1.24 \text{ pM}$). The amount of protein in each sample was calculated by plotting a standard curve of the known concentrations that were made for CK-18 neo epitope M30-antigen vs. measured epitope.

Statistical analysis: The primary aim of the study was to classify five patient groups and one HC group, according to HbA1c and to determine whether they differ in terms of M30-antigen. Based on this information, the sample size was calculated by considering the following pre-acceptances: 5% probability of error, 80% of test power, and $70 \pm 80 \text{ U/L}$ increase on average in M30-antigen compared to healthy individuals, and consequently, at least 160 people had to be included in the study. Since the M30-antigen values of healthy individuals were similar, 24 healthy patients were assigned to the HC group and 28–30 patients were assigned to the other groups.

The appropriateness of numerical variables in a normal distribution that was observed during the study was evaluated by the One-Sample Kolmogorov Smirnov test and the identifying values were calculated as mean and standard deviation ($\text{mean} \pm \text{SD}$). Taking account of the distribution of the values, the one-way ANOVA model and Kruskal Wallis test were used in the comparison of the groups, and then different groups were detected via post-hoc Scheffe test. The relevance between the values was observed by Pearson's correlation coefficient. In addition, the ability of the M30-antigen to diagnose diabetes, according to HbA1c was investigated by ROC analysis. The statistical significance level was approved as $p < 0.05$ and analysis was performed using SPSS V22.0 statistic software (SPSS, Inc, USA).

RESULTS

In total, there were 169 participants classified into an HC group ($n=24$, 14,2%), PD group ($n=28$, 16,5%), and diabetes group ($n=117$, 69,3%). According to analysis, a significant difference was observed between participant's ages, durations of diabetes, BMI, WHR, systolic blood pressure (SBP), and diastolic blood pressure (DBP). In the groups, there was a significant difference in terms of biochemical analysis, including HbA1c, M30-antigen, FBG, PPG, fasting insulin, HOMA-IR, CRP, C-peptide, and uric acid. In terms of IFG, ALT, AST, creatinine and T col no difference was observed. The details of all participants are shown in Table 1.

The relationship between the age of the patients, and the levels of the HbA1c and M30-antigen were evaluated. In addition, diabetic duration of the groups were analyzed in relation to HbA1c and M30- antigen. A significant relationship was not found in the groups in terms of chronological age, diabetic duration, HbA1c levels, and M30-antigen levels. When the diabetes group was analyzed in

terms of diabetic durations a correlation was found with levels of HbA1c, M30-antigen, and C-peptide (Table 2).

When the differences between the groups were analyzed in terms of M30-antigen; it was observed (Table 1) that M30-antigen levels in HC and PD groups were significantly lower than the ones in Gr C and Gr D diabetes groups ($p=0.043$) (Table 3). When all individuals were taken into consideration, a positive and significant relationship was found between HbA1c and M30-antigen levels ($r=0.231$, $p=0.002$) (Table 4).

The analysis of the effects of FBG and PPG on apoptosis showed a positive and significant correlation with M30-antigen of both the parameters. Furthermore, a positive and significant relationship between HOMA-IR and HbA1c levels was found. Moreover, further analysis observed a positive and significant relation between the HOMA-IR and M30-antigen levels (Table 4).

Obesity in the individuals was analyzed according to BMI, and the descriptive statistics for the M30-antigen and HbA1c levels are presented in Table 5. When all individuals and individual groups are analyzed, the analysis showed no correlation between BMI and the M30-antigen levels. BMI was correlated with HOMA-IR. The results showed no relationship between WHR and M30-antigen concentration. However, when all individuals are analysed positive and significant correlation was found between WHR and HbA1c levels. In individual groups, no correlation was found (Table 6). During this study, no correlation between hyperglycemia and C-peptide level was found. The analysis of the C-peptide level and M30-antigen level showed a positive and significant correlation only in the Gr B; however, none of the other groups showed a correlation. A significant and positive relationship was found between insulin and C-peptide levels. A positive and significant correlation was between HOMA-IR and C-peptide (Table 4). A statistically significant and negative correlation was observed between the duration of diabetes and the C-peptide levels in patients with diabetes. However, no correlation was observed between C-peptide and chronological age order (Table 2).

A positive and significant relation between CRP levels and BMI was observed. However, no significant relation was found between CRP levels and WHR (Table 6). A significant relationship was found between HbA1c and HOMA-IR with CRP levels. Taken together, we observed a significant relationship between M30-antigen and CRP levels (Table 4).

In our study, we found a significant correlation between ALT and AST levels, and M30-antigen values. However, no significant correlation was found among M30-antigen, fasting insulin, uric acid, creatinine, microalbuminuria, T col, HDL-c, LDL-c, and TG (Table 7). Additionally, no correlation was found between SBP and DBP with M30-antigen levels. Blood pressure showed a significant correlation with HbA1c, HOMA-IR, and CRP (Table 6). Although there was not a significant correlation between levels of M30-antigen in the HC and PD groups, HbA1c levels were significantly different (Figure 1a). It was suggested that levels of both M30-antigen and HbA1c could effectively differentiate healthy controls from patients with diabetes. Furthermore, ROC analysis suggested that HbA1c is a better marker than M-30 antigen in distinguishing patients with diabetes from healthy individuals (Figure 1b).

Table 1. General and biochemical features of individuals participated in the study

HbA1c Level (%)	HC	PD	Gr A	Gr B	Gr C	Gr D	Gr T	P
	HbA1c< 5.7	5.7≤ HbA1c≤6.4 IFG/IGT, IFG+IGT	HbA1c≤ 6.4	6.5≤HbA1c≤7.9	8.0≤HbA1c≤9.9	10.0≤HbA1c		
Number	24(%14,2)	28(%16,5)	29(%17,1)	30(%17,7)	29(%17,1)	29(%17,1)	169	
Gender (M/F)	13/11	15/14	16/14	14/15	15/14	88/81	-	
Age (year)	47,9±5,75	52,1±8,63	58,1±9,22	56,2±9,55	56,0±7,60	57,1±8,89	54,8±8,98	0.001
Dd (month)	-	-	67±69,8	100±74,8	98,6±83,8	117±80	66±78,7	0.001
BMI (kg/m ²)	27,2±3,11	32,1±5,77	32,8±5,47	33,8±5,51	34,8±5,25	35,6±5,85	32,9±5,83	0.001
WHR (cm)*	0,92±0,07	0,96±0,04	0,98±0,05	0,98±0,06	0,97±0,06	1,00±0,05	0,97±0,06	0.001
SBP(mmHg)	115,4±7,79	122,2±7,63	133,6±24,3	134,6±19,8	135,5±20,6	135,1±19,9	129,9±19,5	0.001
DBP(mmHg)	79,5±4,64	81,8±5,57	83,9±7,24	85,6±7,27	84,1±8,56	87,9±10,39	84,0±7,93	0.001
HbA1c (NGSP) (%)	5,09±0,17	5,73±0,45	5,87±0,34	7,01±0,45	8,86±0,65	11,31±1,14	7,39±2,24	0.001
M30 (U/L)	64,3±19,4	82,0±72,51	109,3±86,16	117,4±78,76	73,6±259,14	163,4±201,12	120,2±150,54	0.002
FBG (mg/dl)	84±5,4	103±8,7	116±17,3	138±17,7	182±40,1	264±54,4	150±67,4	0.001
PPG (mg/dl)	88±10,5	123±30,7	131±41	197±44,2	248±76,5	327±117,8	190±103,5	0.001
FI (μU/mL)	7,9±4,4	14,4±7,3	15,0±8,1	17,9±10,5	16,1±14,6	13,9±8,4	14,4±9,9	0.001
HOMA-IR index	1,65±0,92	3,72±2,15	4,38±2,36	6,29±3,77	7,45±9,46	9,06±6,05	5,60±5,65	0.001
FCP (ng/ml)	1,36±0,52	2,38±0,69	2,26±0,92	2,46±1,17	2,12±1,24	2,15±1,28	2,15±1,07	0.001
CRP (mg/L)	2,0±2,4	2,8±2,4	3,0±2,2	3,8±2,2	5,7±4,4	8,3±8,8	4,3±4,8	0.001
UA (mg/dl)	4,5±0,9	5,2±1,5	5,5±1,2	5,1±2	4,6±1,7	4,1±1,4	4,8±1,6	0.002
ALT (U/L)	29,8±9,7	31,2±12	30,8±17,2	31,9±16,1	34,5±16,8	32,3±13,5	31,8±14,5	0.562
AST (U/L)	23,4±4,8	23,3±4,8	24,8±9,8	26,6±9,8	28,6±14	25,1±9,9	25,4±9,6	0.610
Crn (mg/dl)	0,76±0,15	0,82±0,17	0,72±0,14	0,76±0,18	0,71±0,17	0,70±0,19	0,74±0,17	0.102
MAU (mg)	5,95±4,09	17,8±56,4	29,8±57,1	46,8±65,9	65,6±94,4	53,4±88,2	37,8±70,8	0.001
Tcol (mg/dl)*	176±41	188±36	195±39	212±53	193±43	192±34	193±2	0.072
HDL-c (mg/dl)	51±14	42±9	42±7	43±9	40±10	39±10	43±10	0.009
LDL-c (mg/dl)	93±40	110±34	117±30	132±48	95±44	90±47	107±43	0.014
TG(mg/dl)	123±108	168±97	169±81	211±130	297±523	295±306	214±270	0.001

HC: healthy control; PD: prediabet; Gr A :group A ; Gr B :group B; Gr C: group C; Gr D: group D; Gr T: group Total; Dd: diabetes duration BMI: body mass index; WHR: waist-hip ratio; SBP: systolic blood pressure; DBP: diastolic blood pressure; HbA1c: glycosylated hemoglobin; M30: M30-antigen; FBG: fasting blood glucose; PPG: post prandial glucose; FI:fasting insulin ; HOMA-IR: the homeostatic model of assessment-insulin resistance; FCP: fasting C-peptide; CRP: c-reactive protein; UA: uric acid; ALT: alanine aminotransferase; AST: aspartate aminotransferase; Crn: creatinine; MAU: Microalbuminuria; T col: total cholesterol; HDL-c: high-density lipoprotein; LDL-c: low-density lipoprotein; TG: triglyceride; Data: were expressed as mean±SD , p values (*) oneway ANOVA, other data were calculated by Kruskal Walls tests

Table 2. HbA1c, M30-antigen and C-peptide correlation with diabetes duration and chronologic age*

	HbA1c (NGSP) (%)			M30 (U/L)			C-peptide (ng/ml)		
	r	p	N	r	p	N	r	p	N
HC									
age**	0.241	0.257	24	-0.144	0.502	24	0.018	0.936	23
PD									
age	-0.091	0.645	28	-0.265	0.173	28	0.039	0.848	27
GrA									
age	0.159	0.410	29	-0.276	0.148	29	0.340	0.071	29
dd***	0.159	0.410	29	0.056	0.772	29	0.241	0.207	29
GrB									
age	0.227	0.229	30	-0.092	0.630	30	0.275	0.141	30
dd	-0.108	0.578	29	-0.196	0.308	29	-0.353	0.060	29
GrC									
age	-0.419	0.024	29	-0.377	0.050	29	-0.266	0.164	29
dd	-0.018	0.927	29	-0.287	0.131	29	-0.405	0.029	29
GrD									
age	-0.185	0.337	29	0.135	0.485	29	0.170	0.378	29
dd	-0.053	0.787	29	0.152	0.432	29	-0.153	0.428	29
Gr Diabet									
age	-0.055	0.554	117	-0.133	0.153	117	0.131	0.160	117
dd	0.183	0.049	116	-0.057	0.545	116	-0.203	0.029	116

HC: healthy control; PD: prediabet; Gr A :group A ; Gr B :group B; Gr C: group C; Gr D: group D; Gr Diabet: All groups of diabetics; HbA1c: glycosylated hemoglobin; dd: diabetes duration; (*)Pearson correlation analysis, (**) year, (***) month

Table 3. Distribution of M30-antigen by groups

	M30-Antigen (U/L)			
	N	Mean*	SD	p**
HC	24	64,39 a	19.4	0.043
PD	28	82,07 a	72.51	
GrA	29	109,38 b	86.16	
GrB	30	117,47 ab	78.76	
GrC	29	173,70 bc	259.14	
GrD	29	163,41 bc	201.12	

(*) If the averages have completely different letter from one another, it means that they are significantly different from one another. If they have same or common letters, it means that their difference is not significant. (**)It was assessed with One-Way ANOVA and post hoc Scheffe test. Data: mean± SD, HC: healthy control; PD: prediabet; Gr A :group A ; Gr B :group B; Gr C: group C; Gr D: group D

Table 4. Correlation between biochemical parameters*

	HbA1c	C-peptide	HOMA-IR	Insulin(μU/mL)	FBG(mg/dl)	PPG(mg/dl)	CRP(mg/L)
HbA1c (%) (NGSP)							
r	-	0.055	0.413	0.092	0.909	0.781	0.409
p	-	0.483	0.001	0.236	0.001	0.001	0.001
C-peptide (ng/ml)							
r	0.055	-	0.682	0.821	0.089	0.185	0.058
p	0.483	-	0.001	0.001	0.254	0.018	0.460
HOMA-IR							
r	0.413	0.682	-	0.863	0.5	0.455	0.180
p	0.001	0.001	-	0.001	0.001	0.001	0.022
M30-antigen (U/L)							
r	0.231	0.147	0.205	0.167	0.243	0.319	0.457
p	0.002	0.059	0.008	0.031	0.001	0.001	0.001

HbA1c C-peptide HOMA-IR Insulin(μU/mL) FBG(mg/dl) PPG(mg/dl) CRP(mg/L), FBG: fasting blood glucose; PPG: post prandial glucose; HOMA-IR: homeostatic model of assessment-insulin resistance; CRP: c-reactive protein; HbA1c: glycosylated hemoglobin; (*)pearson correlation analysis

Table 5. Distribution of M30-antigen and HbA1c in terms of obesity degrees*

Obesity degree	BMI (kg/m ²)	Frequency	M30-antigen(U/L)	p	HbA1c (NGSP) (%)	p
Normal weight	18,5-24,9	8(%4,7)	64,7±18,9	,337*	5,8±1,4	,013*
Overweight	25-29,9	44(%26)	126,8±208,5		6,9±2,4	
1° obese	30-34,9	53(%31,4)	97,1±87,5		7,2±2,0	
2° obese	35-39,9	39(%23,1)	133,2±121,1		8,0±2,0	
Morbid obese	40≤	23(%13,6)	163,5±198,4		8,2±3,0	

BMI: body mass index; HbA1c: glycosylated hemoglobin; *It was examined via One-Way ANOVA and post hoc Scheffe test. Data: expressed as mean± SD

Table 6. Comparison of blood pressure and anthropometric parameters with biochemical parameters*

	BMI(kg/m ²)		WHR (cm)		SBP(mmHg)		DBP (mmHg)	
	r	p	r	p	r	p	r	p
M30-antigen (U/L)	0.102	0.191	0.007	0.924	0.056	0.471	0.062	0.424
HbA1c (%)	0.325	0.001	0.219	0.005	0.224	0.004	0.238	0.002
HOMA-IR	0.325	0.001	0.154	0.050	0.166	0.033	0.162	0.037
CRP (ng/ml)	0.187	0.017	0.029	0.713	0.176	0.025	0.237	0.002

HOMA-IR: the homeostatic model of assessment-insulin resistance; CRP: c-reactive protein; HbA1c: glycosylated hemoglobin; *pearson correlation analysis

Table 7. Relation of M30- antigen with biochemical parameters*

	M30- antigen level (U/L)	
	r	p
OGTT 2nd hour (mg/dl)	0.546	0.002
UA (mg/dl)	0.004	0.963
ALT (U/L)	0.503	0.001
AST (U/L)	0.561	0.001
Creatine (mg/dl)	-0.068	0.386
MAU (mg)	-0.012	0.878
T col (mg/dl)	0.111	0.154
HDL-c (mg/dl)	-0.044	0.569
LDL-c (mg/dl)	0.092	0.238
TG (mg/dl)	0.040	0.606

OGTT 2nd: oral glucose tolerance test 2nd hour ; UA: uric acid ALT: alanine aminotransferase; AST: aspartate aminotransferase; MAU: Microalbuminuria; T col: total cholesterol HDL-c: high-density lipoprotein; LDL-c: low-density lipoprotein; TG: triglyceride; *pearson correlation analysis

DISCUSSION

It has been demonstrated through autopsies of pancreatic tissue in patients with T2D that a decrease in β -cell mass is accompanied by an increase in cell apoptosis (13). The study of cellular apoptosis in primary human pancreatic tissue is limited due to the invasive techniques. In this study, this limitation was avoided to some extent by estimating M30-antigen levels in serum (in vivo), which reflects the total apoptosis of single-layer and glandular epithelial cells in the body. This study investigated the relationship between hyperglycemia and apoptosis marker M30-antigen through the comparison of HC subjects and patients with T2D.

Does the increase of hyperglycemia levels escalate apoptosis?

The glucose sensing and subsequent glucose metabolism comprises the mechanism involved in glucose-stimulated insulin secretion by β -cells. Aberrant β -cell functioning results in hyperglycemia by insufficient insulin production(14). ROS is produced because of the exposure of β -cells to glucolipotoxicity that is caused by increased plasma glucose and lipid levels. A perspective claims that ROS produces apoptosis by releasing cytochrome C and activating caspase(15-18). A study observed that apoptotic cell death in diabetic myocardial cells occurs via caspase-3 activation. The study reported that hyperglycemia leads to ROS production in vivo subsequently causing apoptosis of cells in the myocardium (19). Many stimulants such as cytokines, leptin, glucose, sulphonylurea, and free fatty acids can trigger apoptosis in β -cells (4). A second view supports that support the notion that significant and progressive loss in β -cell mass appears before patent hyperglycemia emerges in the patients with the T2D(20). It has been proposed that the loss in β -cell mass is an early stage indicator in the process of T2D development, and it is the primary reason rather than being secondary to hyperglycemia.

They urge that the loss in β cell mass is an early stage in T2D process, and it is a primary reason instead of being secondary for hyperglycemia (13,21). Sulphonylureas have positive insulin secretory effects, they also cause secondary failure decreasing mass and the secretory function of the β -cells because of the continuous increase of Ca^{+2} influx due to a long-term treatment (22).

In a group of patients with morbid obesity selected for bariatric surgery, a significant correlation was observed between HbA1c and M30-antigen levels (23). In a study involving individuals with NAFLD, they claimed that there was not any difference between CK-18 concentrations among the patients having T2D. They also claimed that CK-18 existing in blood will not be affected by apoptotic tendencies in the patients having diabetes(15). Several studies have evaluated the correlation between hyperglycemia and apoptosis, however, it is still unclear whether hyperglycemia induces apoptosis or not. In our study, hyperglycemia level and severity of diabetes were designed on the basis of HbA1c level, regardless of the duration of diabetes (Table 1). When we examined the differences among the groups with reference to M30-antigen, we observed that levels of the M30-antigen in the HC and PD groups were significantly lower than the Gr C and Gr D ($p=0.043$) (Table 3). Furthermore, when all individuals were taken into consideration, there was a significant correlation among M30-antigen and HbA1c levels ($r=0.231$, $p=0.002$) (Table 4). Therefore, it may be concluded that as long as level of HbA1c increases, the level of M30-antigen increases, as well. In other words, when glycemic levels increase, apoptosis increases, too. The PD is a period that hyperglycemia starts, and hyperglycemia severity is lower compared to other groups. In this group, the level of M30-antigen is low in parallel with hyperglycemia that is why it can support the idea that the level of hyperglycemia increases apoptosis.

A significant relationship was found between β -cell mass and FBG concentrations. It has been shown that a decrease in β -cell mass is correlated with an increase in blood glucose level(20). In our study, we report the effects of both parameters FBG and PPG on apoptosis, which revealed a positive correlation with M30-antigen levels (Table 4). Apoptosis may occur as a secondary effect of hyperglycemia and in turn the apoptosis may cause hyperglycemia because of β -cell death. It is thought that uncontrolled hyperglycemia increases M30-antigen levels and in case of emerging of an intense hyperglycemic state, even though as a new-onset, it accelerates the process of apoptosis. Rather than the duration of the diabetes, the level of hyperglycemia in other words its severity may increase the rate of apoptosis.

The relation between insulin resistance and M30-antigen

β -cell hyperplasia develops at the early stage of the T2D, followed by insulin resistance, and impaired insulin secretion, then a decrease occurs in the β -cell mass. In T2D, body produces insulin, but cannot use efficiently, which results in hyperglycemia and subsequently IR. In a study involving morbidly obese patients, a correlation between M30-antigen and HOMA-IR was observed (23). Furthermore, a significant correlation was found between HOMA-IR score and M30-antigen levels in NASH patients (24). In this study, we detected a positive and a significant correlation between HOMA-IR score and HbA1c levels. When the relation between HOMA-IR and M30-antigen is considered, there was also a significant correlation (Table 4). Our results suggest that IR is the key factor causing hyperglycemia. In addition, IR may trigger the process of apoptosis in β -cells.

Relation of the duration of diabetes and chronological age with M30-antigen level

Approximately 90% of individuals over 40 years of age with T2D have early pancreatic β cell loss and a progressive impaired in glucose tolerance (25). Furthermore, it is unclear whether it is a physiological apoptosis period or a pathological one. It was claimed that programmed cell death, in other words, apoptosis are related to the aging effect, as well. On a study focused on patients with breast cancer, it was revealed that apoptosis increases when patient age is higher(26). In another study involving M30-antigen analysis, it was suggested that CK-18 fragments are more sensitive in older patients than younger patients in the diagnosis of NAFLD(27). In our study, we examined whether there is a relation between M30-antigen levels, chronological age, and duration of the diabetes. It was observed that there was no correlation between M30-antigen levels, chronological age, and diabetes duration of the patients (Table 2). Our results provided a contrasting observation to the previous studies that the rate of apoptosis increases with the age. The patient age and the duration of the disease in individuals with diabetes are correlated with HbA1c levels. The duration of diabetes is related to hyperglycemic levels, however, there is no correlation between apoptosis and the duration. When only the diabetics were taken into consideration, a significant, but adverse correlation was observed between the duration and C-peptide levels (Table 2). Furthermore, there was no correlation between HbA1c and C-peptide levels. It can be concluded that in T2D age and duration of the diabetes are not exclusive factors contributing to apoptosis, but

hyperglycemia is also correlated with the duration of the disease and the age.

Relation between anthropometric parameters and M30-antigen

In the studies, BMI and WHR, which are accepted as IR parameters, and their relationship with M30-antigen level was evaluated, a significant positive correlation was found only in individuals with NASH(24,28). In our study, we observed that BMI correlates with HOMA-IR and HbA1c levels, however, we did not observe any relationship between M30-antigen, BMI, and WHR (Table 6). Moreover, we detected a significant correlation between WHR and HbA1c levels (Table 6). We could not find a significant difference between the degree of obesity and M30-antigen levels. The concentration of M30-antigen in morbidly obese individuals was found to be significantly higher compared to the individuals with normal weight. Apart from this finding, we did not observe any significant difference (Table 5). Further comprehensive studies are certainly needed to explore the correlation between obesity and M30-antigen.

β -cell reserve and apoptosis

We also investigated whether hyperglycemia causes apoptosis or vice versa with respect to C-peptide. C-peptide is the optimal standard for the β -cell function, in other words, endogenous insulin secretion (29). The effect of cytosolic and mitochondrial ROS production induced with hyperglycemia on apoptosis was explored in a study. It was identified that mediated C-peptide activation of AMPKa inhibited mitochondrial fission and endothelial cell apoptosis that was caused by ROS production induced by hyperglycemia (30,31). Therefore, C-peptide replacement therapy was presented as encouraging among new treatment options (32).

In our study, we found a significant relation between insulin and C-peptide levels. However, we could not find any correlation between hyperglycemia and C-peptide. The reason for inexistence of the correlation may be IR, in the presence of IR, although the initial hyperglycemia, the C-peptide level is normal because β -cell produces insulin but insulin does not function. In fact, there was also a significant correlation between HOMA-IR and C-peptide levels in our study (Table 4). When only the diabetics were taken into consideration, a statistically significant adverse correlation was found between C-peptide levels and diabetes duration (Table 2). It appears that the C-peptide level decreases as the duration of diabetes increases. These results make it possible to infer that endogenous insulin levels decrease as time goes by. A decrease in C-peptide and insulin levels may be observed in the late stages of T2D because of progressive loss of β -cell. In addition, it also explains the hyperglycemia that occurred due to an increase in the duration of the disease and the increase in the cellular apoptosis that occurred due to hyperglycemia.

Subclinical inflammation and apoptosis

Hyperglycemia increases general oxidative load by increasing various pro-inflammatory cytokines in pancreatic β -cells, resulting in apoptosis through extrinsic and intrinsic pathways (4). CRP, an inflammatory marker, promotes the recognition and elimination of pathogens and increases the clearance of necrotic and apoptotic cells (33). The level of CRP increases in obesity, and this increase is related to BMI and IR(23,27).

In a meta-analysis, it was suggested that increased CRP levels were significantly related to increased T2D risk (34). CRP that is an acute phase indicator, correlate with metabolic syndrome components (35). It is thought that this acute phase response reflects subclinical inflammation that is mainly present, and that this process is responsible for progressively developing DM (36). In this study, we observed a significant relation between CRP levels and BMI (Table 6). In addition, a significant correlation was observed between CRP levels and HbA1c levels as well as between CRP levels and HOMA-IR (Table 4). We also observed a significant correlation between levels of M30-antigen and CRP ($r=0.471$, $p=0.001$) (Table 4). IR leads to hyperglycemia, and in turn it causes an inflammatory condition. CRP increases as an inflammatory response indicator. In addition, that suggests apoptosis can be triggered because of this developing inflammatory condition. The limitation of this study is that because of financial constraints, the high sensitive CRP was not analyzed.

Aminotransferases and M30-antigen relation

In a study focused on the patients with NAFLD and T2D, a positive correlation was discovered between AST and ALT levels, and serum CK-18 concentrations (28). In addition, during a study which was designed to distinguish NASH with simple steatosis by using CK-18 fragments, a significant correlation was found even if the connection between AST and ALT values and M30-antigen levels was weak (37). In our study, we found a significant correlation between levels of ALT and AST with M30-antigen. However, the study lacks in examining the patients in terms of NAFLD thoroughly.

Will M30-antigen be useful while diagnosing Diabetes Mellitus?

The HbA1C level, which is used in T2D diagnosis, was initially solely during the follow-up on the treatment. International Specialist Committee, constituted by the ADA, The European Association for the Study of Diabetes, and International Diabetes Federation, reported in 2009 that HbA1c test could be used as one of the diagnostic criteria of DM (38, 39). However, illnesses, which shorten the life of erythrocytes, renal failure, and chronic over consumption of alcohol prevent the test to be used for the purposes of screening, diagnosis, and follow up (40). Therefore, the question arises if M30-antigen can be used to diagnose T2D and PD illnesses according to HbA1c. In this study, it was observed that the levels of M30-antigen could not distinguish HC and PD

When we analyze the results of HbA1c and M30-antigen in terms of distinguishing diabetes, from the healthy control group, it is possible to say that these two markers can be used for separating both groups. ; ROC analysis has shown that HbA1c is more effective than M-30 antigen in the segregation of patients with diabetes from healthy individuals (Figure 1a, Figure 1b).

CONCLUSIONS

This study concludes that when there is a rise in glycemic level, M30-antigen level increases. Therefore, when glycemia level increases, apoptosis increases consequently. Hence, it might be appropriate to infer that apoptosis occurs as a

secondary manifestation to hyperglycemia, and the apoptosis causes the loss of β -cells and aggravates hyperglycemia. When diabetes is not controlled, it increases M30-antigen levels, and even if the duration of the disease is short, the hyperglycemia accelerates the process of apoptosis.

Considering the findings in this study, it can be stated that IR is the real cause that leads to hyperglycemia. In the IR an inflammatory condition is started and as a response to the inflammatory condition the CRP increases. This inflammatory state triggers apoptosis, concurrently. The C-peptide and insulin deficiencies may be observed in late stages of T2D because of progressive loss of β -cells. Moreover, M30-antigen is less successful than HbA1c in terms of diagnosing diabetes and prediabetes.

As a result, we should develop T2D treatment strategies by focusing on breaking or preventing IR occurrence, which causes hyperglycemia. Apoptosis is not a reason but is observed as a response of hyperglycemia, and our results support this opinion. Further studies should explain the effect of medical treatment on apoptosis and relation of diabetes complications with apoptosis.

Acknowledgments: The authors thank Prof. Dr. Azmi Yerlikaya and Talip Kurumemet for initial collaboration on the manuscript.

Author contributions: **IB, TY, HA;** Literature search and study design, statistical analyzes, **IB;** Writing article and revisions

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive and specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical issues: All authors declare originality of research.

REFERENCES

1. International Diabetes Atlas [Internet]. Brussels: IDF; [2019; cited 2021 Feb 19]. Available from: <https://www.diabetesatlas.org/en/sections/worldwide-toll-of-diabetes.html>
2. Allen DA, Yaqoob MM, Harwood SM. Mechanisms of high glucose-induced apoptosis and its relationship to diabetic complications. Vol. 16, Journal of Nutritional Biochemistry. 2005. p. 705–13. Available from: <https://doi.org/10.1016/j.jnutbio.2005.06.007>
3. Kahn SE, Cooper ME, Del Prato S. Pathophysiology and treatment of type 2 diabetes: perspectives on the past, present, and future. Lancet [Internet]. 2014 Mar;383(9922):1068–83. Available from: <https://linkinghub.elsevier.com/retrieve/pii/S0140673613621546>
4. Anuradha R, Saraswati M, Kumar KG, Rani SH. Apoptosis of Beta Cells in Diabetes Mellitus. DNA Cell Biol [Internet]. 2014;33(11):743–8. Available from: <http://online.liebertpub.com/doi/abs/10.1089/dna.2014.2352>
5. Ueno T, Toi M, Bivén K, Bando H, Ogawa T, Linder S. Measurement of an apoptotic product in the sera of breast cancer patients. Eur J Cancer. 2003;39:769–74. Available from: [https://doi.org/10.1016/S0959-8049\(02\)00865-1](https://doi.org/10.1016/S0959-8049(02)00865-1)
6. Wieckowska A, Zein NN, Yerian LM, Lopez AR, McCullough AJ, Feldstein AE. In vivo assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. Hepatology [Internet]. 2006;44(1):27–33. Available from: <http://doi.wiley.com/10.1002/hep.21223>

7. Linder S, Havelka AM, Ueno T, Shoshan MC. Determining tumor apoptosis and necrosis in patient serum using cytokeratin 18 as a biomarker. *Cancer Lett* [Internet]. 2004 Oct 8 [cited 2015 Dec 13];214(1):1–9. Available from: <http://www.sciencedirect.com/science/article/pii/S0304383504005002>
8. Barak V, Goike H, Panaretakis KW, Einarsson R. Clinical utility of cytokeratins as tumor markers. *Clin Biochem* [Internet]. 2004 Jul 1 [cited 2019 Feb 7];37(7):529–40. Available from: <https://www.sciencedirect.com/science/article/pii/S0009912004001316>
9. Chen J, Zhu Y, Zheng Q, Jiang J. Serum cytokeratin-18 in the diagnosis of non-alcoholic steatohepatitis: A meta-analysis. *Hepatol Res*. 2014;44(8):854–62. Available from: <https://doi.org/10.1111/hepr.12197>
10. Tsutsui M, Tanaka N, Kawakubo M, Sheena Y, Horiuchi A, Komatsu M, et al. Serum Fragmented Cytokeratin 18 Levels Reflect the Histologic Activity Score of Nonalcoholic Fatty Liver Disease More Accurately Than Serum Alanine Aminotransferase Levels. *J Clin Gastroenterol* [Internet]. 2010 Jan [cited 2017 Dec 4];44(6):440–7. Available from: <http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00004836-900000000-99432>
11. Hoffmanová I, Sánchez D, Hábová V, Anděl M, Tučková L, Tlaskalová-Hogenová H. Serological Markers of Enterocyte Damage and Apoptosis in Patients With Celiac Disease, Autoimmune Diabetes Mellitus and Diabetes Mellitus Type 2. *Physiol Res* [Internet]. 2015 [cited 2017 Nov 18];64:537–46. Available from: <http://www.biomed.cas.cz/physiolres>
12. Chamberlain JJ, Rhinehart AS, Shaefer CF, Neuman A. Diagnosis and Management of Diabetes: Synopsis of the 2016 American Diabetes Association Standards of Medical Care in Diabetes. *Ann Intern Med* [Internet]. 2016 Apr 19 [cited 2019 Feb 7];164(8):542. Available from: <http://annals.org/article.aspx?doi=10.7326/M15-3016>
13. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC. Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes* [Internet]. 2003;52(January):102–10. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/12502499>
14. Li JJ, Lee SHS-K, Kim DK, Jin R, Jung D-S, Kwak S-J, et al. Mechanisms of high glucose-induced apoptosis and its relationship to diabetic complications. *Diabetes* [Internet]. 2014 Jan 1 [cited 2015 Dec 13];44(1):1078–87. Available from: <http://joijlc.jst.go.jp/JST.JSTAGE/jphs/11072FP?from=CrossRef>
15. Kaneto H, Katakami N, Matsuhisa M, Matsuoka T. Role of Reactive Oxygen Species in the Progression of Type 2 Diabetes and Atherosclerosis. *Mediators Inflamm* [Internet]. 2010;2010:1–11. Available from: <http://www.hindawi.com/journals/mi/2010/453892/>
16. Shirakawa J, Amo K, Ohminami H, Orime K, Togashi Y, Ito Y, et al. Protective effects of dipeptidyl peptidase-4 (DPP-4) inhibitor against increased β cell apoptosis induced by dietary sucrose and linoleic acid in mice with diabetes. *J Biol Chem*. 2011;286(29):25467–76. Available from: [https://www.jbc.org/article/S0021-9258\(19\)48537-2/fulltext](https://www.jbc.org/article/S0021-9258(19)48537-2/fulltext)
17. Liu Z, Stanojevic V, Brindamour LJ, Habener JF. GLP1-derived nonapeptide GLP1(28-36)amide protects pancreatic β -cells from glucolipotoxicity. *J Endocrinol* [Internet]. 2012 May 1 [cited 2018 Jan 8];213(2):143–54. Available from: <http://joe.endocrinology-journals.org/cgi/doi/10.1530/JOE-11-0328>
18. Power SE, O'Toole PW, Stanton C, Ross RP, Fitzgerald GF, O'Keefe J, et al. Intestinal microbiota, diet and health. *Br J Nutr* [Internet]. 2014 Feb 12 [cited 2017 Feb 3];111(3):387–402. Available from: http://www.journals.cambridge.org/abstract_S0007114513002560
19. Cai L, Li W, Wang G, Guo L, Jiang Y, Kang YJ. Hyperglycemia-induced apoptosis in mouse myocardium: mitochondrial cytochrome C-mediated caspase-3 activation pathway. *Diabetes* [Internet]. 2002 Jun 1 [cited 2018 Jan 1];51(6):1938–48. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/12031984>
20. Ritzel RA, Butler AE, Rizza RA, Veldhuis JD, Butler PC. Relationship Between β -Cell Mass and Fasting Blood Glucose Concentration in Humans. *Diabetes Care* [Internet]. 2006;29(3):717–8. Available from: <http://care.diabetesjournals.org/content/29/3/717.full>
21. DeFronzo RA. Banting Lecture. From the triumvirate to the ominous octet: a new paradigm for the treatment of type 2 diabetes mellitus. *Diabetes* [Internet]. 2009 Apr [cited 2018 Oct 28];58(4):773–95. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/19336687>
22. Kim J-Y, Lim D-M, Park H-S, Moon C-I, Choi K-J, Lee S-K, et al. Exendin-4 Protects Against Sulfonylurea-Induced β -Cell Apoptosis. *J Pharmacol Sci* [Internet]. 2012 [cited 2018 Feb 10];118(1):65–74. Available from: <http://joijlc.jst.go.jp/JST.JSTAGE/jphs/11072FP?from=CrossRef>
23. Civera M, Urios A, Garcia-Torres ML, Ortega J, Martinez-Valls J, Cassinello N, et al. Relationship between insulin resistance, inflammation and liver cell apoptosis in patients with severe obesity. *Diabetes Metab Res Rev* [Internet]. 2010 Mar 10 [cited 2018 Nov 24];26(3):187–92. Available from: <http://doi.wiley.com/10.1002/dmrr.1070>
24. Hussien HI, Pasha HF, Mostafa NA. Apoptotic Shards of Cytokeratin 18 Point to Steatohepatitis in Patients with non Alcoholic Hepatic Steatosis [Internet]. Vol. 79, *Med J Cairo Univ*. 2011 [cited 2018 Nov 24]. 2011;79(2):199–205 [cited 2018 Nov 24]. Available from: <http://www.medicaljournalofcairouniversity.com>
25. Maiese K. Programming apoptosis and autophagy with novel approaches for diabetes mellitus. *Curr Neurovasc Res* [Internet]. 2015 [cited 2017 Nov 18];12(2):173–88. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/25742566>
26. Holmqvist P, Lundström M, Stål O. Apoptosis and Bcl-2 expression in relation to age, tumor characteristics and prognosis in breast cancer. South-East Sweden Breast Cancer Group. *Int J Biol Markers* [Internet]. 1999 [cited 2018 Jan 8];14(2):84–91. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/10399627>
27. Chen J, Zhu Y, Zheng Q, Jiang J. Serum cytokeratin-18 in the diagnosis of non-alcoholic steatohepatitis: A meta-analysis. *Hepatol Res*. 2014;44(8):854–62. Available from: <https://onlinelibrary.wiley.com/doi/abs/10.1111/hepr.12197>
28. Mai, Murase-Mishiba Y, Bessho M, Miyawaki M, Imbe H, Tsutsumi C, et al. The cytokeratin-18 fragment level as a biomarker of nonalcoholic fatty liver disease in patients with type 2 diabetes mellitus. *Clin Chim Acta* [Internet]. 2014;433:184–9. Available from: https://ac.els-cdn.com/S0009898114001260/1-s2.0-S0009898114001260-main.pdf?_tid=e9b95836-bf8e-11e7-830c-00000aab0f6c&acdnat=1509600798_dea392d4cd2dff138df2bb1684b23590
29. Jones AG, Hattersley AT. The clinical utility of C-peptide measurement in the care of patients with diabetes. *Diabetic Medicine* [Internet]. 2013 Jul 23;30(7):803–17. Available from: <https://onlinelibrary.wiley.com/doi/10.1111/dme.12159>
30. Bhatt MP, Lim Y-C, Kim Y-M, Ha K-S. C-peptide activates AMPK α and prevents ROS-mediated mitochondrial fission and endothelial apoptosis in diabetes. *Diabetes* [Internet]. 2013 Nov [cited 2018 Jan 1];62(11):3851–62. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/23884890>
31. Bhatt MP, Lim Y-C, Hwang J, Na S, Kim Y-M, Ha K-S. C-peptide prevents hyperglycemia-induced endothelial apoptosis through inhibition of reactive oxygen species-mediated transglutaminase 2 activation. *Diabetes* [Internet]. 2013 Jan [cited 2018 Jan 1];62(1):243–53. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22923476>
32. Waldhäusl W. C-peptide as a remedy for diabetic microangiopathy? *Diabetes* [Internet]. 2013 Jan [cited 2018 Jan 1];62(1):39–40. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/23258913>

33. Gershov D, Kim S, Brot N, Elkon KB. C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity. *J Exp Med* [Internet]. 2000 Nov 6 [cited 2018 Jan 31];192(9):1353–64. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/11067883>
34. Wang X, Bao W, Liu J, Ouyang Y-Y, Wang D, Rong S, et al. Inflammatory markers and risk of type 2 diabetes: a systematic review and meta-analysis. *Diabetes Care* [Internet]. 2013;36(1):166–75. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/23264288>
35. Kopp HP, Kopp CW, Festa A, Krzyzanowska K, Kriwanek S, Minar E, et al. Impact of Weight Loss on Inflammatory Proteins and Their Association With the Insulin Resistance Syndrome in Morbidly Obese Patients. *Arterioscler Thromb Vasc Biol* [Internet]. 2003 Jun 1 [cited 2019 Feb 9];23(6):1042–7. Available from: <https://www.ahajournals.org/doi/10.1161/01.ATV.0000073313.16135.21>
36. Festa A, D'Agostino Jr R, Howard G, Mykkanen L, Tracy RP, Haffner SM. Chronic Subclinical Inflammation as Part of the Insulin Resistance Syndrome: The Insulin Resistance Atherosclerosis Study (IRAS). *Circulation* [Internet]. 2000;102(1):42–47. Available from: <https://doi.org/10.1161/01.CIR.102.1.42>
37. Yilmaz Y, Dolar E, Ulukaya E, Akgoz S, Keskin M, Kiyici M, et al. Soluble forms of extracellular cytokeratin 18 may differentiate simple steatosis from nonalcoholic steatohepatitis. *World J Gastroenterol* [Internet]. 2007 Feb 14 [cited 2017 Oct 20];13(6):837–44. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17352011>
38. International Expert Committee TIE. International Expert Committee report on the role of the A1C assay in the diagnosis of diabetes. *Diabetes Care* [Internet]. Virginia :ADA;2009;32(7):1327–34. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/19502545>
39. Nathan DM, Buse JB, Davidson MB, Ferrannini E, Holman RR, Sherwin R, et al. Medical management of hyperglycaemia in type 2 diabetes mellitus: a consensus algorithm for the initiation and adjustment of therapy. *Diabetologia* [Internet]. 2009;52(1):17–30. Available from: <http://link.springer.com/10.1007/s00125-008-1157-y>
40. Mostafa SA, Davies MJ, Srinivasan BT, Carey ME, Webb D, Khunti K. Should glycated haemoglobin (HbA1c) be used to detect people with type 2 diabetes mellitus and impaired glucose regulation? *Postgrad Med J* [Internet]. 2010;86(1021):656–62. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/20956395>