Endocrinology & Metabolic Syndrome
ISSN: 2161-1017

Siomos et al., Endocrinol Metab Syndr 2017, 6:1
DOI: 10.4172/2161-1017.1000259

Research Article Open Access

Prothrombotic and Endothelial Inflammatory Markers in Greek Patients with Type 2 Diabetes Compared to Non-Diabetics

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Received date: December 12, 2016; **Accepted date:** January 23, 2017; **Published date:** February 13, 2017

Abstract

Objective: To evaluate specific factors of coagulation and endothelial inflammatory markers namely, thrombomodulin, soluble receptor of the protein C (sEPCR), factor VIII, plasminogen activator inhibitor 1, Von Willebrandt factor, fibrinogen, fibrinogen dimers (d-dimers), high sensitivity C-reactive protein and homocysteine in a subset of Greek subjects with and without Type 2 (T2) Diabetes. Design: 84 subjects, of which 44 patients with T2 diabetes, were included in the randomized comparative prospective cross sectional study. The subjects were split into a T2 diabetics group and a group of healthy controls of similar age, anthropometric profiles and similar gender distribution.

Results: A total of 47 variables and biomarkers together with indicators for metabolic profiles, clinical history, as well as detailed anthropometric profiles and traditional risk factors, were evaluated. Dipeptidyl peptidase-4 (DPP4), Insulin, use of Sulfonylurea, high HBA1c and glucose levels, were clearly statistically differentiated in the two groups, while no other biomarkers including the new potential indicators were found to be different. High values of thrombomodulin and homocysteine were correlated with a rise in creatinine and thus seem to affect renal function in the diabetic patients group while in the non-diabetics group the correlations are different with sEPCR having a relative strong negative correlation in renal function as measured with The Modification of Diet in Renal Disease, in agreement with the latest international findings.

Conclusions: The presence of T2 diabetes in conjunction with age clearly correlates with problems in renal function, thrombomodulin and homocysteine could serve as indicators for renal damage in diabetics but not in healthy individuals. sEPCR on the other hand could be a potential generic indicator for renal damage. Thrombomodulin and sEPCR as prothombotic agents, did not show any indication that they can be utilised as markers for the prevention and/or treatment of thrombotic complications in diabetic patients.

Keywords: Diabetes; Thrombomodulin; sEPCR; Prothrombotic markers; Endothelial inflammation

Introduction

T2 diabetes is a high prevalence syndrome characterized by high blood glucose levels due to inability/failure of insulin production by the body, limited action of the produced insulin, or a combination of both situations. [1] Dealing with diabetes is a daily challenge for the clinician. Despite tremendous achievements in the diagnosis, monitoring and treatment of diabetes, diabetic patients continue to experience cardiovascular complications that result in death at an elevated rate.

The prevalence in 2015 reached 59.8 million just in Europe alone, representing 9.1% of the adult population. Today the population of patients with diabetes worldwide is estimated at 387 million, a figure that far exceeds initial forecasts. In Greece the disease prevalence in 2015 was 7.5%. [1] Prevalence of medication-prescribed diabetes was 7.0% going up to 8.2% in adults, and 30.3% in those more than 75 years old in the Greek population based on real-world data from the nation-wide prescription database. [2] The major complication of diabetes is microangiopathy (retinopathy and nephropathy), macroangiopathy (coronary heart disease, strokes, peripheral vascular disease) and neuropathy [3,4]. In Greece, diabetes is the leading cause of vision loss, end stage renal failure and the primary cause of amputation and erectile dysfunction with a very high cost for the patient [5,6]. Diabetes unfortunately coexists with other serious conditions such as dyslipidemia, hypertension and obesity increasing the risk of complications with the modern lifestyle further exacerbating its morbidity [5].

The study of blood coagulation is a promising scientific area of research with many new discoveries. A question that arises is what happens to patients with T2 diabetes at the blood coagulation level and how the markers of inflammation and pro - coagulant factors change, eventually leading to thrombotic events. The application of new knowledge in blood coagulation in diabetes could be of significantly help in the prevention and treatment of thrombotic complications in diabetic patients [7-9]. Blood coagulation has been previously studied,

as have been proinflammatory agents in T2 diabetic patients, but these studies have not included the recently discovered agents such as thrombomodulin and the Soluble Endothelian Receptor Protein C (sEPCR) [10,11]. Thrombomodulin, an endothelian transmembrane protein, heavily involved the regulation of inflammation though binding to thrombin, acts as an anticoagulant and is a vital cofactor for thrombin-mediated activation of protein C, which is promoted further by the endothelial cell protein C receptor (EPCR), its soluble form circulates in plasma and inhibits activated protein C anticoagulant activity [12,13].

In this study we focused on specific factors of coagulation and endothelial inflammation markers. Specifically, thrombomodulin, [11,14] the soluble receptor of the protein C [15] (sEPCR), factor VIII, [16] plasminogen activator inhibitor 1 (PAI 1) [17], von Willebrandt factor (VWF), [18-20] fibrinogen, fibrinogen dimers (-dimers), high sensitivity C-reactive protein (hsCRP) [21] and homocysteine [22] in a subset of Greek subjects.

This is the first study to examine thrombomodulin and sEPCR together wih the other indicators of thrombosis and endothelial inflammation (PAI 1, D-dimers, VWF, VIII, FIB) in a population of patients with diabetes in the Greek area. There is very little known, even at international level, for sEPCR and thrombomodulin in small scale studies [10, 11,14,15].

Methods

Subjects

A randomized comparative prospective cross sectional observational case-control study was conducted at the 3rd Department of Internal Medicine of Aristotle University of Thessaloniki in "Papageorgiou" General Hospital of Thessaloniki in October 2012. Recruitment was random with all subjects that came to the Department during 1st to the 31st of October, 200 in total, were asked to participate in the study. Consenting subjects were split into two groups, a Type 2 diabetics group (Group 1) and a non-Diabetics control group (Group 2). Subjects with a history of cancer, family history of thrombophilia, use of oral contraceptives, history of miscarriages, a recent history of coronary heart disease and stroke, end stage renal disease were excluded from the study, Figure 1 has the study inclusion exclusion flow chart. The final study population comprised 84 subjects that gave their informed consent and did not meet the exclusion criteria. Group 1 comprises 44 subjects with Type 2 Diabetes, 24 male and 20 female, with an average age of 61, while the Control group (Group 2) included 40 subjects without type 2 diabetes, 19 male and 21 female with an average age of 59. All subjects underwent a complete physical examination, height, weight (SECA 754, Germany), and waist circumference measurements to calculate the BMI along with blood pressure (mm Hg) (SK Welch Allyn). A complete personal and family medical history was taken, including information such as the duration and type of diabetes, concomitant disease, medication, smoking, alcohol consumption. Blood samples from all participants underwent comprehensive biochemical testing in order to have a metabolic profile for each subject, comprising measurements of levels of glucose(mg/dl), HDL(mg/dl), LDL (mg/dl), triglycerides(mg/dl), cholesterol(mg/dl) (Auto analyzer-Architect 8000 c, Abbott USA), and proinflammatory factors hs-CRP(mg/L) and fibrinogen(g/L) (Dade® Fibrinogen Determination Kit, Dade Behring GMbH, Germany), glycosylated hemoglobin (HbA 1c/HbA2, mmol/mol) (HPLC Menarini-Akray HA 8160 Japan), urea (mg/dl), creatinine (mg/dl), K (mmol/L), Na (mmol/L) (Autoanalyzer -Architect 8000 c, Abbott USA), for the assessment of renal function and calculation of the glomerular filtration rate (GFR - MDRD) as well as CBC and full urine analysis (XT-4000i ™ Automated Hematology Analyzer, Sysmex Japan). Furthermore, assessment of thrombotic agents PAI 1(ng/ml), D-dimers (μg/ml), Thrombomodulin(ng/ml), sEPCR (ng/ml) VWF (VWF: Ag %), VIII(VII: Ag %), were performed by ELISA (using respective Asserachrom enzyme monoclonal Immunoassay kits, Stago France, BCS XP System Siemens Germany) with additional testing for Total L-Homocycteine(μmol/l) also performed (FPIA AxSYM, Abbott USA). All blood and biochemical tests were conducted at the Hematology Laboratory-Haemostasis Unit of the same hospital.

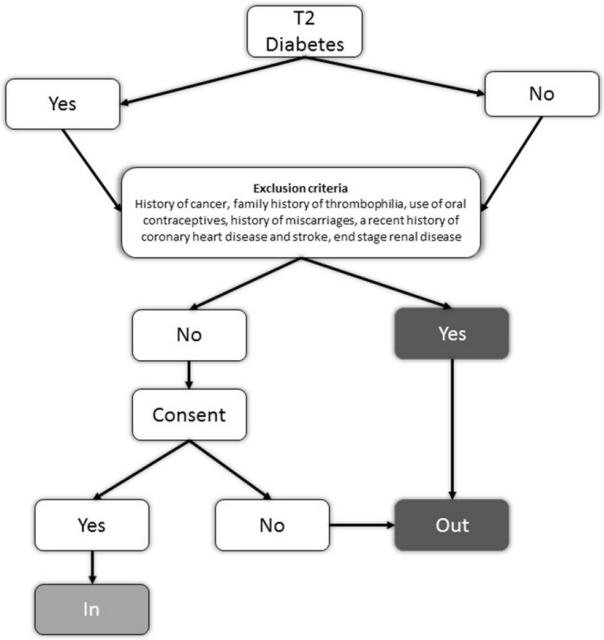

Figure 1: Flow chart with exclusion criteria of the patients and control subjects for the study

Statistical Analysis

Permutation-based Monte-Carlo permutation test statistical tests [23] were used in most cases with N=2000 permutations in each case. All variables with discrete values were treated as qualitative variables. The independence test used was the permutation-based Pearson's χ^2 test. For continuous variables permutation-based T-tests (independent samples, non-directional), non-directional Wilcoxon rank sum tests and two-sample Kolmogorov-Smirnov (non-parametric) tests were conducted where appropriate with the above mentioned number of permutations. All analyses were 2-sided, Shapiro-Wilk test was used to assess for normality of continuous variables and a FDR (corrected P value) of ≤ 0.05 was considered statistically significant. A multivariate Monte-Carlo permutation-based version of Hoteling's test with independent samples assuming unequal covariance was performed between thrombomodulin, Fibrinogen, VIII, VWF, PAI, and sEPCR and disease status. Power analysis has been performed with the G*Power tool version 3.1.3 [24]. For the continuous tests the effect sizes d of 0.2, 0.5, 0.8 are considered small, medium, and large. For the χ^2 test the effect size of 0.1, 0.3, and 0.5 are considered small, medium,

and large respectively. All statistical analysis, was done using Matlab, Release 2011α, The MathWorks, Inc., Natick, Massachusetts, United States, G*Power, [24] v 3.1.3, R, v 3.0.2 (R packages pspearman, v 0.2-5, mass, v7.3-30,nortest, v1.0-2). Plots and graphs were created using R Packages, lattice v0.20-27, plotrix, v3.5-5 and ggplot2, v0.9.3.1 [25].

Results

A total number of 47 variables and biomarkers together with indicators for metabolic and clinical history profiles, as well as detailed anthropometric profiles and traditional risk factors, were evaluated and are listed in Table 1. The multivariate test, assuming multivariate normality of the distribution did not result in more discoveries, (p-value=0.1530).

	Diabetics		Non-Diabetics		Total population	
	Mean	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**
Age (Years)	61.886	58.28-65.49	59.275	56.01-62.54	60.643	58-23-63.05
Disease Duration (Years)	11.295	9.562			5.94	8.907
Smoking (Years)	8.977	13.695	13.05	16.216	10.917	14.997
BMI	31.477	29.95-32.83	29.05	27.40-30.67	30.321	29.17-31.36
Waist circumf (cm)	106.273	102.9-109.65	100.125	95.42-104.83	103.345	100.48-106.21
Systolic pressure (mmHg)	139.818	134.19-145.44	129.675	126.05-133.30	134.988	131.47-138.51
Diastolic pressure (mmHg)	78.841	75.93-81.75	82	78.80-85.20	80.345	78.21-82.48
Pulse Rate (b/min)	74.386	70.78-78.00	72.675	70.23-75.12	73.571	71.39-75.75
MDRD	98.514	92.05-104.98	94.993	92.59-102.26	96.837	93.97-98.50
HCT (μmol/l)	41.545	40.49-41.45	42.45	41.38-43.29	41.976	41.20-42.59
INR	0.98	0.96-1.00	0.988	0.96-1.01	0.983	0.97-1.00
Appt (sec)	30.711	29.46-31.96	30.068	28.23-31.90	30.405	29.33-31.47
HBA1c (mmol/mol)	6.811	6.38-7.24	5.4	5.32-5.47	6.139	5.86-6.411
HbA2 (mmol/mol)	2.039	1.78-2.29	2.1	1.95-2.25	2.068	1.92-2.22
Glucose (mg/dl)	119.182	105.08-133.29	89.05	84.19-93.91	104.833	96.56-113.10
Urea (mg/dl)	37.159	33.56-40.75	35.35	32.84-37.86	36.298	34.10-38.49
Creatinine (mg/dl)	0.787	0.72-0.85	0.758	0.72-0.79	0.773	0.74-0.81
Cholesterole (mg/dl)	189.727	177.62-201.83	205.125	191.16-219.09	197.06	187.91-206.21
Triglycerites (mg/dl)	155.205	137.28-173.13	144.425	119.41-169.44	150.071	135.19-164.96
HDL (mg/dl)	51.636	48.02-55.25	53.9	49.58-58.22	52.714	49.97-55.46
LDL (mg/dl)	112.023	102.02-121.76	124.35	111.59-137.11	117.893	110.02-125.77
SGOT (mg/dl)	21.045	18.50-23.60	20.8	18.73-22.87	20.929	19.30-22.56
SGPT (mg/dl)	24.114	19.18-29.05	24.275	19.09-29.46	24.19	20.69-27.69
K (mmol/l)	4.461	4.32-4.60	4.243	4.20-4.50	4.357	4.31-4.51
Na (mmol/l)	139.773	138.98-140.56	140.725	140.21-141.24	140.226	139.74-140.71
D-DIMER (μg/ml)	0.491	0.320-0.662	0.365	0.308-0.422	0.431	0.338-0.524
Fibrinogen (g/l)	4.054	3.70-4.40	3.553	3.28-3.82	3.815	3.59-4.04
hsCRP (mg/l)	0.33	0.221-0.438	0.223	0.156-0.289	0.279	0.21-0.34

Homocysteine (μmol/l)	14.593	13.29-15.89	14.188	12.99-15.39	14.4	13.53-15.27
PAI1 (ng/ml)	22.207	16.75-27.66	25.925	20.35-32.82	23.977	20.20-28.32
Thrombomodulin (ng/ml)	27.773	24.799-30.747	27.253	24.69-29.81	27.525	25.59-29.463
VIII (%)	130.955	119.22-142.69	133.55	123.31-150.64	132.19	125.01-142.55
VWF (%)	132.455	124.22-140.68	132.225	127.01-144.22	132.345	128.11-139.77
sEPCR (ng/ml)	161.227	134.67-187.78	148.575	126.78-177.98	155.202	138.92-175.23

Table 1: Descriptive statistics for the diabetic, non-diabetic groups and total population.

The initial statistical comparison focused on the descriptive statistics (Tables 1 and 2) of all variables between the two groups. Both groups were found to have similar age distribution and gender ratio and similar anthropometric profiles (Tables 1 and 2).

		Diabetics		**Non-Diabetics**		**Total Population**	
	Group	**Abs. Freq.**	**%**	**Abs. Freq.**	**%**	**Abs. Freq.**	**%**
Gender	Male	24	54.55	19	47.5	43	51.19
	Female	20	45.45	21	52.5	41	48.81
Coronary Disease	Yes	7	15.91	1	2.5	8	9.52
	No	37	84.09	39	97.5	76	90.48
Hypertriglyceridemia	Yes	3	6.82	2	5	5	5.95
	No	41	93.18	38	95	79	94.05
Stroke	Yes	1	2.27	4	10	5	5.95
	No	43	97.73	36	90	79	94.05
Dyslipidaemia	Yes	27	61.36	24	60	51	60.71
	No	17	38.64	16	40	33	39.29
Hypertension	Yes	31	70.45	20	50	51	60.71
	No	13	29.55	20	50	33	39.29
Smoke status	Yes	9	20.45	16	40	25	29.76
(current smoker)	No	21	47.73	19	47.5	40	47.62
Prescribed statins	Yes	24	54.55	23	57.5	47	55.95
	No	20	45.45	17	42.5	37	44.05
Prescribed DPP4	Yes	34	77.27	0	0	34	40.48
	No	10	22.73	40	100	50	59.52
Prescribed	Yes	17	38.64	8	20	25	29.76
Anticoagulants	No	27	61.36	32	80	59	70.24
Prescribed	Yes	10	22.73	0	0	10	11.9
insulin	No	34	77.27	40	100	74	88.1
Prescribed	Yes	7	15.91	3	7.5	10	11.9
Omega-3 lipids	No	37	84.09	37	92.5	74	88.1
Sulfonylurea	Yes	22	50	0	0	22	26.19

	No	22	50	40	100	62	73.81

Table 2: Qualitative statistics for the diabetic and non-diabetic groups.

It is evident from the data of (Table 3), that there are no differences in the two groups in terms of age, gender, smoking status, BMI, and non-diabetic medication and only showed statistical difference with an adjusted p-value (FDR)<0.05 in the variables DPP4, Insulin, Sulfonylurea, HBA1c and glucose that are indeed only present in the diabetic group, as expected [2]. All other variable comparisons showed no significant statistical difference between the two groups.

Variable	(T-test/χ2)	(RS/Perm)	K-S	FDR adjusted p
DPP4	0	0	NaN	0
Insuline	0.00132	0	NaN	0
Sulfur	0	0	NaN	0
HBA1c	0	0	0	0
Glycose	0.00018	5E-06	0	4.8E-05
Age	0.28463	0.29222	0.32137	0.51255
Gender	0.51881	0.6605	NaN	0.84214
Systolic Pressure	0.00364	0.01533	0.10028	0.11167
Diastolic Pressure	0.14346	0.25281	0.26083	0.47773
BMI	0.02793	0.02975	0.10028	0.1686
Smoke Status	0.04627	0.0395	NaN	0.20145
Smoke Years	0.79955	0.4803	0.81767	0.76548
Smoking Years	0.21588	0.19118	0.81767	0.39001
Coronary Disease	0.03653	0.0535	NaN	0.24179
Cholesterol	0.09464	0.05696	0.08099	0.24179
Hypertension	0.05523	0.072	NaN	0.26229
Stroke	0.13493	0.19	NaN	0.39001
Waist circumference	0.03245	0.06163	0.11128	0.24179
Pulse Rate	0.43957	0.70307	0.48531	0.87455
Hyperlipidaemia	0.72503	1	NaN	1
Dyslipidaemia	0.8983	1	NaN	1
HbA2	0.68224	0.01402	0.10566	0.11167
LDL	0.12067	0.07991	0.04875	0.27171
Anticoagulants	0.06207	0.093	NaN	0.27361
Urea	0.41621	0.63115	0.43668	0.84214
Fibrinogen	0.02723	0.02788	0.03008	0.1686
Na	0.04943	0.09076	0.22875	0.27361
a PTT	0.55309	0.12003	0.28397	0.30607
CRP HS	0.10113	0.13726	0.43668	0.31819
K	0.28008	0.18898	0.30618	0.39001
PAI1	0.28621	0.25406	0.50743	0.47773
VWF	0.59366	0.52575	0.61957	0.81253
SEPCR	0.63155	0.56534	0.6058	0.84214
Homocysteine	0.64697	0.86488	0.89027	0.93848
VIII	0.4989	0.62181	0.71604	0.84214
TM	0.79152	0.85779	0.91457	0.93848
HCT	0.19911	0.26228	0.50209	0.47773
HDL	0.41634	0.33975	0.45259	0.55894
D-DIMER	0.17903	0.09771	0.60724	0.27361
MDRD	0.79102	0.64496	0.56941	0.84214
Creatinine	0.41438	0.64759	0.53638	0.84214
SGPT	0.96379	0.78448	0.60724	0.93848
Prescribed Omega Lipids	0.2346	0.3015	NaN	0.51255
Statins	0.7853	0.839	NaN	0.93848
PLT	0.90276	0.85782	0.90281	0.93848
SGOT	0.88192	0.86086	0.91457	0.93848
INR	0.61255	0.8894	0.76796	0.94499

Table 3: Statistical analysis of all the variables.

Correlations between variables in the diabetics group

Thrombomodulin has a very strong positive correlation (r=0.730 p. adj<0.000001) with creatinine in the diabetics group. Equally strong is homocysteine's correlation with creatinine with a positive correlation (r=0.670, p. adj<0.00001). High values of thrombomodulin and homocysteine are correlated with a rise in creatinine and thus affect renal function of the diabetic patients as seen in (Figure 2A). In the non-diabetics group the correlations are different. sEPCR is shown to have a relative strong negative correlation (r=-0.449 p. adj=0.0417) in renal function as measured with MDRD shown in (Figure 2B).

Total population correlations

Treating the two groups as a single population other correlations became evident, most important shown in (Figure 2C). Factor VIII is seen to be strongly correlated with age (r=0.419, p. adj<0.001). Also hsCRP is positively correlated with the subjects weight (r=0.352, p. adj<0.01), more so if the BMI is considered (r=0.51, p. adj <0.00001). Also evident, is the effect that thrombomodulin has to the renal function in the whole population, as it has a relative strong negative correlation with MDRD measurements (r=-0.510, p. adj<0.00001) and

a strong positive correlation (r=0.640, p. adj <0.000000001) with creatinine. Similarly a negative correlation exists with homocysteine and MDRD (r=-0.410, p. adj<0.001) coupled with a very strong positive correlation with creatinine r=0.500, p. adj<0.00001). Factor VIII, on the other hand, is only negatively correlated with MDRD (r=-0.322, p. adj<0.01). Fibrinogen also shows a negative correlation, albeit weaker, (r=0.380 p. adj<0.01) with INR.

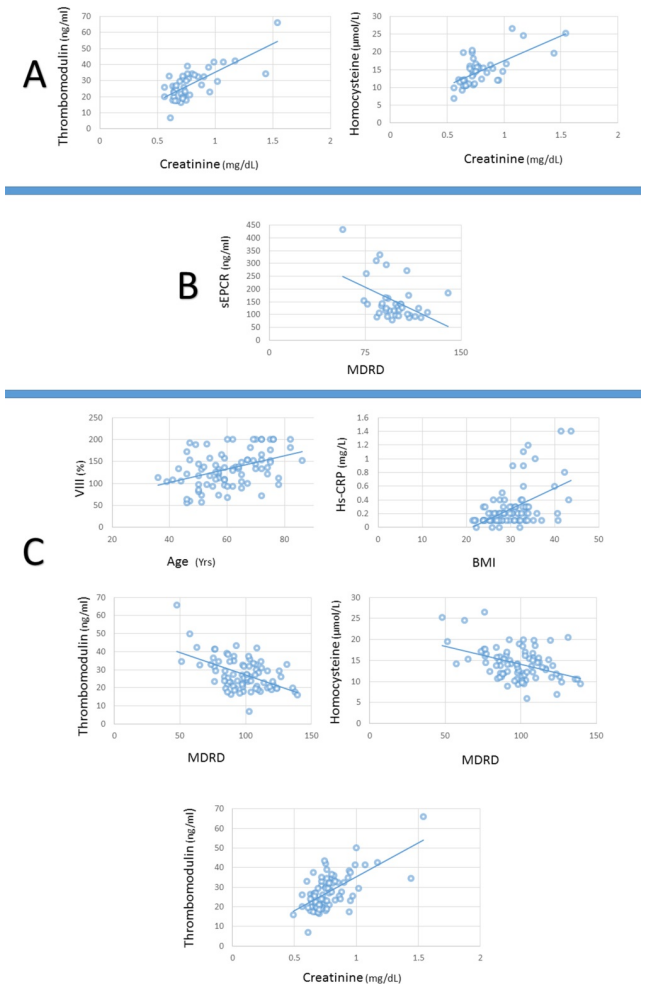

Figure 2: A) Correlations in the diabetics group for Thrombomodulin and Creatinine (r=0.730 p. adj<0.000001) and Homocysteine and Creatinine (r=0.670, p. adj< 0.00001). **B)** Correlations in the non- diabetics group for sEPCR (r=-0.449 p.adj=0.0417) with renal function as measured with MDRD. **C)** Correlations for the total population for Factor VIII with age (r=0.419, p. adj<0.001), CRP with the subjects weight (r=0.352, p.adj<0.01), and with BMI (r=0.51, p. adj<0.00001).

Discussion

Very little is known for indicators such as thrombomodulin and sEPCR about their potential role as diagnostic markers in T2 diabetes, to date there are few studies that have been published and for smaller numbers of patients. [10,11,14,15] The present study evaluated thrombomodulin [14,15] and sEPCR, amongst other well established indicators of thrombosis and endothelial inflammation, namely, [15] factor VIII, [16] (PAI 1), [17](VWF), [18-20] fibrinogen, d - dimers, hsCRP [21] and homocysteine [22] in a subset of Greek subjects with and without T2 diabetes, of similar age, similar distribution in terms of gender and nearly identical anthropometrically. A total number of 47 variables including biomarkers together with indicators for metabolic and clinical history profiles, as well as detailed anthropometric profiles and traditional risk factors, were evaluated.

The biomarkers associated with the onset and progression of T2 diabetes, DPP4, Insulin, use of Sulfonylurea, high HBA1c and glycose levels, were clearly statistically differentiated in the two groups, while no other biomarkers including thrombomodulin and sEPCR were found to be different.

Interestingly, high values of thrombomodulin and homocysteine, in the diabetic patients group, were correlated with a rise in creatinine [26] and thus could act as markers for renal function in T2 diabetes. Furthermore sEPCR was shown to have a relative strong negative correlation with renal function status as measured with MDRD in the non-diabetics group, indicating its value as another potential marker for renal function which is in agreement with the latest international findings [27].

The strengths of the current study include the complete concordance between the two groups in age, anthropometric elements, smoking habits and relevant clinical history profiles as well as the extensive number of factors tested, and the detailed and strict statistical analysis of the data. The main limitation of the study is the relative small number of subjects under investigation.

In conclusion the presence of T2 diabetes in conjunction with age, clearly correlates with problems in renal function, with thrombomodulin and homocysteine serving as indicators for renal damage in diabetics but not in healthy individuals as it has been previously extensively described [27]. sEPCR on the other hand could be a potential generic indicator for renal damage in agreement with very recent research. Thrombomodulin and sEPCR as prothombotic agents, did not show any indication that they can be utilised as markers for the prevention and/or treatment of thrombotic complications in diabetic patients a conclusion that clearly a larger scale study could strengthen.

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