

## **Preliminary study of the fibrin structure in hypertensive, dyslipidemic and type 2 diabetic patients.**

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**Key words:** polymerization, permeation, compaction, fibrin structure, fibrin lysis, hypertension, dyslipidemia, type 2 diabetes.

**Abstract.** Individuals with hypertension, dyslipidemia or diabetes are at a higher risk to suffer cardiovascular disease than other people; while impaired fibrin structure/function may contribute to further raise the cardiovascular risk in the former. The purpose of this work was to study the fibrin network and fibrin degradation properties in hypertensive (HT) patients, pharmacologically treated,  $124 \pm 11$  mmHg, systolic blood pressure, and  $70 \pm 10$  mmHg, diastolic blood pressure,  $n = 12$ ; metabolic dyslipidemic patients (DL), cholesterol:  $5.7 \pm 1.5$  mmol/L,  $n = 10$ ; patients with type 2 diabetes mellitus (T2D), fasting plasma glucose:  $8.8 \pm 2.2$  mmol/L,  $n = 10$ ; and a control group of healthy individuals,  $n = 9$ . The fibrinogen concentration was determined by the gravimetric method. Fibrin network formation and porosity were assessed by turbidity and permeation techniques, respectively; fibrin elastic properties were evaluated by compaction and fibrin lysis, by turbidity after addition of external tPA prior to plasma clotting. Fibrinogen concentration was significantly higher only in T2D patients ( $p = 0.004$ ), compared to the control group. The fibrin polymerization and lysis processes were similar for all patient and control groups. Permeation was significantly slower in DL and T2D patients,  $p = 0.022$  and  $0.0002$ , respectively, whereas the compaction coefficient was significantly smaller in T2D patients,  $p = 0.0015$ . Our results suggest that the fibrin structure was altered in DL and T2D patients, probably due to the increased cholesterol and glycation, respectively.

## **Estudio preliminar de la estructura de la malla de fibrina en pacientes hipertensos, dislipidémicos y diabéticos tipo 2.**

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**Palabras clave:** polimerización, permeabilidad, compactación, estructura de la fibrina, lisis de la fibrina, hipertensión, dislipidemia, diabetes tipo 2.

**Resumen.** Individuos hipertensos, con dislipidemia o diabetes tipo 2 tienen un mayor riesgo de sufrir enfermedades cardiovasculares, y la alteración en la estructura/función de la malla de fibrina aumenta su riesgo. El propósito del presente trabajo fue estudiar la estructura de la malla de fibrina y su degradación en pacientes hipertensos (HT), tratados farmacológicamente, con una presión arterial sistólica de  $124 \pm 11$  mmHg y una presión arterial diastólica de  $70 \pm 10$  mmHg,  $n = 12$ ; pacientes con dislipidemia metabólica (DL), colesterol  $5,7 \pm 1,5$  mmol/L,  $n = 10$ ; pacientes con diabetes mellitus tipo 2 (DT2), glicemia en ayunas:  $8,8 \pm 2,2$  mmol/L,  $n = 10$ ; y un grupo control de individuos sanos,  $n = 9$ . Se determinó la concentración de fibrinógeno por el método de la pesada. La formación de la malla de fibrina y su porosidad fue estudiada mediante las técnicas de turbidimetría y permeabilidad, respectivamente; las propiedades elásticas por compactación y la degradación de la fibrina por turbidimetría, añadiendo externamente el activador tisular del plasminógeno (tPA) antes de la coagulación del plasma. Se encontró que la concentración de fibrinógeno fue significativamente mayor solamente en los pacientes DT2 ( $p = 0,004$ ), en comparación con el grupo control. El proceso de polimerización y degradación de la fibrina de los pacientes fue similar a la del grupo control. La permeabilidad estuvo disminuída significativamente en los pacientes DL y DT2,  $p = 0,022$  and  $0,0002$ , respectivamente, mientras que la compactación fue significativamente mayor solamente en los pacientes DT2,  $p=0,0015$ . Nuestros resultados sugieren que la estructura de la malla de fibrina estuvo alterada en los pacientes DL y DT2, probablemente debido al aumento en los valores de colesterol y glicemia, respectivamente.

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## **INTRODUCTION**

Several important factors have been determined that may contribute to overall cardiovascular risk. Among them, impaired endothelial function leads to atherogenesis, which is a local and systemic inflammatory response that triggers plaque formation, and eventually may end up in plaque fissures or rupture and thrombus formation (1, 2). In spite of the improved knowledge

of the atherosclerotic process, the prediction of cardiovascular risk still relies mainly on classical risk factors like hypertension, hypercholesterolemia, diabetes, smoking, advanced age and gender (3). Abnormalities of the vessel walls, of hemostatic and fibrinolytic factors, of platelets and of blood flow, can precipitate and may explain most of the thrombotic complications. The vascular endothelium keeps blood fluidity, modulates blood coagulation, promotes or

prevents vascular growth, modulates inflammation and regulates vasomotor tone (4). The renin-angiotensin and the kallikrein-kinin systems are powerful regulators of these processes.

Hypertension, hypercholesterolemia and diabetes may initiate endothelial damage. Essential hypertension is a heterogeneous disorder that develops most likely due to several overlapping subsets of pathophysiological mechanisms (5). Although hypertension imposes increased pulsatile stresses on blood vessels, the major complications in hypertensive patients are thrombotic rather than hemorrhagic events (6). Likewise, metabolic dyslipidemia is characterized by high circulating triglycerides (TG) and low HDL-cholesterol levels (4), and it is tightly correlated with both hyperinsulinemia, even in the absence of diabetes (7), and with non-alcoholic fatty liver (8). Type 2 diabetes and its preceding signals, such as impaired glucose tolerance and syndrome of insulin resistance, are associated with a highly increased risk of thrombosis. Although the effects of traditional risk factors for atherosclerosis, like hypertension and hypercholesterolemia are increased in type 2 diabetes, these factors account for only half of the observed increased risk for cardiovascular disease (CVD) (9). Additional risk factors have been implicated, such as alterations of fibrinolysis and coagulation, secondary to insulin resistance (10-12). Moreover, recently it has been demonstrated that hyperinsulinemia inhibits fibrinolysis irrespective of glucose concentrations, whereas hyperglycemia stimulates coagulation irrespective of insulin concentrations (13). Fibrinogen is regarded as an independent risk factor of cardiovascular disease, and it happens to be a marker of coagulation and inflammation, and also influence fibrinolysis (14). It is well known that clots formed at high fibrinogen concentration show a peculiar

clot structure characterized by thin fibers, reduced pore size and increased tensile strength (15, 16); also, this clot structure is degraded at a lower rate by plasmin, its physiological enzyme (15).

The aim of the present study was to investigate the structure of *in vitro* fibrin clots from patients diagnosed as hypertensive, dyslipidemic or type 2 diabetes. The fibrin network formation and lysis was studied by turbidity, clot porosity by permeation and their tensile strength by the compaction technique.

## MATERIALS AND METHODS

All chemicals used were Analar® Grade. Bovine thrombin was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Trasylol was acquired from Bayer (Germany), tissue-type plasminogen activator (tPA) from American Diagnostica (Greenwich, Connecticut, USA) and soya lecithin from Now Natural Foods (Bloomington, Illinois, USA).

## Subjects

Forty-one subjects were randomly selected from the Internal Medicine, Diabetes, Cardiology and Emergency Care units of the Francisco Antonio Rísquez Hospital, Caracas, Venezuela, from January to July 2005. They were of the "mestizo" ethnic type and only one caucasian (dyslipidemic); black people were not included in this study. Blood and urine analyses, electrocardiograms (ECG), chest RX, echocardiograms and ultrasonographies were performed. Subjects were classified as hypertensive-stage I (HT), blood pressure  $\leq 140/90$  mmHg, treated with diuretic drugs, angiotensin-converting enzyme inhibitors,  $\beta$ -blockers, angiotensin-receptor blockers, calcium channel blockers, vasodilators and others,  $n = 12$ . Patients with metabolic dyslipidemia (DL) had LDL  $< 2.6$

mmol/L, HDL <1.1 mmol/L and triglycerides >1.7 mmol/L, without lipid lowering therapy, normoglycemic and normotensives (systolic BP 120 to 139 mmHg and/or diastolic BP 80 to 89 mmHg),  $n = 10$ . Type 2 diabetes (T2D) patients with fasting plasma glycemia less than 11.1 mmol/L, treated with hypoglycemic agents or insulin, normotensive and normal lipids profile,  $n = 10$ ; and a control group of  $n = 9$ .

Subjects with microvascular complications (history of retinopathy, proteinuria/microalbuminuria on analyses of urine samples provided at the time of recruitment) and/or macrovascular complications (history of coronary artery disease, cerebrovascular disease or peripheral vascular disease, at recruitment), comorbidity with other pathologies (thyroid, infections, hematologic, hepatic), pregnant women, alcoholic and smoking habits, were excluded. None of the subjects were taking anticoagulant (warfarin or heparin) or antiaggregant medication (aspirin or clopidrogel).

All subjects signed an informed consent and ethical approval was obtained from the Ethics Committee of the Francisco Antonio Rísquez Hospital.

### Blood sampling

Fasting (12 hours) venous blood samples were drawn with minimal stasis, discarding the first 3 mL of blood and collected into 3.8% sodium citrate. The blood was centrifuged at  $1400 \times g$  at  $4^{\circ}\text{C}$  for 20 min. Trasylol® was added to the separated plasma at 200 units/mL, except to the plasma to be used for fibrinolysis experiments. Aliquots of plasma were kept at  $-80^{\circ}\text{C}$  until further use. The various studies were performed during the following 7 days after blood samplings. Plasma fibrinogen was quantified by means of the gravimetric method of Ingram (17).

### Polymerization

Plasma samples were diluted (1:10) with Tris-buffered saline (TBS) (50 mM Tris, 0.15 M NaCl, pH 7.4) and clotted with a solution of 0.6 units/mL of bovine thrombin and 20 mM  $\text{CaCl}_2$  (final concentrations). Fibrin polymerization was followed by turbidity. The increase in optical density (OD) was recorded at 350 nm every 15 sec for 15 min in a Genesys 6 spectrophotometer, Spectronic Instruments (Rochester, NY, USA). Fibrin polymerization curves were characterized by measuring three parameters: the lag phase, the slope or the maximum rate of increase in OD with time, and the final or maximum turbidity. These parameters were calculated from the averaged values of three replicates.

### Internal fibrinolysis

The procedure was essentially the same as for fibrin polymerization with the difference that  $0.5 \mu\text{g/mL}$  of tPA was mixed before adding the thrombin- $\text{CaCl}_2$  solution. The change in OD was recorded at 350 nm every 15 sec during 60 min or after the OD reached a value close to zero. In order to evaluate the internal fibrin lysis process, two parameters were measured: the time required to dissolve 50% of the clot (based on the maximum turbidity reached) and the rate of fibrin lysis, that is, the slope of the linear part of the descending limb of the curve after the maximum turbidity. Three replicates of each sample were performed.

### Permeation

Permeation experiments were performed essentially as described elsewhere (18). Briefly,  $100 \mu\text{L}$  of undiluted plasma were mixed with a solution of 1 unit/mL of bovine thrombin and 20 mM  $\text{CaCl}_2$  (final concentrations). The mixture was immediately transferred to a plastic column (inter-

nal diameter: 0.2 cm). The columns were left in a moist environment at room temperature for two hours and then the top part of the column was filled with TBS. The clot was perfused with buffer, to wash out plasma proteins that had not been incorporated into the formed clots, before starting the flux measurements. The flux was calculated from the weight of the drops that percolated in a given time, which is equivalent to the volume of a drop with a density of 1 g/mL (approximately the same as that of water at room temperature). Six recordings for each clot (three replicates of each sample) were taken.

### Compaction

The compaction of fibrin has been inversely correlated to the Young's modulus of elasticity of the network and with the final strength at the breaking point (19). Fibrin networks were prepared essentially as described elsewhere (20), with some minor modifications. Samples of 450  $\mu$ L of plasma were clotted with 50  $\mu$ L of thrombin-CaCl<sub>2</sub> (1 unit/mL and 20 mM respectively, final concentrations) in 1.5 mL Eppendorf microcentrifuge tubes, previously coated with a solution of 20% lecithin in carbon tetrachloride to render the surface non-adherent. The tubes were left overnight at room temperature. Then, clots were centrifuged at 12500 rpm in a Beckman Microfuge 11 (USA) from 5 to 60 sec. The volume of the fluid expelled from the network was measured with a 100  $\mu$ L Hamilton syringe and expressed as a percentage of the initial volume of the network. Three replicates of each sample were measured.

### Statistical analyses

Regression analysis was performed between the results of polymerization (final turbidity), fibrin degradation (50% of lysis), permeation, compaction coefficient (at 60

sec) and age, BMI and gender. Since the multiple correlation coefficients were  $< 0.5$ , the means were not adjusted for age, BMI and gender. Differences between the mean values of the results of the various measurements carried out on each group of subjects were evaluated using the Mann-Whitney U test. Spearman's Correlation Coefficient was used to evaluate the association between fibrinogen concentration and kinetic parameters of fibrin formation, and between fibrinogen concentration, fibrin lysis rate and the slope of the fibrin lysis process. Statistical significant differences were accepted at  $p < 0.05$ .

## RESULTS

### Subjects characteristics

Of the subjects's parameters evaluated throughout the present study, the ages of the DL and T2D patients were similar to controls; however, HT patients were older than controls. Fibrinogen concentration was higher only in T2D patients ( $p < 0.05$ ). Table I summarizes these features of the studied subjects. Of the HT and DL patients 83% and 80%, respectively, were women.

### Fibrin polymerization

The fibrin polymerization process, evaluated measuring the lag phase, rate and maximum turbidity, were similar in the controls and the three groups of patients studied: HT, DL and T2D,  $p > 0.05$ . There was a great variability within the subjects in each group, reflected by the high standard deviation values. The highest final turbidity values were found in T2D patients. These results are shown in Table II. The correlation coefficient analyses performed between the three parameters of fibrin polymerization and fibrinogen concentration, in patients and controls, revealed that there was no association between these variables.

**TABLE I**  
GENERAL FEATURES OF CONTROL SUBJECTS AND HYPERTENSIVE (HT), DYSLIPIDEMIC (DL), OR TYPE 2 DIABETIC PATIENTS (T2D)

Parameter	Control	HT	DL	T2D
Sex (M/F)	4/5	2/10	2/8	6/4
Age in years	50 ± 8	62 ± 11 (p = 0.0104)	55 ± 9	54 ± 12
Fg (g/L)	3.02 ± 0.49	3.84 ± 1.26	3.36 ± 0.77	4.73 ± 1.83 (p = 0.004)
BMI (Kg/m <sup>2</sup> )	26 ± 4	25 ± 2	25 ± 4	23 ± 2
Cholesterol (mmol/L)	4.5 ± 0.2	4.7 ± 0.6	5.7 ± 1.5	4.6 ± 0.6
Triglyceride (mmol/L)	0.9 ± 0.02	1.2 ± 0.4	1.9 ± 1.0	1.7 ± 0.3
FPG (mmol/L)	4.4 ± 0.6	5.0 ± 0.5	4.9 ± 0.4	8.8 ± 2.4
Systolic BP (mmHg)	119 ± 13	124 ± 11	115 ± 11	116 ± 11
Diastolic BP (mmHg)	70 ± 10	74 ± 8	68 ± 11	71 ± 9

BMI: Body mass index. FPG: Fasting plasma glucose. BP: Blood pressure. Data are given as means ± SD. The one-sided p-value is showed in brackets in those cases that the results were found statistically significant.

**TABLE II**  
PLASMA FIBRIN POLYMERIZATION, FIBRINOLYSIS AND PERMEATION IN HYPERTENSIVE (HT), DYSLIPIDEMIC (DL) OR TYPE 2 DIABETIC SUBJECTS (T2D), IN COMPARISON WITH CONTROLS

	Control (n=9)	HT (n=12)	DL (n=10)	T2D (n=10)
Polymerization				
Lag phase (sec)	60 ± 80	100 ± 74	83 ± 56	113 ± 54
Slope (ΔOD/sec) × 10 <sup>-3</sup>	2.0 ± 1.5	1.6 ± 1.4	1.3 ± 1.5	2.6 ± 2.8
Maximum <i>t</i> (OD units)	0.170 ± 0.091	0.122 ± 0.050	0.155 ± 0.117	0.207 ± 0.218
Fibrinolysis				
50% Lysis(sec)	660 ± 312	1187 ± 929	743 ± 267	671 ± 340
Slope (ΔOD/sec) × 10 <sup>-3</sup>	0.44 ± 0.31	0.46 ± 0.25	0.35 ± 0.26	0.11 ± 0.12
Permeation				
Flux (g/sec) × 10 <sup>-4</sup>	2.6 ± 1.5	1.5 ± 1.1	1.6 ± 0.6 (p = 0.022)	0.8 ± 0.6 (p = 0.0002)

OD: optical density. Values are presented as means ± SD. The one-sided p-value is showed in brackets in those cases that the results were found statistically significant.

### Internal fibrinolysis

The fibrin lysis process was evaluated by the calculation of the slope (in the linear part of the OD decrease as a function of time) and by the time required for 50% of

clot lysis. There were no differences between these two parameters when comparing the patients with the control group. The great variability between the subjects in each group is reflected by the large stan-



dard deviation values, as was the case for the polymerization process. Although the slope of fibrin lysis was diminished in T2D patients compared to the other groups of patients and controls, the difference was not statistically significant. The results of these tests are shown in Table II. The results of clot lysis of two of the DL patients were not included in this Table, as their times for 50% lysis surpassed 3600 sec.

When correlation analysis between the 50% of clot lysis, the rate of fibrin lysis and fibrinogen concentration was performed, it was found that only in HTA and T2D patients there was a positive correlation between those parameters.

### Permeation

The porosity of the fibrin network was quantified by measuring the rate of buffer percolation through the clot. All the three groups of patients showed diminished rates in relation to the control group, but only in DL and T2D patients, the differences were statistically significant ( $p = 0.022$  and  $0.0002$ , respectively) in comparison with the controls. These results are shown in Table II.

### Compaction

The percentage of compaction, an indirect measure of the grade of compressibility of the fibrin network, related to the fibrin network structure, was decreased, only in T2D patients ( $p = 0.0015$ ), from 10 sec up to 60 sec follow-up time. In HT and DL patients, the compaction values were similar to controls (Fig. 1). This means that the fibrin networks of T2D patients were the most rigid compared to the other groups.

## DISCUSSION

The present study included patients who had been diagnosed for hypertension (HT), dyslipidemia (DL), or type 2 diabetes mellitus (T2D) and a control group of

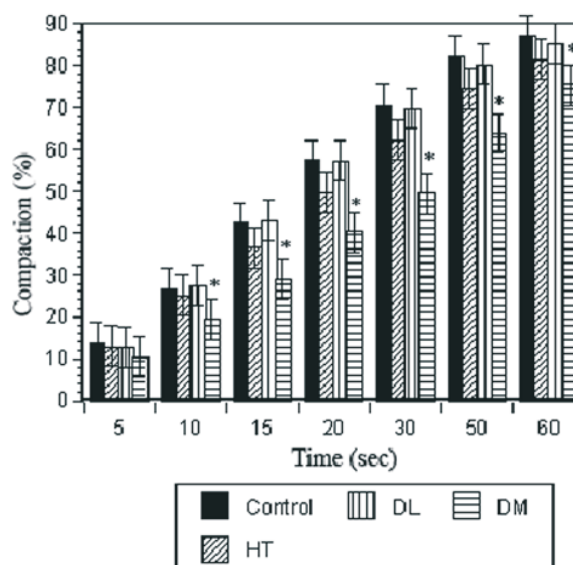


Fig. 1. Compaction of fibrin networks at various centrifugation times. The bars represent means  $\pm$  SD and correspond to controls (■), HT patients (//), DL patients (||) or T2D patients (≡). \*The compaction values of T2D patients were significantly lower ( $p < 0.05$ ) than those of the other groups, over the range from 10 to 60 seconds.

healthy subjects, that were normotensive, normoglycemic and showed normal lipidic profiles (cholesterol and triglycerides less than  $5.13$  mmol/L and  $1.69$  mmol/L, respectively). The control group was tested as a reference, in order to compare fibrin formation, lysis and elastic properties. This is the first time that the characteristics and features of fibrin networks are examined in hypertensive and/or dyslipidemic patients. Even though this work was carried out using a small number of patients, it revealed clearly that patients diagnosed for type 2 diabetes show unique features of the properties of their fibrin networks.

The group of patients with the highest values of fibrinogen was the T2D. It ought to be pointed out that despite diabetes patients were under pharmacological treatment, fibrinogen levels did not normalize.

Interestingly, it has been stated that fibrinogen levels often are increased in diabetic patients, especially in those with vascular complications or altered metabolic control (21). Fibrinogen represents a reactant of acute phases, showing a large increase throughout inflammatory processes. Hypertension, dyslipidemia and diabetes may be regarded as conditions that induce a chronic inflammatory state on the walls of blood vessels (21, 22).

The process of formation of fibrin network showed large individual differences within all groups (both patients and controls), as evidenced by the high values of standard deviations. Although differences were not statistically significant, polymerization was faster in diabetic patients than in the other groups or the controls, possibly due to the increased fibrinogen levels in the former.

Nair and collaborators (23) had already reported that in poorly controlled diabetic patients, with a mean glucose value of 15 mmol/L, the mass/length ratio of fibrin (which is directly proportional to the thickness of the fibrin fibers) and its permeability (a measure of the pore size of the mesh) were diminished, while the tensile strength of the fibers, as measured by compaction remained unchanged. On the other hand, recent studies such as that of Pitiers and collaborators (24), on type 2 diabetic patients with average glucose levels of 14.6 mmol/L, did not detect differences in polymerization, permeability or fibrin network compaction between those patients and healthy controls. In our study, both the tensile strength (measured by compaction) and the permeability of fibrin from diabetic patients were significantly decreased. Such observed differences may be due to the selection of the various groups or the controls, or the chosen coagulation conditions for each study.

Our findings revealed that the degradation of the fibrin network was much slower, though not significantly so, in the DL and T2D patients than in the other groups; also, in two of the DL patients the dissolution process was so slow, that their data were not included in the Results. A recent work by Dunn et al. (25) in which they evaluated the lysis process starting with purified fibrinogen, in 150 subjects with type 2 diabetes has shown that the disaggregation of the fibrin mesh was significantly slower in those patients. It must be pointed out that fibrin fibers formed from plasma samples are two to three times thicker than the purified ones (26) and hence, their lysis may be much faster than that of fibrin networks formed from purified fibrinogen. Various studies, both *in vivo* and *in vitro*, have shown that high blood glucose levels induce the non-enzymatic glycosilation of fibrinogen in the lysine residues (27-29) and that this population of glycosilated fibrinogen molecules is responsible for the structural alterations and resistance to lysis of the fibrin network of diabetic patients (30). These parameters tend to show larger alterations in these patients when the blood glucose levels are not thoroughly controlled (31). Therefore, it seems likely that if our diabetic patients had not been on hypoglycemic treatment, such differences would have emerged more clearly. The available literature does not mention comparable studies using dyslipidemic patients such as those examined by us. It is known that lipoprotein (a), due to its structural resemblance of plasminogen, competes with it for the linkage to the lysine residues exposed on fibrin (32, 33); thus, a slower lysis of the fibrin network of such patients is to be expected. Furthermore, the high lipid content reduced the pore size of the fibrin network, reflected in the diminished permeation values.



The way that fibrin is build up determines its structure, and fibrinolysis speed is related to fibrin structure (34). Furthermore, the fibrin elastic properties are both related to fibrin structure and fibrin cross-linking by activated factor XIII (35). If fibrin structure is altered in such an extent that affects the fibrinolytic process, in addition to an altered endothelium phenotype, the occlusion of the blood vessel is more probable and the reestablishment of the vessel patency slower.

To conclude, the present work confirms others studies that indicated that the fibrin network structure is less porous in type 2 diabetic patients than in other people. Moreover, our findings also revealed that the tensile strength of the network increases in these patients, whereas in the hypertensive or the dyslipidemic ones is not significantly altered in relation to healthy controls. It may well be that by choosing patients with higher cholesterol or triglycerides levels, further differences can be detected in comparison with otherwise healthy and normolipemic individuals.

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