Research Article

P-Selectin as a Platelet Activation Marker and Cardiovascular Risk Prediction Factor. Differences between its Two Isoforms Using Flow Cytometry and Elisa Analyses

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Abstract

Background

Cardiovascular diseases are the main cause of death in developed countries. Increased platelet activation and reactivity are critical processes in arterial thrombogenesis and are cardiovascular disease precursors. Although there is much research done related to platelet activation markers, at present a strongly conclusive prognosis marker is not yet verified.

Methods

In this study we analyzed samples of 57 volunteers by flow cytometry and ELISA to determine the levels of mCD62p, sCD62p and CD40L as platelet activation markers with predictive significance for cardiovascular risk.

Result

The mean percentage value of platelet activation in diabetic patients with ischemic cardiopathy was the highest, although no statistical difference was found in data from flow cytometry analysis. Statistically significant changes were found in sCD62p (p =0.004) and CD40L (p =0.045) values when comparing ischemic females and males, respectively. Among women, presence of ischemia caused significant differences in CD40L values (p =0.023). Pearson correlation test was negative among CD62p isoforms in all samples tested.

Conclusion

This is the first report in which both isoforms from the same volunteers were simultaneoulsy evaluated A sexual dimor-

phism was detected for sCD62p and CD40L as platelet activation markers by ELISA assay. Our results were more consistent with Reynolds' cardiovascular risk classification. Our investigation affords valuable information regarding the investigation of new anti-aggregation therapies as well as treatments involving either double anti-aggregation therapy or threefold therapy including anticoagulants.

Keywords: Platelets; P-selectin; CD40L

Introduction

Cardiovascular diseases are the main cause of death in developed countries [1]. Twenty seven percent of total deaths were caused by cardiovascular diseases, according to 2012. Uruguayan Health State Department reports [2]. Diabetes mellitus (DM) is frequently associated with vascular complications. DM patients have 2 to 4 times more probabilities to suffer macrovascular diseases and DM abnormal metabolic conditions are responsible for changes leading to atherosclerosis [3-6].

Increased platelet activation and reactivity are crucial mechanisms in arterial thrombogenesis and are accepted as cardiovascular disease precursors. Although there is a variety of studies regarding platelet activation markers [1,4-23], at present a reliable prognosis marker is not yet validated. It is well-known that DM patients suffer platelet aggregation and exhibit decreased sensitivity to anti-aggregation agents. Moreover, chronic hyperglycemia is known as an *in vivo* platelet activation promoting factor. Hyperglycemia produces an alteration on the platelet membrane increasing receptors' expression, such as P-selectin and GPIIB/IIIa[3].

P-selectin (CD62p) is one of the several cellular adhesion molecules that was identified by using monoclonal antibodies (MoAb) specific for thrombin-activated platelets. CD62p is part of the alpha granules membrane inside the platelet and it is expressed on the platelet membrane immediately after activation (mCD62p). There is also a soluble form (sCD62p) that differs in approximately 3 kDa and lack the trans-membrane domain [24-30]. Both isoforms (mCD62p and sCD62p) promote clotting, whereas high sCD62p concentrations selectively inhibit mCD62p adhesion, competing with the ligand. Therefore, therapeutic sCD62p could be used in treatments for different hematologic and vascular pathologies as an anti-thrombotic agent. Hence, the accumulated evidence suggests that CD62p can be used as: a) a platelet activity marker on the membrane; b) a platelet marker on plasma; c) a predictive molecule for some pathologies and; d) a possible therapeutic target [17,26,30-32].

Protein CD40L is a member of the Tumor Necrosis Factor family (TNF) and is part of the platelet membrane. Once activated, *in vitro* platelets express a considerable quantity of membrane CD40L. This process is also observed in *in vivo* thrombus. Therefore high levels of sCD40L in blood are predominantly caused by activated platelets [33-40]. In the present work mCD62p, sCD62p and CD40L were evaluated as platelet activation markers with a predictive value for cardiovascular risk.

Materials and Methods

A transverse study, case-control double-blind comparison was performed. We selected 57 volunteers that were classified as 40 patients from a public and a private hospital, and 17 healthy controls, all aged between 39 and 78 years old. For this study, the following inclusion criteria were taken in consideration: a) type 2 DM was diagnosed according to the "American Diabetes Association" (ADA, 2011); b) presence of ischemic coronary disease was demonstrated and documented by one or both of the following conditions: 1) diagnosis criteria of the "Report of the ACC/AHA Task Force on Practice Guildelines Committee"; 2) coronary angiography analysis. To perform catheterization, critical injuries were considered as those presenting a significant reduction of the blood flow determined as decrease of either 50% of diameter or at least 70% of section of blood vessel, respectively. Two negative ergometry studies were understood as absence of cardiovascular episodes.

Exclusion patient criteria employed were: a) patients with no ischemic coronary disease studies performed and b) putative controls with renal failure.

In agreement to the physician team patients were analyzed and classified as follows: a) 17 DM individuals with ischemic coronary disease, b) 11 DM patients without ischemic coronary disease; c) 12 non-DM individuals with ischemic coronary disease and; d) 17 healthy control volunteers.

Control volunteers were selected according to the ensuing criteria: a) absence of diabetes, b) no history of blood transfusions, c) no intake of medication or drugs interfering with platelet activation, d) no previous or present cancer disease; and e) no dyslipidemia or uncontrolled hypertension. Furthermore, liver function studies, WBC counts and corporal temperature controls were also performed.

Once the patients were selected, physicians were instructed to begin the preparatory studies in each health center to ensure similar conditions among patients at the time when the biological sample was obtained. Under the supervision and control of the treating physicians, all drugs which could interfere with platelet aggregation were suspended seven days prior to blood collection (warfarin, clopidogrel and aspirin). Therapeutic doses were similar in all patients.

Since elevated postprandial lipemia affects P-selectin and CD40L expression [41,42], 12 hours of fasting as well as 15-30 min of rest was strictly required to volunteers prior to blood

extraction. Peripheral blood was drained with a BD Vacutainer butterfly device 21G x ¾" x 7" from 14.00 to 17.00 hours, in order to discard circadian variations. Blood was collected in Vacutainer tubes of 4,5 mL citrated at 0.129 M (3.8%). The first 2 mL of the collected blood were discarded in order to avoid platelet activation artifacts.

All samples were analyzed by flow cytometry (FC) and ELISA.

Flow cytometry

Blood samples were labeled with mouse antiCD62p MoAb conjugated with Phycoerythrin (PE) and antiCD61 conjugated with Peridinin Chlorophyll Protein Complex (PerCP) (ab42795 and ab91130, Abcam, Cambridge, Massachusetts, respectively). CD61 was used as a platelet marker and CD62P as platelet activation marker. Double positive events were considered as activated platelets in FC studies. The antibody titer was performed with 0.025 U mL^{-1} of thrombin for 5 minutes. 5 μL of blood sample was taken and diluted in 40 μL of PBS. Each sample was incubated for 30 min at room temperature using 5 μL of each MoAb diluted in 100 μL of 10 mM, pH 7 PBS. Finally the specimen fixation was performed with 1% formaldehyde 100 μL (pH 7). The entire immunollabeling procedure never exceeded two hours. To perform FC analysis the volume was adjusted to 400 μL.

FC measurements were carried out using a FACSVantage flow cytometer (Becton Dickinson, USA) equipped with an argon laser emitting at 488nm (100 mW). The flow cytometer was adjusted with 1 μm beads to optimize small event detection. 10.000 platelets were analyzed per sample. Data acquisition of each subject was in triplicate, obtaining a mean value of relative platelet activation for each sample analyzed. Two different regions (R2 and R3) were defined in logarithmic amplified Forward Scatter (FSC) vs Side Scatter (SSC) dot plots to exclude cell debris and larger blood cells (Figure 1). The R2 region contained only single platelets, which were determined considering their size in comparison with 1 μm beads and SSC patterns. The R3 region, comprised the R2 region as well as larger events than single platelets which could correspond to platelet aggregates or aggregates of other cellular groups. To evaluate platelet activation a gate in R2 or R3 was made, and dot-plots of CD62P vs CD61 signals were used to quantify double positive events considered as activated platelets.

ELISA

Anti CD62p and anti CD40L antibodies were used to recognize platelet activation. The following commercial kits were utilized: RayBio Human P-Selectin ELISA kit to quantify CD62p levels and RayBio Human CD40 Ligand ELISA kit to quantify CD40L levels (RayBiotech Inc, Norcross, Georgia, USA). All tests were performed according to manufacturer´s guidelines. Optical density measurement was performed at 450 nm in a

Thermo Scientific VarioskanFlash spectrometer attached to Thermo Skanlt Software for Varioskan Flash version 2.4.3. Each sample was analyzed in triplicate, obtaining a mean value and a standard deviation.

Statistical analysis

A descriptive and inferential statistical analysis was performed using the statistical package SPSS10.0.

A single tail Student *t* test was used to compare measurements and to probe the hypothesis that patients present marker values higher than controls. Levene test for variance equality and Pearson correlation coefficient were utilized. For each case, a 95% of confidence interval, a 5% of confidence, and an 80% power were considered.

Ethical considerations

The project was approved by the Ethics Committee of the Public Health Department of State and by the corresponding offices of each participant institution and the informed consent was obtained from each participant.

Results

In our study we included samples obtained from 57 volunteers that were classified as: a) 17 (29.8%) patients with ischemic coronary disease, b) 11 (19.3%) DM patients without ischemic coronary disease, c)12 (21.1%) non-DM patients with ischemic coronary disease, and d) 17 (29.8%) healthy control volunteers.

Flow cytometric analysis of samples labeled with CD61 and CD62P; and ELISA analysis with CD40L and CD62P reflected the following results:

In the FSC and SSC dot plots two different regions (R2 and R3) were delineated. R2 contained single platelets, while R3 contained R2 data plus others events exhibiting higher size and complexity probably due to the presence of other cell types aggregated to platelets (Figure 1). For statistical analysis only R2 region data was taken into consideration.
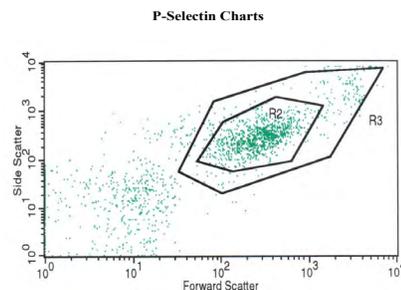

Figure 1. Chart of Forward Scatter and Side Scatter. Each point represent one event of 10.000 adquiered. We considered positive value for both markers (CD61 y CD62p). R2 represents the region containing

the activated platelets and R3 the region containing activated platelets plus platelet aggregates and other cellular aggregates plus platelets.

We analyzed CD61 and CD62p markers by flow cytometry in the four groups (Figure 2). Absolute values obtained showed disparities between the group with the double condition (DM with ischemic cardiopathy) and the others. Although the mean percentage values in the double condition group was higher when compared to the other groups. No statistically significant differences were observed (p=0.892) when Student *t* test with Levane correction was used. Among other causes, this difference could be related to the large disparities observed in the group presenting the double condition (Figure 3).
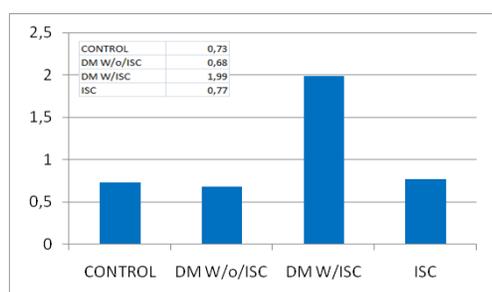
Figure 2. Mean percentages of platelet activation for each group measured in mCD 62p and CD 61by flow cytometry. (W/o/ISC=without ischemic; W/ISC=with ischemic; DM=Diabetes Mellitus; ISC=ischemic).
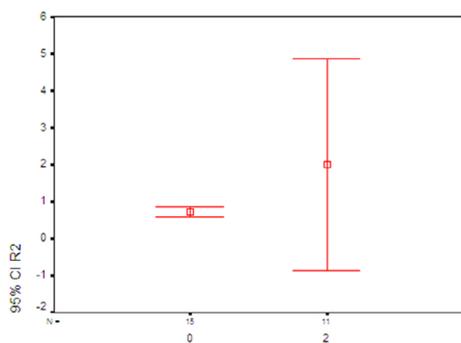

Figure 3. Mean values and disparities of control group (0) and diabetic group having suffered an ischemic event (2).

Due to the high dispersion of the double condition group and thinking that diabetes could be a counfusing variable, we decide to reorder the sample in two groups: patients with and without ischemic cardiopathy (Figure 4). This new arrangement did not evidence statistical differences among groups (p=0.707).

ELISA analysis showed diverse markers' behaviors according the sex of the volunteers (Figures 5 to 8). While sCD62p value is higher in women who did not suffer ischemic coronary

disease, difference in men is higher when suffered ischemic coronary disease (Figures 5 and 6). However, these patterns changed completely for CD40L marker (Figures 7 and 8). Even statistically significant variations were seen between women who suffered ischemic coronary disease and those who did not suffer it (Figure 7), as indicated the unilateral 0.023 p-value for a 0.05 significance level (n=10 for both groups).
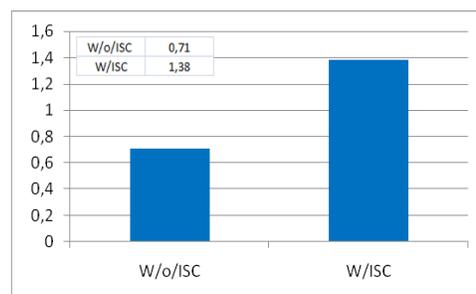
Figure 4. Mean percentajes of platelet activation for the group of patients who suffered an ischemic event and for the group which did not suffer an ischemic event, measured in CD62p and CD61 by flow cytometry. (W/o/ISC=without ischemic; W/ISC=with ischemic).
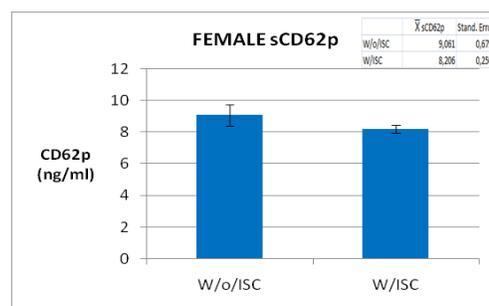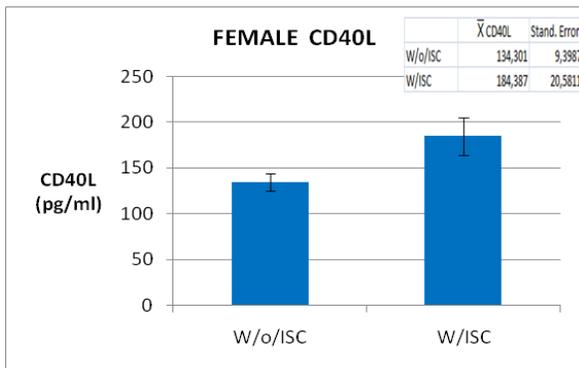

Figure 5. Female mean averages and standard error grouped together considering if they had suffered ani schemic coronary event or if they had not, measured in sCD62p ng/mL by ELISA. (W/o/ISC=without ischemic; W/ISC=with ischemic).
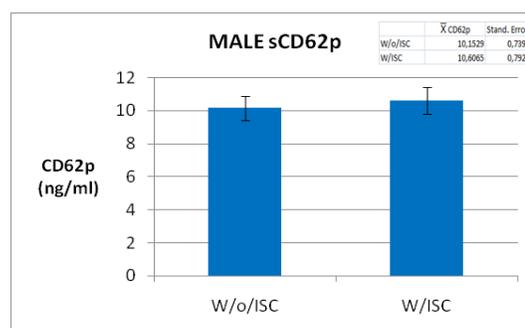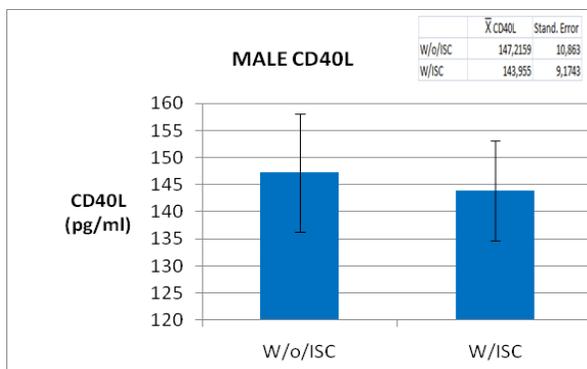

Figure 6. Male mean averages and standard error grouped taking into account if they had suffered an ischemic coronary event or if they had

not, measured in sCD62p ng/mL by ELISA. (W/o/ISC=without ischemic; W/ISC=with ischemic).
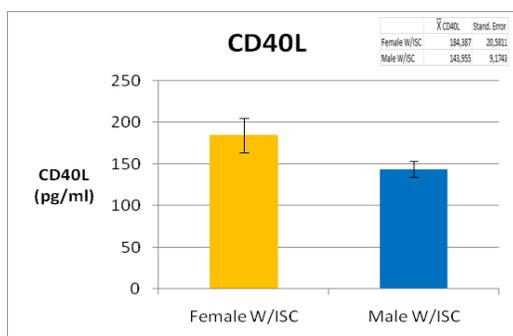

Figure 7. Femalemean averages and standard error grouped taking into account if the had suffered an ischemic event or if they had not, measured in CD40L pg/mL by ELISA. Unilateral p-value=0.023 for a 0.05 significance level. (W/o/ISC=without ischemic; W/ISC=with ischemic).
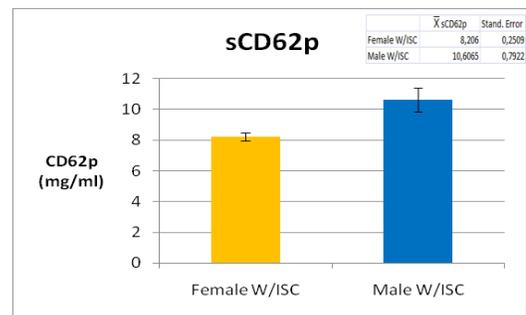

Figure 8. Male mean averages and standard error grouped considering if they had suffered an ischemic event or if they had not, measured in CD40L pg/mL by ELISA. (W/o/ISC=without ischemic; W/ISC=with ischemic).

We decided to re-arrange the samples by sex due to the differential behavior observed: a) men with and without ischemic cardiopathy and b) women with and without ischemic cardiopathy (Figures 9 and Figure 10).

**Figure 9.** Mean averages and standard error for females and males

with ischemic coronary disease measured in CD40L pg/mL by ELISA p-value=0.045 for a 0.05 significance level. (W/o/ISC=without ischemic; W/ISC=with ischemic).

Figure 10. Mean average and standard error for females and males with ischemic coronary disease measured in sCD62p ng/mL by ELISA p-value=0.004 for a 0.05 significance level. (W/o/ISC=without ischemic; W/ISC=with ischemic).

Statistically significant differences were observed for both soluble markers between females and males suffering ischemic coronary disease. For sCD40L, p-value 0.045 (Figure 9) and for sCD62 p-value 0.004 were obtained (Figure. 10) (n=10 and n=20, respectively).

No Pearson correlation was found between both isoforms of P-selectin by ELISA and FC data (Figure 11). However, in one patient a very high mCD62p value in region R3 was detected (see arrow in Figure 11).
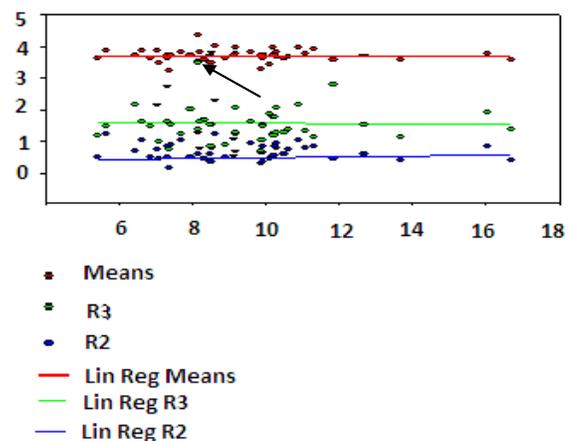
Figure 11. Correlation between both techniques for region R2, R3 and average mean, calculated with Pearson´s correlation coefficient. The arrow indicated one patient with a very high mCD 62p value in region R3. (X axis = ELISA, Y axis = FC)

Discussion

The following particularities were seen taking into account of the patient data provided by the physician teams:

a) The highest values of CD62p and CD40L were observed in patients with severe ischemic coronary disease that all underwent surgical revascularization or angioplasty. These results are in accordance with those obtained by Osmancik et al. (2007) [22].

b) Patients suffering ischemic coronary disease for a long period of time (more than 10 year) seemed to be more stable. Therefore time elapsed since the occurrence of the ischemic episode could be relevant.

c) We also verified that, in general, values increased with the age of patients.

d) Although diabetes and platelet aggregation and activation medication was similar for all patients, dysplidemic patients were controlled with different drugs; therefore they should be considered within the exclusion criteria. There is evidence of the effects of different statins as protective factors in the prevention of coronary episodes in DM patients [43,44]; hence, this medication group could be a distortion circumstance.

During the past years, differences between ischemic coronary disease expressions have been studied. During decades there was a belief that women had ischemic coronary diseases at older ages than men due to their preventive hormone profile. Taking into account this hypothesis, in the 1990s the hormone replacement therapy was used; however, scientific evidence discarded it. Women who underwent this therapy suffered coronary disease in the same percentage as women who did not. Nevertheless, there is scientific evidence that there are physiopathological differences in the configuration of coronary disease depending on the sex of the patient. Disparities between females and males do not imply that coronary disease in women could be milder than in men; in fact mortality is higher. It has been also observed that women have less anatomic and functional lesions; however their mortality rate is equal or even higher than in men. It is worth mentioning that the number of symptoms is higher in women than in men, which leads to a higher number and larger periods of hospitalization. Also, sudden death associated with ischemic coronary disease is more frequently seen in women [45]. Our results have shown a sexual dimorphism for sCD62p and CD40L. It is not possible to compare this fact since no studies stratified by sex have been carried out using both markers. In consequence, based on our results, Reynolds´ classification of cardiovascular risk would be more realistic.

CD40L secretion plays a central role in the inflammatory process. It is also known that during the reproductive period of time, females are prone to inflammatory processes due to their hormone profile or due to suffer more autoimmune diseases related to inflammation. Inflammation is associated with microvascular dysfunction and the latter one with endothelial dysfunction which is closely connected with platelet activation. Furthermore, inflammation process is important in the atherosclerosis pathogenesis, in plaque instability and plays a role in several atherosclerotic pathologies [38,46-48].

According to our results, inflammation must be taken as an additional exclusion criterion in the control group. Determination of reactive C protein, alpha TNF or some interleukin such as IL-1 as inflammation markers is recommended.

The mCD6p molecule determines and stabilizes platelet aggregation. After platelet activation, mCD6p is secreted into plasma losing the trans-membrane domain but preserving the epitope that recognizes mCD62p ligand. This soluble P-selectin is not a mere degradation output; instead it has an inhibitor capacity competing with the membrane isoform in its interaction with receptors. sCD62p selectively inhibits neutrophil adhesion to the endothelium. Thus, sCD62p therapeutic dosage could be used as an anti-thrombotic agent in several vascular pathologies. Moreover, in animal models, specific inhibitors and ligand inhibitors were used and their anti-thrombotic efficacy was demonstrated. However, other studies suggested that the procoagulant properties of this selectin could be used for treatment of hemorrhagic diseases such as hemophilia. Therefore, we could expect worthy results in further investigations related to anti-thrombotic and/or anti-hemorrhagic therapies [17,25-27,29-31,49].

Although no relation was found between FC and ELISA data, in one patient a very high mCD62p value in region R3 was detected. This fact suggests a high platelet activity together with the conformation of platelet aggregates. It is of great importance to highlight that this patient underwent a cardiovascular event during the following days after blood collection; therefore the treating physician decided to intensify the treatment. If we could have drawn a new blood sample during the following days, we could have confirmed the sCD2p increased concentration, presenting new evidence about mCD62p-sCD62p system. This analysis was not possible due to the lack of necessary authorizations. Although there are investigations presenting molecules sCD62p [25,30,50,51] and mCD62p [52-55] as platelet activation markers, up to now no research has obtained results for both isoforms with samples of the same patient.

It would be interesting to continue with this investigation as in last years the anti-aggregation therapy has acquired increasing relevance due to the application of new anti-aggregation drugs (ticagrelor, prasugrel). In parallel, it could fournished valiable information to the discussion involving the benefits of double anti-aggregation medication compare to threefold therapy including anticoagulants.

Finally one of the limiting factors in this research was the impossibility to obtain a higher number of paired patients and control volunteers in the Uruguayan population (3.5 million). Some of the reasons were:

1. Medication withdrawal: Difficulty to find patients who could stop taking platelet anti-aggregation drugs during the established period of time;

2. Age-matched controls: Type 2 DM patients are generally elderly people; therefore it was difficult to find aged-matched controls. Most people of that age group present one or more exclusion factors;

3. Differential incidence: Documented incidence of coronary diseases is larger in males than in females, so there is less number of females which could be considered for this study.

4. The multifactorial nature of these pathologies and the long asymptomatic period of time that frequently precede them.

Conclusions

In this research we found that:

a) It is the first time that platelet activation is studied in Uruguay by FC. We found differences in mCD62p molecule in the different groups analyzed, presenting no statistically significant differences;

b) Comparing both sexes, ELISA assays reflected statistically significant changes in sCD62p and CD40L values;

c) A sexual dimorphism was detected, sex of patients should be considered when deciding which platelet activation marker will be used in future studies;

d) Is the first time that both CD62p isoforms were evaluated in samples from the same subjects, therefore is imposible to compare with other references;

e) There is a need to narrow the inclusion factors for each group analyzed.

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