Biochemical Markers with Low-grade Inflammation as Predictors of Thrombotic Events in Antiphospholipid Syndrome

CLAUDIA GAVRIS[1], VLADIMIR POROCH[2]*, LAURENTIU SIMION[3]*, ADRIAN BARACAN[1], ELENA TOADER[4,5], ALINA MIHAELA PASCU[1]

[1]Transilvania University of Brasov, Faculty of Medicine, 56 Nicolae Balcescu Str., 500019, Brasov, Romania
[2]Grigore T. Popa University of Medicine and Pharmacy, Faculty of Medicine, 16 Universitãii Str., 700115, Iasi, Romania
[3]Carol Davila University of Medicine and Pharmacy, 37 Dionisie Lupu Str., 020021, Bucharest, Romania
[4]Grigore T. Popa University of Medicine and Pharmacy, Faculty of Medicine, Department of Bioethics and Medical Ethics, 16 Universitatii Str., 700115, Iasi, Romania
[5]St. Spiridon Emergency Hospital, Institute of Gastroenterology and Hepatology, 1 Independentei Blv., 700111, Iasi, Romania

The study aimed to test the inflammation hypothesis in antiphospholipid syndrome (APS) by assessing biochemical markers of inflammation and platelet activation. Forty one patients with APS were compared to 40 controls. High-sensitivity C-reactive protein (hs-CRP) (as inflammatory biomarker), P-selectin and soluble CD40L (sCD40L) (as platelet activation markers) were measured by ELISA at enrolment and after 12 months follow-up. Serum hs-CRP, P-selectin and sCD40L levels were significantly higher in patients with APS compared to controls. P-selectin was significantly higher in APS patients with recurrent or acute thrombosis compared to APS patients with no recurrent thrombotic events. Serum hs-CRP and anticardiolipin antibodies (aCL) and were independent predictors of thrombosis in APS. In conclusion, persistent increased hs-CRP titres demonstrated low-grade inflammation in APS. Serum biomarkers as aCL and hs-CRP were independent thrombotic cumulative risk predictors in patients with APS.

Keywords: Antiphospholipid syndrome, biochemical markers, high-sensitivity C-reactive protein, P-selectin, soluble CD40L, anticardiolipin antibodies, recurrent thrombosis

Antiphospholipid syndrome (APS) is histopathologicaly defined by the presence of vascular thrombosis in the absence of inflammation signs.

However, recently published data reported evidence for a pro-inflammatory status in APS [1-4].

An antiphospholipid antibody-induced endothelial proinflammatory response has been demonstrated by the up-regulation of adhesion molecules (vascular cell adhesion molecule-1 –VCAM-1 and E-selectin), and the synthesis and secretion of proinflammatory cytokines. Additionally, platelet activation and increased leukocyte adhesion to activated endothelium was reported [4-8].In catastrophic APS (affecting less than 1% of APS patients with invariant evolution towards a life-threatening condition), the systemic inflammatory response caused by excessive cytokine release from necrotic tissues was described as a possible new pathogenic mechanism.

Another example of inflammation as a basic pathogenic mechanism in APS is the already described role of complement in recurrent pregnancy losses in APS patients. Briefly, complement activation products may cause an imbalance in vascular growth factors required for normal pregnancy evolution, causing an insufficient placental infusion responsible for complications [9-17].

Inflammasome activation has been demonstrated in vivo in a mouse model. Moreover, cells with an increased expression of caspase-1 and NLR family pyrin domain containing 3 (NLRP3), and high (up to three-fold) serum concentration of interleukin-1b (IL-1β) have been described in APS patients. These findings could suggest chronic inflammasome activation and influence of cofactors independent from antiphospholipid antibodies (aPL) on APS pathogenesis [18-20].

However, the impact of inflammation in triggering pathogenic mechanisms in APS is still not yet defined.

The study aimed to investigate the hypothesis of low-grade inflammation in APS in correlation with the risk of evolution with thrombotic events by assessing specific pro-inflammatory and platelet activation biochemical markers.

Experimental part

Methods. Study population

The ambispective (retrospective and prospective for a 12 months follow-up period), non-randomized cohort study, included 41 patients diagnosed with APS in the Haematology, Neurology, Rheumatology and Clinical Immunology Departments of the County Clinical Emergency Hospital of Brasov (group A), and 40 healthy volunteers without risk of thrombosis (control group). The study was approved by the local Ethic Committee and all the included patients signed an informed consent [21] and the experimental procedures were carried out in accordance with the mandatory principles of the ethics [22, 23].

The study population included 14 patients with primary antiphospholipid syndrome (PAPS), 20 patients with APS secondary to systemic lupus erythematosus (LES secondary APS) and 7 patients with APS secondary to collagen diseases other than LES (non-LES secondary APS).

The diagnosis of APS was established according to revised Sapporo criteria [24].

Inclusion criteria: patients with primary and secondary APS with at least one clinical and one laboratory criterion. In the moment of inclusion patients were retrospectively evaluated for a history of thrombotic events, and prospectively for the evolution of new thrombotic episodes over a 12 months follow-up period (the number and the type of thrombosis: arterial, venous, arterial and venous, single or multiple).

*email: vlader2000@yahoo.com; Phone: +40723271972; lasimion@yahoo.com; Phone: +40722350308

The occurrence of at least two thrombotic events (arterial and/or venous and/or obstetric complications), either at the time of inclusion or during the follow-up period was considered as recurrent thrombosis.

There was no patient with catastrophic APS (clinical evidence of multiple organic disease in a short period of time). It is important to highlight that a patient with LES-secondary APS from the study group had a normal progression of a pregnancy during the follow-up, which is a rare condition.

Assessment of biochemical markers

Serum IgG or IgM anticardiolipin antibodies (aCL), serum P-selectin, soluble CD40L (sCD40L), and high-sensitivity C reactive protein (hs-CRP) levels were assessed at baseline (V1) and after 12 months follow-up (V2), as follows:

- aCL screening and typing (IgG, IgM) (ELISA, Quanta reagent, LiteInova Diagnostics Inc San Diego, Bio-Rad PR1100, normal value < 18 UI/mL). aCL titres were expressed in IgG (GPL) or IgM (MPL) phospholipid units. Titres > 40 GPL or MPL were considered moderate-high, while titres < 40 GPL or MPL were considered to be low.

- Determination of lupus anticoagulant (LA) was performed using the following coagulation tests: Kaolin coagulation time, activated partial thromboplastin time (aPTT), prothrombin time (PT) and diluted Russell venom viper time (dRVVT).

- Assessment of hs-CRP (immunoturbidimetric, Diagnostic Systems International reagent, Hitachi 717, normal value < 0.5 mg/L). The method is recommended for samples with concentrations < 20 mg/L (range 0.05 – 20 mg/L).

- Evaluation of platelet activation markers was performed by a solid phase ELISA technique (Human s-P-selectin, R & amp; D Systems, Minneapolis, USA) to determine the soluble P-selectin serum levels, normal values < 113 ng/mL and

- The soluble form of CD40L was assessed by a solid phase ELISA technique (Human sCD40L, R & amp; D Systems, Minneapolis, USA), normal values < 11451 pg/mL.The seven patients with an acute thrombotic event during the follow-up were included in the moment of admission for the thrombotic complication. The assessment of the biochemical markers of platelet activation and inflammation was performed in the same time with aCL titres (within the first two to three days after diagnosing the thrombotic event).

Thrombosis Diagnosis

The diagnosis of thrombosis was performed by ultrasound for deep vein thrombosis, angio computed tomography for pulmonary thromboembolism, arteriography for peripheral arterial occlusion, and computed tomography for cerebral thrombosis.

Statistics

Statistical analysis was performed using the SPSS 22.0.0 statistical package (SPSS Inc., Chicago, IL). Continuous quantitative variables were expressed as median (25^{th} percentile; 75^{th} percentile) (in case of non-normal distribution) or mean ± standard deviation (SD) (for normal distributed variables), and nominal qualitative values as number (n) and percentage (%). Chi-squared (χ^2) test was used to compare categorical values. The analysis of variance (the independent samples *t*-test/ANOVA) was utilized to compare means, and Man-Whitney U test to compare medians, as appropriate. A *P*-value < 0.05 was considered statistically significant. Correlation of continuous quantitative variables was assessed by Pearson test, and Spearman test was used to study correlations of qualitative variables. Independent variables associated to recurrent thrombosis, such as aCL, serum P-selectin, sCD40L, and hs-CRP were considered for multivariate regression analysis. Logistic regression analysis was used to investigate whether high titre of aCL, hs-CRP, P-selectin, sCD40L could independently predict a thrombotic event.

Results and disscussions

The study group included 14 patients with PAPS (7 females) with a mean age of 45.5±27 years and a mean disease age of 3.5 years, 20 patients with LES-secondary APS (16 women), with an average age of 47±27 years and a mean disease age of 5.6 years, and 7 patients with non-LES secondary APS (4 women) with an average age of 46±21 years and a mean disease age of 4.6 years.

In the 41 patients group with APS, 28 had a history of recurrent thrombosis (two thrombotic events at least): 12 patients with PAPS (4 patients with arterial recurrences, 6 patients with venous recurrences, 1 patient with arterial and venous recurrence, and 1 patient with recurrent abortion), 13 patients with LES-secondary APS (9 patients with arterial recurrences, 2 patients with venous recurrences, and 2 with recurrent abortions), and 3 patients with non-LES secondary APS (2 patients with arterial recurrences, 1 with venous recurrences).

V1 hs-CRP levels were significantly higher in patients with APS compared to controls (table 1). There was also a significant difference between V2 hs-CRP values in the study group versus the control group (table 2). Persistance of elevated serum levels of hs-CRP in APS group (both at V1 and V2) reveals the presence of a low-grade inflammation and confirms the hypothesis of a pro-inflammatory status in APS patients. In APS inflammation is considered as a possible second hit in the two-hit pathogenic scenario of platelet activation.

V1	Study group (APS patients)	Controls	*p*
Biomarker	n = 41	n = 40	
hs-CRP (mg/L)	30.95 (7.26; 29.75)	0.05 (0.03; 0.19)	0.03
P-selectin (ng/mL)	195.40 (94.51; 316.27)	102.92 (86.06; 109.17)	< 0.0003
sCD40L (pg/mL)	16871.60 (5727.90; 25670.40)	4031.79 (2710.62; 4301.50)	0.000008

Values are expressed as median (25^{th} percentile; 75^{th} percentile)

Table 1
SERUM BIOCHEMICAL MARKERS AT BASELINE (V1) IN THE STUDY GROUP VS. CONTROLS

V2	Study group (APS patients)	Controls	p
Biomarker	n = 41	n = 40	
hs-CRP (mg/L)	21.07 (10.21; 19.27)	0.05 (0.02; 0.14)	0.037
P-selectin (ng/mL)	119.52 (73.79; 278.59)	101.96 (85.04; 108.25)	0.019
sCD40L (pg/mL)	11682.75 (3720.12; 13687.07)	4031.39 (2337.17; 4289.75)	0.045

Values are expressed as median (25th percentile; 75th percentile)

Table 2
SERUM BIOCHEMICAL MARKERS AFTER 12 MONTHS FOLLOW-UP (V2) IN THE STUDY GROUP VS. CONTROLS

There was also a significant difference in serum P-selectin levels between APS patients and controls, both at V1 and at V2 (table 1, 2). sCD40L was significantly higher in APS patients compared to controls, both at V1 and at V2 (table 1, 2). High persisting serum levels of P-selectin and sCD40L during follow up demonstrates platelet activation in APS patients.

Moreover, in APS patients serum hs-CRP was significantly correlated to aCL titres (R = 0.38; p = 0.014) and to P-selectin levels (R = 0.296; p = 0.05).

In APS study group, values of V1 hs-CRP were higher in patients with a history of recurrent thrombosis compared to patients without a history of recurrent thrombosis, but not statisticaly significant (table 3). At V2 (after 12 months follow-up) the serum hs-CRP values were higher in the group of patients with APS and recurrent thrombotic events versus the group of APS patients without recurrent thrombotic events, without statistical significantion (table 4).

Instead, at baseline (V1) P-selectin was significantly higher in APS group with a reccurent thrombosis in history compared to APS patients without a history of recurrent thrombosis (table 3). Serum P-selectin was also significantly increased in patients with APS and recurrent throbosis compared to those without any recurrent thrombotic event during the follow-up (V2) (table 4).

No significant differences were observed in sCD40L serum levels between the group of APS patients with recurrent thrombosis compared to those without recurrrent thrombotic events, neither at the inclusion (V1) neither after the 12 months follow-up period (V2) (table 3, 4).

These results support the conclusion of other published reports that already demonstrated a platelet activation in APS [8, 25-27].

At the inclusion (V1), serum hs-CRP was higher in APS patients with an acute thrombotic event than in patients with a history of recurrent thrombotic events [66.09 (24.07; 71.34) mg/L versus 27.11 (12.76; 38.26) mg/L, values expressed as medians (25th percentile; 75th percentile)], but without statistic significance (p = 0.24). Neither the titres of sCD40L were significantly different between the two APS patients subgroups at V1 [17976.54 (6117.78; 29274.38) versus 14856.76 (4227.34; 25267.07) pg/mL, p = 0.18, values expressed as medians (25th percentile; 75th percentile)]. Instead, a statistic significant difference in serum P-selectin levels was observed between the two subgroups [281.76 (152.72; 379.23) compared to 91.54 (61.45; 264.89) ng/mL, p = 0.21, values expressed as medians (25th percentile; 75th percentile)].

Multiple logistic regression of the independent variables correlated with the likelihood of developing a recurrent thrombotic event in APS patients revealed that serum aCL and hs-CRP were independent predictors of thrombosis in APS patients from the studied group: Odds Ratio 1.0902, 95% Confidence Interval (C.I.): 0.9893-1.2014, p = 0.0006, respectively, 1.1215, 95% C.I.: 0.9844-1.2778, p = 0.0006. Serum hs-CRP was the best predictor of thrombosis risk in APS patients enrolled in our study group.

Moreover, the higher the aCL or hs-CRP serum values, the higher the risk of thrombosis in APS patients from our study group. A combination of an elevated aCL titer with increasing hs-CRP levels expressed a cumulative prediction

Table 3
SERUM BIOCHEMICAL MARKERS OF LOW-GRADE INFLAMMATION AND PLATELET ACTIVATION AT BASELINE (V1) IN APS PATIENTS WITH RECURRENT THROMBOSIS IN HISTORY COMPARED TO THOSE WITHOUT RECURRENT THROMBOSIS IN HISTORY FROM THE STUDY GROUP

V1	APS patients with recurrent thrombosis	APS patients without recurrent thrombosis	p
Biomarker	n = 28	n = 13	
hs-CRP (mg/L)	27.11 (12.25; 20.03)	18.26 (11.63; 19.97)	0.21
P-selectin (ng/mL)*	212.20 (126.83; 316.05)	94.51 (78.01; 316.20)	0.04
sCD40L (pg/mL)	17282.37 (6125.93; 28837.62)	15875.53 (4871.20; 23621.71)	0.15

Values are expressed as median (25th percentile; 75th percentile)

Table 4

SERUM BIOCHEMICAL MARKERS OF LOW-GRADE INFLAMMATION AND PLATELET ACTIVATION AT V2 IN APS PATIENTS WITH RECURRENT THROMBOSIS COMPARED TO THOSE WITHOUT THROMBOSIS DURING THE FOLLOW-UP

V2	APS patients with recurrent thrombosis	APS patients without recurrent thrombosis	*p*
Biomarker	n = 28	n = 13	
hs-CRP (mg/L)	19.65 (11.21; 21.76)	17.48 (10.93; 20.03)	0.23
P-selectin (ng/mL)*	209.43 (124.32; 319.45)	92.87 (69.75; 295.67)	0.04
sCD40L (pg/mL)	16927.68 (5716.34; 27867.45)	15178.89 (4723.87; 22345.56)	0.17

Values are expressed as median (25^{th} percentile; 75^{th} percentile)

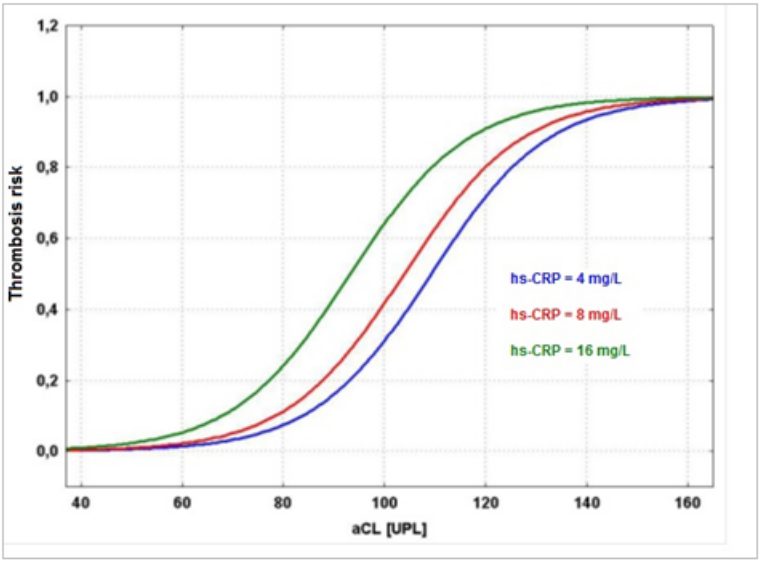

Fig.1. Cumulative thrombosis risk prediction of serum aCL in combination with incresing hs-CRP levels in the APS patients group

risk of developing a thrombotic event in APS patients (fig. 1).

Conclusions

Increased and persisting serum levels of biochemical markers hs-CRP, P-selectin, and sCD40L observed in the APS group of patients enrolled revealed a low-grade inflammation and a platelet activation in APS. Serum biomarkers aCL and hs-CRP were independently associated with an increased risk of thrombosis in APS patients. hs-CRP proved to be the best independent predictor for the evolution with a thrombotic event in our APS study group. High serum aCL predicted cumulative thrombotic risk with increasing hs-CRP serum levels in APS patients.

References

1.DA SILVA SARAIVA, S., FERNANDES CUSTÓDIO, I., DE MORAES MAZETTO, B., PEREIRA COLLELA, M., VINÍCIUS DE PAULA, E., APPENZELLER, S., ANNICHINO-BIZZACHI, J., ORSI, F.A., Thrombosis Research, **136**, nr. 6, 2015, p.1174-1178.
2.SCIASCIA, S., BALDOVINO, S., SCHREIBER, K., SOLFIETTI, L., RADIN, M., CUADRADO, M.J., MENEGATTI, E., ERKAN, D., ROCCATELLO, D., Clin. Mol. Allergy, **14**, nr. 6, 2016, DOI 10.1186/s12948-016-0043-2.
3.CHATURVEDI, S., MCCRAE, K.R., Hematology, **1**, nr. 1, 2015, p. 55-60. doi:10.1182/asheducation-2015.1.53.
4.SALMON, J.E., GIRARDI, G., LOCKSHIN, M.D., Nature Reviews Rheumatology, **3**, 2007, p. 140-147, doi:10.1038/ncprheum0432
5.AMES, P.R.J., ANTINOLFI, I., CIAMPA, A., BATUCA, J., SCENNA, G., LOPEZ, L.R., DELGADO ALVES, IANNACCONE, J.L., MATSUURA, E., Rheumatology, **47**, 2008, p. 1832–1837.
6.ZARBOCK, A., POLANOWSKA-GRABOWSKA, R.K., LEY, K., Blood Rev., **21**, 2007, p. 99–111.
7.PROULLE, V., FURIE, R.A., MERRILL-SKOLOFF, G., FURIE, B.C., FURIE, B., Blood, **124**, nr. 4, 2014, p. 611–622.
8.GIANNAKOPOULOS, B., KRILIS, S.A., N. Engl. J. Med., **368**, nr. 11, p. 1033–1044.
9.SALMON, J.E., GIRARDI, G., J. Reprod. Immunol., **77**, 2008, p. 51–56.
10.BALAN, G., PELIN, A.M., MACOVEI, L.A., CONDRATOVICI, A.P., CONDRATOVICI, C.P., BUSILA, C., Rev.Chim. (Bucharest), **68,** no. 3, 2017, p. 608
11.ALEXA, A.I., CANTEMIR, A., ANTIOCH, I., BALMUS, I.M., COJOCARU, S., GARDIKIOTIS, R., LUCA, A., FILIP, M.A., ABABEI, D.C., ZAMFIR, C.L., Rev.Chim. (Bucharest), **68,** no. 2, 2017, p. 350
12.SCHAAS, B.A., IVAN, S., TITIANU, M., CONDRATOVICI, C.P., MAIER, A., SCHAAS, C.M., Mat. Plast. **54,** no. 1, 2017, p. 133
13.CANTEMIR, A., ALEXA, A.I., CIOBICA, A., TOADER, E., BALMUS, I.M., COJOCARU, S.I., CHISELITA, D., COSTIN, D., Rev.Chim. (Bucharest), **67,** nr. 9, 2016, pp. 1725-1727.
14.ANCUTA, C., ANCUTA, E., CHIRIEAC, R., ANTOHE, M., IORDACHE, C., Rev.Chim. (Bucharest), **68,** no. 2, 2017, p. 369
15.CIOLOCA, D.P., FOIA, L., HOLBAN, C., TRANDAFIRESCU, M., POROCH, V., MAXIM, D., JIPU, R., COSTULEANU, M., TOMA, V., Rev.Chim. (Bucharest), **67,** no. 12, 2016, p. 2409
16.TOMA, V., CIOLOCA, D.P., FORNA, D.A., HURJUI, L., BOTNARIU, G.I., NECHIFOR, I.E., BOGDAN, M., COSTULEANU, M., SIMION, L., HOLBAN, C., Rev. Chim. (Bucharest), **67,** no. 12, 2016, p. 2545

17.NAVOLAN, D., VLADAREANU, S., CIOHAT, I., STOIAN, D., BADIU, D., CRAINA, M., TOMOVIC, M., BIRSASTEANU, F., CRACIUNESCU, M., Rev. Chim. (Bucharest), **67,** no. 6, 2016, p. 1224
18.MULLA, M.J., SALMON, J.E., CHAMLEY, L.W., BROSENS, J.J.,BOERAS,C.M., KAVATHAS, P.B., ABRAHAM, V.M., PLoS One, **8**, nr. 6, 2013, p. e65237, doi:10.1371/journal.pone.0065237.
19.MULLER-CALLEJA, N., KOHLER, A., SIEBALD, B., CANISIUS, A., ORNING, C., RADSAK, M., STEIN, P., MÖNNIKES, R., LACKNER, K.J., Thromb. Haemost., **113**, nr. 5, p. 1071-1083.
20.LOOD, C., TYDEN, H., GULLSTRAND, B., STURFELT, G., JÖNSEN, A., TRUEDSSON, L., et al., PLoS One, **9**, nr. 6, 2014, p. e99386. doi: 10.1371/journal.pone.00993.
21.AGHEORGHIESEI CORODEANU, D.T., POROCH, V., A possible theoretical model of ethical system management in healthcare institutions, 6th LUMEN International Conference on Rethinking Social Action Core Values, 16-19 April 2015, Iasi, Romania, Rethinking Social Action. Core Values, p. 33-41.
22.TOADER, E., TOADER, T., Revista Romana de Bioetica, **10**, no. 3, 2012, p. 66.
23. TOADER, E., Revista Romana de Bioetica, **8**, no. 2, 2010, p. 157.
24.MIYAKIS, S., LOCKSHIN, M.D., ATSUMI, T., et al., J. Thromb. Haemost., **4**, 2006, p. 295–306.
25.DE GROOT, P.G., Blood, **124**, nr. 4, 2014, p. 475-476.
26.LOPEZ, A.G., ORTEGA O.L., SAAVEDRA, M.A, MENDEZ CRUZ, R., FLORES, J.R., GARCIA DE LA PENA, M., Reumatol. Clin., **9**, nr. 4, 2013, p. 216–220.
27.GAVRIS, C.M., MOGA, S., DIMA, L., HOGEA, M., PASCU, A., Archives of Balkan Medical Union, **52**, nr. 2, 2017, p. 162-165.

Manuscript received:10.01.2017