Cryptogenic stroke and seronegative antiphospholipid syndrome: a case series of patients with positivity for "non-criteria" antiphospholipid antibodies

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SUMMARY

Cerebrovascular events (CE) are one of the most common and severe events in antiphospholipid syndrome (APS), a condition characterized by thrombosis and circulating anti-phospholipid antibodies (aPL). Seronegative APS (SN-APS) refers to a group of patients with clinical features of APS but persistently negative tests for "criteria aPL": anti-cardiolipin antibodies (aCL) and anti-$\beta_2$glycoprotein I antibodies detected by enzyme-linked immunosorbent assay (ELISA), and the lupus anticoagulant detected by clotting assays.

We report a series of five cases of SN-APS in young or middle-aged patients who tested positive for "non-criteria" aPL. We retrospectively collected cases of SN-APS patients who experienced CE without an identified cause despite an extensive diagnostic work-up and tested negative for criteria aPL. All the patient sera were tested for aCL by immunostaining on thin-layer chromatography (TLC) and anti-vimentin/cardiolipin (aCL/Vim) by ELISA. We identified five cases of female patients aged 21 to 58 years, evaluated at the Rheumatology Unit and/or Stroke Unit/Emergency Department of the Sapienza University Hospital of Rome, "Policlinico Umberto I". All patients presented a clinical history suggestive of APS. All the patients tested positive for aCL by TLC-immunostaining, and one patient was positive for aCL/Vim. In young or middle-aged patients with cryptogenic CE and a clinical history suggestive of APS, the use of new diagnostic tools for identifying aPL, if validated in future studies, could represent an important step in the prompt diagnosis of APS.

***Key words**: Cryptogenic IS, SN-APS, aCL/Vim, TLC-immunostaining.*

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INTRODUCTION

Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterized by thrombosis, pregnancy morbidity, and circulating anti-phospholipid antibodies (aPL) (1). aPL have been detected in around 14% of ischemic stroke (IS) patients of all ages (2). IS in young subjects generates huge interest among physicians, especially when no cause can be identified despite an appropriate diagnostic work-up (cryptogenic IS) (3). Considering that more than 20% of IS in patients younger than 45 years are associated with APS (3), this condition should be considered in the diagnostic framework of these patients.

Moreover, it is possible to find individuals with clinical signs suggestive of APS but who are persistently negative for "criteria aPL": anti-cardiolipin antibodies (aCL) and anti-$\beta_2$glycoprotein I antibodies (aβ_2GPI) detected by enzyme-linked immunosorbent assay (ELISA), and the lupus anticoagulant (LA) detected by clotting assays (4). The term "seronegative APS" (SN-APS) has been proposed to refer to this group of patients (3). New antigenic targets or method-

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ological approaches to detect aPL in SN-APS have been proposed (5). Among others, the cardiolipin/vimentin (CL/Vim) complex has been identified as a "novel" antigenic target of aPL (6). In addition, aCL have been detected in SN-APS patients through immunostaining on thin-layer chromatography (TLC) plates (7).
Herein, we report a series of five cases of CE in young or middle-aged patients, evaluated at our hospital between June 2017 and August 2018, who tested positive at least twice 12 weeks apart for "non-criteria aPL": aCL/Vim by ELISA and aCL by TLC-immunostaining (6).

METHODS

In this case series, we included all consecutive patients who presented to the shared rheumatology and neurology outpatient clinic of the Sapienza University of Rome with clinical features of APS, despite the evidence of persistently negative tests for the detection of conventional aPLs (aCL, aβ_2GPI, and LA tests) in at least two consecutive occasions, 12 weeks apart between tests. Blood samples were obtained from each patient, and the sera were stored at -20 °C. They were analyzed for the detection of aCL using TLC-immunochromatography, while the ELISA test was employed to identify anti-CL/Vim IgG, as previously described (6). This study was approved by ethics committees of the "Sapienza" University of Rome, and participants gave written informed consent in accordance with Helsinki Declaration.

CASE REPORT 1

A 21-year-old female taking oral contraceptives developed bilateral iliac deep vein thrombosis and pulmonary thromboembolism. Therefore, contraceptive therapy was discontinued, and oral vitamin K antagonist (VKA) was prescribed for 3 months, followed by antiplatelet therapy. Over 8 eight years, she experienced multiple transient ischemic attacks. Carotid ultrasonography was normal. Dyslipidemia and diabetes mellitus were excluded. Thrombophilic screening showed no mutation of methylentetrahydrofolate reductase (MTHFR), factor II, or factor V, while antithrombine III, homocysteine, protein S, and protein C were within the normal range. Antinuclear antibodies (ANA), anti-double stranded DNA antibodies (anti-dsDNA), anti-extractable nuclear antigens (anti-ENA), aCL, aβ_2GPI antibodies, and LA were undetectable on several occasions. In suspicion of SN-APS, "non-criteria" aPL tests were performed, revealing positivity for aCL by TLC-immunostaining and anti-CL/Vim by ELISA. Therefore, combined therapy with VKA and low-dose acetylsalicylic acid (LDA) was started. During 3 years of therapy, she did not present new events.

CASE REPORT 2

A 25-year-old female, with a history of livedo reticularis (LR) and migraine, suffered from an IS involving the middle cerebral artery (MCA) during oral contraceptive therapy. Transesophageal echocardiography (TEE) showed a patent foramen ovale, which was treated with transcatheter closure. Thrombophilia panel was performed: the patient had a homozygous MTHFR mutation with elevated serum homocysteine levels. Oral contraceptive therapy was stopped, and folate therapy was started. ANA, anti-dsDNA, anti-ENA, aCL, aβ_2GPI antibodies, and LA were persistently negative. At the age of 27, she experienced a pregnancy miscarriage at 11 weeks' gestation, and one year later she had a full-term pregnancy with a prophylactic dose of low molecular weight heparin (LMWH). 6 years later, she had a left capsule-lenticular IS. Carotid ultrasonography showed a right internal carotid artery (ICA) irregular plaque with stenosis <30%. Laboratory findings excluded dyslipidemia or diabetes mellitus, and electrocardiogram (ECG) monitoring did not detect atrial fibrillation (AF). Fabry disease was also excluded. The patient was discharged on LDA. Two years later, a new IS occurred due to a left intracranial ICA occlusion involving the M1 MCA segment (Figure 1).

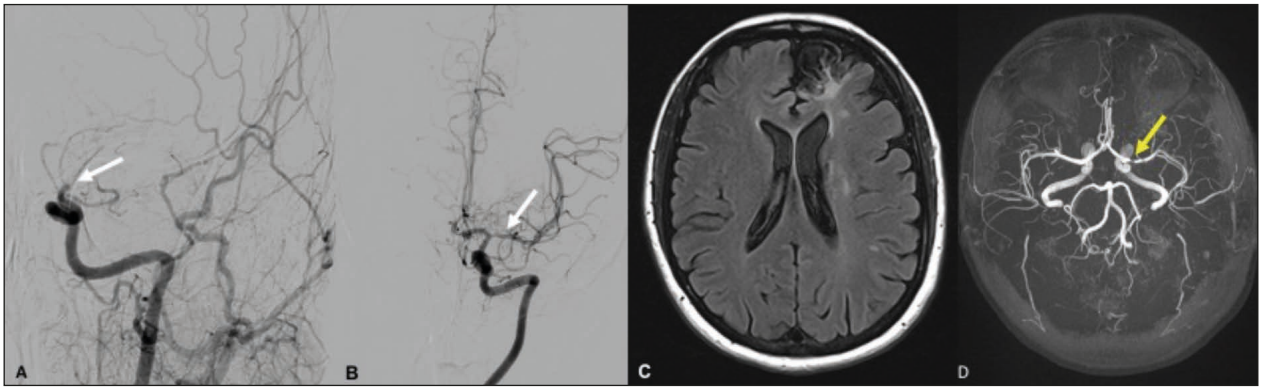

Figure 1 - Radiological representative findings in patient #2. A, B) Endovascular recanalization of the left internal carotid terminus (white arrow in A) (A before and B after the mechanical thrombectomy) with residual focal middle cerebral artery (MCA) M1 segment stenosis (white arrow in B); C) fluid attenuated inversion recovery magnetic resonance imaging (MRI) shows a left chronic frontal and a left periventricular infarct with encephalomalacia and multiple small left white matter hyperintensities; D) focal stenosis of the M1 segment of the left MCA (yellow arrow) was confirmed at the time of fight MRI sequence several months later.

Endovascular thrombectomy was successfully performed with residual MCA stenosis (Figure 1 A and B). During hospitalization, paroxysmal AF with a high ventricular rate was recorded, and dabigatran was started in combination with LDA. Further recurrent IS of the left cerebral hemisphere occurred within the next year without relevant consequences.
Magnetic resonance angiography confirmed the stenosis of the left MCA M1 segment (Figure 1D).
Since all known causes of thrombophilia were ruled out, an SN-APS was hypothesized. Serum TLC-immunostaining was performed, and the patient turned out to be positive for aCL. Therefore, VKA was started in association with LDA. During the next 3-year follow-up, no further thrombotic events occurred.

CASE REPORT 3

A 35-year-old female with a medical history of migraine and smoking habit presented an IS involving the MCA without relevant consequences. LDA was started. Thrombophilic screening resulted negative. aCL, aβ_2GPI, ANA, anti-dsDNA, anti-ENA, and LA were persistently negative. She reported eight pregnancy miscarriages over the next 9 years, despite antiplatelet therapy. However, she had never received the combination therapy with LDA and LMWH during pregnancy. SN-APS was hypothesized, and the patient tested positive for aCL *via* TLC-immunostaining. The patient continued taking LDA as secondary prevention therapy without experiencing new events. However, she preferred not to become pregnant again.

CASE REPORT 4

A 54-year-old female, with a history of LR and pregnancy miscarriage, on therapy with tamoxifen for breast cancer, presented to our Emergency Department with a left MCA IS. She was successfully treated with intravenous thrombolysis and endovascular thrombectomy (Figure 2 A-D). TEE did not show any embolic sources. Computed tomography angiography of neck and intracranial vessels did not reveal stenosis or dissections. Laboratory findings excluded dyslipidemia or diabetes mellitus. Arterial blood pressure and continuous ECG monitoring were normal. Thrombophilic screening, including MTHFR, factor II, and factor V, showed no mutation; anti-thrombin III, homocysteine, protein S, and protein C were within range. ANA, anti-dsDNA, anti-ENA, aCL, aβ_2GPI antibodies, and LA were negative on more occasions. Tamoxifen was stopped and therapy with LDA was start-

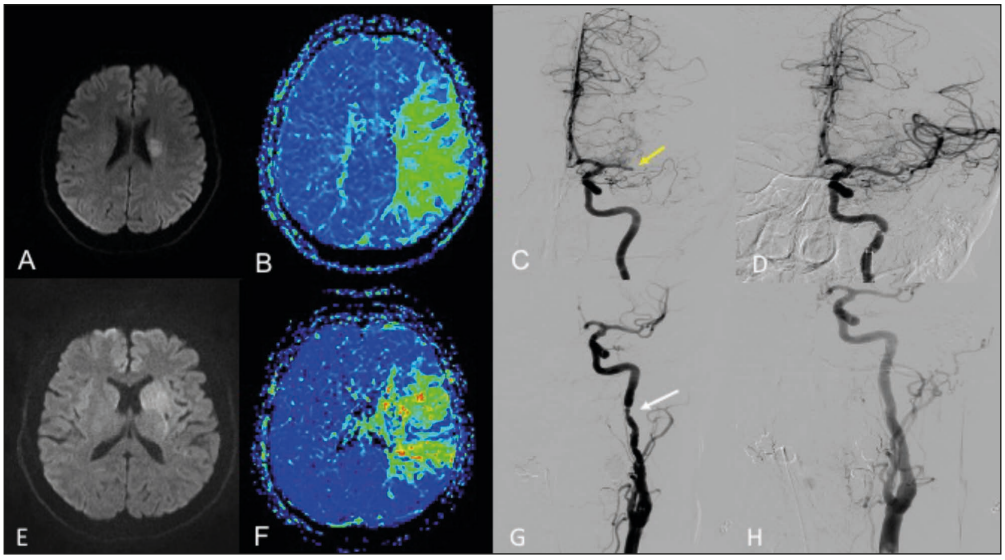

Figure 2 - Radiological representative findings in patient #4. A-D) First ischemic stroke: A) left acute periventricular ischemic lesion on diffusion weighted imaging (DWI); B) large perfusion deficit in the territory of the left middle cerebral artery (MCA) [prolonged mean transit time (MTT) at the perfusion imaging derived maps], suggesting the presence of salvageable tissue; C) angiographic occlusion evidence of the left distal M1 segment before mechanical thrombectomy (yellow arrow); D) recanalization of the distal M1 segment of the left MCA after endovascular procedure. E-H) Second ischemic stroke: E) left caudate head and internal capsule infarct on DWI; F) perfusion imaging (prolonged MTT) shows a large zone of ischemic penumbra in the territory of the left MCA; G) spasm of the left internal carotid artery (ICA) (white arrow) early after mechanical recanalization of the ipsilateral MCA; H) resolution of the ICA vasospasm after intraarterial nimodipine administration.

ed. One month later she developed femoral venous thrombosis, treated for 3 months with LMWH and LDA. One year later she had an IS recurrence (Figure 2 E-H) with occlusion of the left MCA, treated with mechanical thrombectomy with complete recanalization of the vessel. Notably, at the end of the endovascular procedure, a moderate spasm of the left ICA and MCA was evident. Intraarterial administration of nimodipine in the left ICA led to a clear improvement of the angiographic vasospasm. The patient was discharged on clopidogrel in place of LDA. After one month, a new IS event occurred. Considering the venous thrombotic event and since all known causes of IS had been ruled out, an SN-APS was hypothesized. Since she tested positive for aCL by TLC-immunostaining, antiplatelet therapy was replaced by VKA. During the following 2 years of follow-up, she did not report any other vascular thrombotic events.

CASE REPORT 5

A 58-year-old female with a medical history of hypercholesterolemia suffered from an acute right MCA IS. In addition, brain magnetic resonance imaging (MRI) showed several small areas of chronic ischemia in the semi-oval center. After 3 months, she had a focal seizure without new ischemic lesions on MRI. Thrombophilic screening showed no mutation of MTHFR, factor II, and factor V, and antithrombin III, homocysteine, protein S, and protein C were within the normal range. ANA, anti-dsDNA, anti-ENA, aCL, aβ_2GPI antibodies, and LA were persistently negative. Carotid ultrasonography did not reveal hemodynamically significant stenosis. All known causes of IS were ruled out, and a SN-APS was hypothesized. Therefore, aCL test by TLC-immunostaining was performed and resulted positive. She continued taking LDA as secondary prophylaxis without recurrences during the next three years.

DISCUSSION AND CONCLUSIONS

In this case series, we describe the diagnostic work-up and the clinical management of five SN-APS patients with cryptogenic CE and positivity for "non-criteria" aPL, either directed against new antigenic targets (aCL/Vim) or identified by a new methodological approach (TLC-immunostaining). These tests, if validated in future studies, could support APS diagnosis and therefore guide the subsequent pharmacological treatment. In the described cases, switching patients from LDA to AVK (the gold standard therapy in thrombotic APS) and avoiding the use of direct oral anticoagulants (DOACs) may have led to the prevention of new ischemic events, including IS.

The mechanisms of CE associated with APS are thrombotic and embolic. A prothrombotic state is determined through different pathways, including aPL interference with endogenous anticoagulant mechanisms, binding and activation of platelets, interacting with endothelial cells and expression of adhesion molecules and tissue factor, and activation of the complement cascade (8). Cerebrovascular disease frequently involves small arteries, leading to lacunar and subcortical IS. Moreover, intracranial arterial occlusions or stenosis in APS patients can occur (9) (three cases in our report). Furthermore, another pathological mechanism underlying CE in APS is cardiogenic cerebral embolism, due to left valve heart disease and Libman-Sacks endocarditis, patent foramen ovale, and atrial septal aneurysm (10).

All patients in our cohort exhibited clinical characteristics strongly suggestive of APS, and we ruled out other causes for their neurological manifestations. This comprehensive evaluation was the result of the strict collaboration between rheumatologists and neurologists in our outpatient clinic. The high suggestiveness of APS is the result of the combination of several clinical aspects: three out of five patients were younger than 40 years, and all patients had at least one other criterion or extra-criteria clinical manifestation beyond the cerebrovascular event (venous thrombotic events, pregnancy morbidity, migraine, LR, and epilepsy). These aspects taken together support the clinical suspicion that CE was due to aPL, since other manifestations that can be explained by a diagnosis of APS are concomitant and other causes of CE have been excluded.

Despite APS is usually considered a cause of juvenile stroke, it can also occur in the elderly (11). Considering the aforementioned evaluations, we suspected an SN-APS diagnosis and tested for non-criteria aPL, encouraged by previous studies, that identified these antibodies in nearly half of SN-APS patients (7, 12). Zohoury *et al.* reported the high sensitivity of aCL/Vim and anti-phosphatidylserine/prothrombin in SN-APS patients (13). Furthermore, in our previous study on SN-APS patients, the double positivity (aCL by TLC-immunostaining plus aCL/Vim) showed a likelihood positive ratio of 8 to present mixed thrombotic and obstetrical features. In addition, in SN-APS patients, aCL by TLC-immunostaining was associated with brain MRI ischemic changes and migraine, and aCL/Vim was associated with the presence of LR and thrombocytopenia (6). APS patients who tested positive for CL/Vim IgG showed a higher prevalence of pregnancy morbidity and thrombocytopenia (14).

aPL could be considered a mosaic of antibodies directed against phospholipids or cofactors other than CL or β_2GPI (such as anti-annexin V, anti-annexin II, anti-prothrombin, anti-protein S): these non-criteria aPL could help to identify SN-APS patients (5).

All the patients tested positive for aCL by TLC-immunostaining, and only one tested positive for aCL/Vim by ELISA. The latest classification criteria recommend the ELISA for aPL testing (4). In those patients in whom criteria aPL tested negative by ELISA and there is a strong clinical suspicion of APS, TLC-immunostaining might be a useful alternative method. In fact, TLC-immunostaining is a laboratory approach that provides different antigenic exposure than ELISA (7), and could detect aPL in ELISA-negative cases, supporting diagnosis and therapeutic management.

The latest 2019 European Alliance of Associations for Rheumatology recommendations for APS indicate VKA treatment [international normalized ratio (INR) 2-3] over LDA in arterial secondary thromboprophylaxis and, in patients with recurrent events, recommend VKA treatment (INR 3-4) or VKA (INR 2-3) plus LDA (15). Previously, the treatment of arterial thrombosis in APS patients was controversial, and it has long been debated whether to use LDA or VKA. Some of our patients presented recurrent arterial thrombosis despite LDA.

Due to the need to monitor INR during VKA therapy and the young age of several patients, the treatment with DOACs of APS patients was taken into consideration. However, a trial on APS subjects treated with rivaroxaban or warfarin was prematurely concluded due to the higher rate of thromboembolic and hemorrhagic complications in the rivaroxaban group. Consequently, EULAR guidelines on APS do not recommend DOACs in APS patients with arterial thrombosis (15).

Considering that CE represents a life-threatening manifestation of APS, it is fundamental to identify those "hidden" APS patients who may benefit from long-term secondary thromboprophylaxis with AVK. In our patients, the detection of "non-criteria" aPL supported the diagnosis, and we switched patients from LDA to VKA, preventing new ischemic events. In two patients, we did not change LDA to VKA due to a long period without ischemic events during LDA treatment.

Therefore, the use of new diagnostic tests may help clinicians in making a prompt diagnosis of APS in patients with cryptogenic IS. If these novel tests are validated, this may lead to an improvement in the clinical management and prognosis of such patients.

Contributions

All the authors made a substantial intellectual contribution, read and approved the final version of the manuscript, and agreed to be accountable for all aspects of the work.

Conflict of interest

The authors declare no potential conflict of interest.

Ethics approval and consent to participate

This study was approved by the ethics committees of the Sapienza University of Rome.

Informed consent

Participants gave written informed consent in accordance with the Helsinki Declaration.

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Availability of data and materials

Data are available from the corresponding author upon request.

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