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Anti-beta 2 glycoprotein I antibodies in centenarians

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Abstract

Background: Non-organ-specific autoantibodies are present in centenarians without evidence of autoimmune diseases but conflicting or no data on anti-phospholipid and anti-phospholipid binding proteins were reported.

Objective: To investigate the presence and antigen specificity of anti-phospholipid and anti-phospholipid binding proteins in centenarians.

Methods: Seventy-seven centenarians, 70 adult controls, 65 unselected elderly subjects, and 38 old SENIEUR volunteers were investigated. Anti-cardiolipin, anti-human β2glycoprotein I, and lupus anticoagulant were detected. Antigen specificity was assayed against plates coated with anionic, neutral and cationic phospholipids and β2glycoprotein I-dependence was also evaluated.

Results: 54.3% of the centenarians were positive for IgG and 8.6% for IgM anti-β2glycoprotein I antibodies, while only 20.7% centenarians were positive for anti-cardiolipin IgG and 2.59% for IgM; none resulted positive for lupus anticoagulant. Anti-cardiolipin positive sera cross-reacted with negatively charged phospholipids and displayed decreased binding to serum-free cardiolipin-coated plates that was restored by human β2glycoprotein I or fetal calf serum.

Conclusions: Centenarians display high reactivity against human β2glycoprotein I but low binding to the bovine molecule in the anti-cardiolipin assay. In spite of the presence of antibodies comparable to those found in patients with the anti-phospholipid syndrome, no vascular events were reported suggesting the presence of unknown protective factors and/or the lack of triggering factors.

Keywords: Ageing; Centenarians; Anti-phospholipid antibodies; β2Glycoprotein I; Thrombosis

1. Introduction

Centenarians display a characteristic autoantibody profile, being organ-specific autoantibodies absent and non-organ-specific autoantibodies increased without any full-blown autoimmune disease (Mariotti et al., 1992; Candore et al., 1997). In addition, non-organ-specific autoantibodies increase with age, but it is still debated whether aPL are also produced and associated with the clinical manifestations of the APS (Candore et al., 1997; Piette and Cacoub, 1998; Levine et al., 2004).

Abbreviations: aPL, anti-phospholipid antibodies; APS, anti-phospholipid syndrome; LA, lupus anticoagulant; aCL, anti-cardiolipin antibody; β2GPI, β2glycoprotein I; ELISA, solid-phase enzyme-linked immunosorbent assay; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcoline; Sph, sphingomyelin; NHS, normal human sera; FCS, fetal calf serum; oxLDL, oxidized LDL; IL, interleukin.

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Anti-phospholipid antibodies make up a heterogeneous group of autoantibodies diagnosed as LA or aCL, associated with recurrent thrombosis, pregnancy loss, thrombocytopenia and thought to be pathogenic (Levine et al., 2002). Rather than to be directed against PLs only, these antibodies are specific for PL-binding protein (Levine et al., 2002; Meroni and Riboldi, 2001; de Groote et al., 2002; Roubey, 2000). Among them, β2GPI does represent the most important one (de Groote et al., 2002). It has been widely accepted that aCL detectable in APS require the presence of serum PL-binding proteins, mainly β2GPI, when detected by solid-phase assay (Levine et al., 2002; Meroni and Riboldi, 2001; de Groote et al., 2002; Roubey, 2000). Such an antigen specificity does allow to distinguish them from aPL occurring in infectious diseases which are not usually associated with the clinical manifestations of the syndrome (Levine et al., 2002). In this regard, there is no information on the aPL antigen specificity in centenarians and no data on the occurrence of anti-β2GPI autoantibodies in these subjects (Candore et al., 1997).

Interestingly, centenarians have an increased prevalence of high-risk genetic markers of hypercoagulability (Mari et al., 1995; Mannucci et al., 1997), and are paradoxically characterized by low HDL-cholesterol and relatively high triglyceride levels, which together are considered to be strong risk factors for atherothrombosis (Baggio et al., 1998). It has been suggested that protective mechanisms might counteract these risk factors, allowing them to age successfully and to escape major thrombotic diseases (Baggio et al., 1998).

Taking into account the age-associated immune dysfunction leading to autoimmunity, we investigated the presence of aPL in a total of 250 individuals of different ages, including 77 centenarians. We also characterized the antibodies, in order to assess whether the lack of thrombotic events in these subjects can be related to the antibody antigen specificity or to the paradoxical status that does not induce thrombosis in centenarians in spite of the presence of factors that are risk factors for the general population.

2. Materials and methods

2.1. Subjects and patients

A total of 250 individuals living in Northern Italy were studied: $n=77$ centenarians [20 males and 57 females, age 102 ± 1.5 years (mean $\pm$ SD); group 1]; $n=70$ randomly selected, apparently healthy, young control subjects [23 males and 47 females, age 35.8 ± 9.3 years (group 2)]; $n=65$ unselected elderly subjects [31 males and 34 females, age 70.2 ± 5.3 years (group 3)]; and 38 old volunteers [14 males and 24 female, age 70.9 ± 4.3 years (group 4)] selected according to the SENIEUR Protocol (Ligthart et al., 1984). Centenarians were selected according to the classification proposed by Franceschi et al. (2000).

APS patients were diagnosed according to the Sapporo's criteria (Wilson et al., 1999).

Approval for these studies were obtained from the Institutional Revue Board of the University of Milan and informed consent was obtained according to Declaration of Helsinki.

2.2. Anti-phospholipid antibodies

aCL were detected by ELISA and values expressed as IgG/IgM aPL Units (GPL/MPL, respectively; values were considered positive when >10 GPL or MPL) or as low, medium and high positivities as described (Tincani et al., 2001).

Anti-anionic (PS), -neutral (PE, PC), -cationic PL (Sph) activity was evaluated by ELISA as described (Allegri et al., 1990; Tincani et al., 1996; Di Simone et al., 2000).

β2GPI was purified from NHS, and anti-β2GPI antibodies were detected by ELISA as described (Tincani et al., 1996; Di Simone et al., 2000; Balestrieri et al., 1995). Sera were considered positive if OD values were higher than the 95th percentile of 50 normal healthy controls (0.130 for IgG and 0.280 for IgM, respectively).

To evaluate the β2GPI-dependence of aCL positive sera, aCL assays were performed in the absence of FCS, using gelatin only (0.5%; Sigma-Aldrich) in the blocking buffer as well as after addition of human β2GPI (5 μg/ml) or FCS (10%) as described (Di Simone et al., 2000).

2.3. Lupus anticoagulant

LA was detected by activated thromboplastin time and by Kaolin clotting time carried out with 0.2% kaolin suspension in saline (Exner et al., 1978).

2.4. Statistical analysis

The association with abnormally high aPL levels were evaluated in logistic regression. Odds ratios and 95% confidence intervals (CIs) were reported in centenarians, young healthy subjects, unselected elderly subjects and old SENIEUR volunteers.

3. Results

3.1. Prevalence of aCL and LA in centenarians

Fig. 1 shows the values of IgG (Fig. 1A) and IgM (Fig. 1B) aCL in the different groups. Sixteen out of 77 (20.7%) centenarians displayed IgG positivities in comparison to 0/70 in young healthy subjects, 3/65 (4.6%) in unselected elderly subjects and 4/38 (10.5%) in the old SENIEUR volunteers, respectively. The association with IgG aCL was significantly higher ($p=0.0001$) in centenarians than in young healthy subjects and in unselected elderly

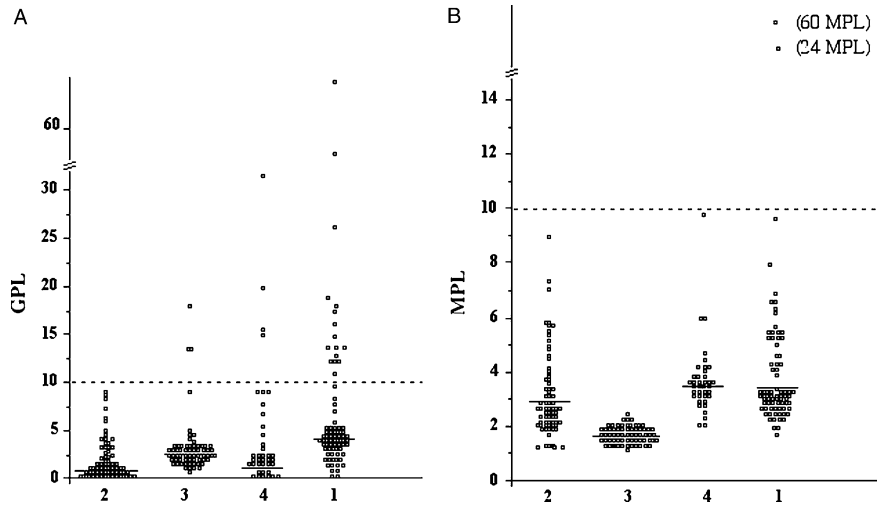

Fig. 1. Prevalence of IgG (A) and IgM (B) aCL in centenarians and control groups: (1) centenarians; (2) healthy young subjects; (3) unselected elderly subjects; (4) old volunteers, selected according to the SENIEUR Protocol (13). The dashed line indicates the cut off limit of normal values; the mean values are represented by horizontal lines. Values are expressed as GPL or MPL units.

subjects [$p=0.01$ (Odds ratio 5.4 CI 95% 1.5–19.6)] and tended to be higher than in old SENIEUR volunteers [$p=0.140$ (Odds ratio 2.4 CI 95% 0.7–7.7)]. Two samples only from centenarians resulted positive for IgM aCL.

Fifty-nine plasmas were available for LA evaluation and all resulted negative. Ten out of these 59 samples resulted positive in the IgG aCL assay (8/10 low and 2/10 medium positive).

3.2. Antigen characterization of aCL in centenarians

Samples positive for IgG aCL from centenarians have been tested against plates coated with PL with different electric charges. Table 1 shows representative results of 10 selected sera (six positive and four negative for IgG aCL) against plates coated with anionic (PS), cationic (Sph) and neutral (PE, PC) PLs. Samples negative for aCL did not display any binding even to plates coated with other anionic PLs. While all the aCL positive sera reacted with negatively (PS), only few displayed borderline reactivity with neutral (PE, PC) and none with positively (Sph) charged molecules. One serum from centenarians positive also for IgM aCL (24 MPL) displayed a binding activity to PS (0.421 OD value; mean + 3SD of 50 normal controls = 0.198 OD value). The remaining aCL positive sera from centenarians displayed comparable results (data not shown). As previously reported, sera from APS patients reacted with plates coated with anionic but not with neutral or positive PLs (data not shown) (Di Simone et al., 2000; Harris et al., 1985).

Forty-six sera from centenarians have been tested for anti-human β2GPI IgG and IgM antibodies; 25/46 (54.3%) and 4/46 (8.6%) sera, respectively, displayed IgG and IgM anti-β2GPI values higher than the normal controls. Only 4/38 (10.5%) old SENIEUR volunteers displayed low anti-β2GPI IgG positivities (lower than 0.320 OD values), while just one unselected elderly subject resulted borderline positive; no positivities for IgM anti-β2GPI antibodies were found (data not shown). Fig. 2 shows the analytical

Table 1
IgG aCL positive sera from centenarians display a cross-reactivity with anionic phospholipids

Subject	Anti-CL	Anti-PS IgG	Anti-PC IgG	Anti-PE IgG	Anti-Sph IgG
1	97	**0.980 ± 0.152**	**0.231 ± 0.02**	**0.201 ± 0.05**	0.001 ± 0.001
2	12	**0.324 ± 0.101**	0.041 ± 0.04	0.05 ± 0.02	0.021 ± 0.05
3	19	**0.452 ± 0.115**	0.092 ± 0.005	0.06 ± 0.02	0.034 ± 0.02
4	16	**0.402 ± 0.102**	0.045 ± 0.002	0.101 ± 0.03	0.025 ± 0.02
5	54	**0.758 ± 0.161**	0.157 ± 0.04	**0.197 ± 0.01**	0.017 ± 0.01
6	10	**0.284 ± 0.022**	0.035 ± 0.03	0.04 ± 0.03	0.028 ± 0.01
7	4	0.123 ± 0.098	0.161 ± 0.054	0.191 ± 0.045	0.187 ± 0.052
8	3	0.106 ± 0.047	0.120 ± 0.085	0.154 ± 0.074	0.162 ± 0.068
9	5	0.078 ± 0.021	0.157 ± 0.079	0.098 ± 0.021	0.097 ± 0.031
10	7	0.111 ± 0.075	0.115 ± 0.073	0.078 ± 0.052	0.140 ± 0.075

Values are expressed in GPL units for aCL and in OD values (mean ± SD of triplicate experiments) for anti-PS, anti-PC, anti-PE and anti-Sph antibodies. Normal values for anti-PS, anti-PE, anti-PC and anti-Sph antibodies were, respectively, lower than 0.154, 0.194, 0.197, 0.201 (mean + 3 SD of 50 normal human sera). Positive results are typed in bold.

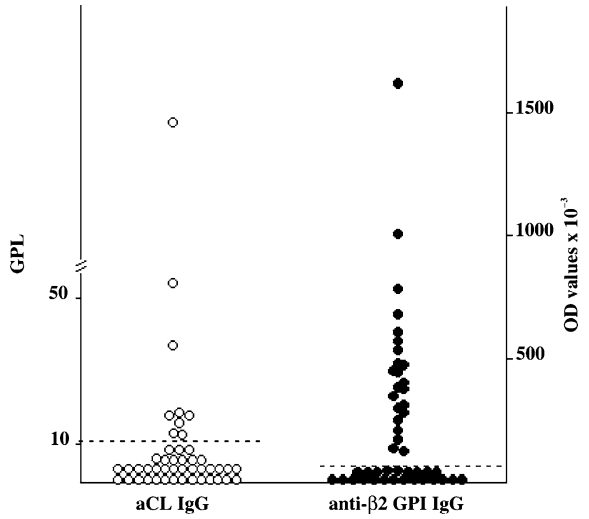

Fig. 2. Anti-cardiolipin and anti-human β2GPI IgG antibodies in sera from centenarians. Values are expressed as GPL units or as OD$\times 10^{-3}$ values. The dashed lines indicate the cut off limit of normal values (10 GPL for aCL and 0.130 OD for anti-β2GPI assay, respectively).

data of anti-β2GPI and aCL IgG; interestingly most of the samples reacted with human β2GPI but not with CL-coated plates and the reactivity against β2GPI was high in almost half of the samples. Only two out of four IgM anti-β2GPI positive sera also tested positive in the aCL assay (data not shown).

The presence of a reactivity against human β2GPI suggests a cofactor dependence for the aCL activity detected in centenarians. In order to demonstrate such a dependence, two positive samples from APS patients or from aCL positive centenarians (two IgG positive and one IgM positive) were tested with plates blocked with human β2GPI or with gelatin. The binding activity of the sera from centenarians declined when tested on plates without β2GPI (i.e. blocked with gelatin), and the binding was restored by the addition of purified human β2GPI (5 μg/ml) in a manner quite comparable to that found with the two APS reference sera. Fig. 3 shows the results of representative samples. Experiments carried out with the addition of FCS (10%) as source of bovine β2GPI gave comparable results (data not shown).

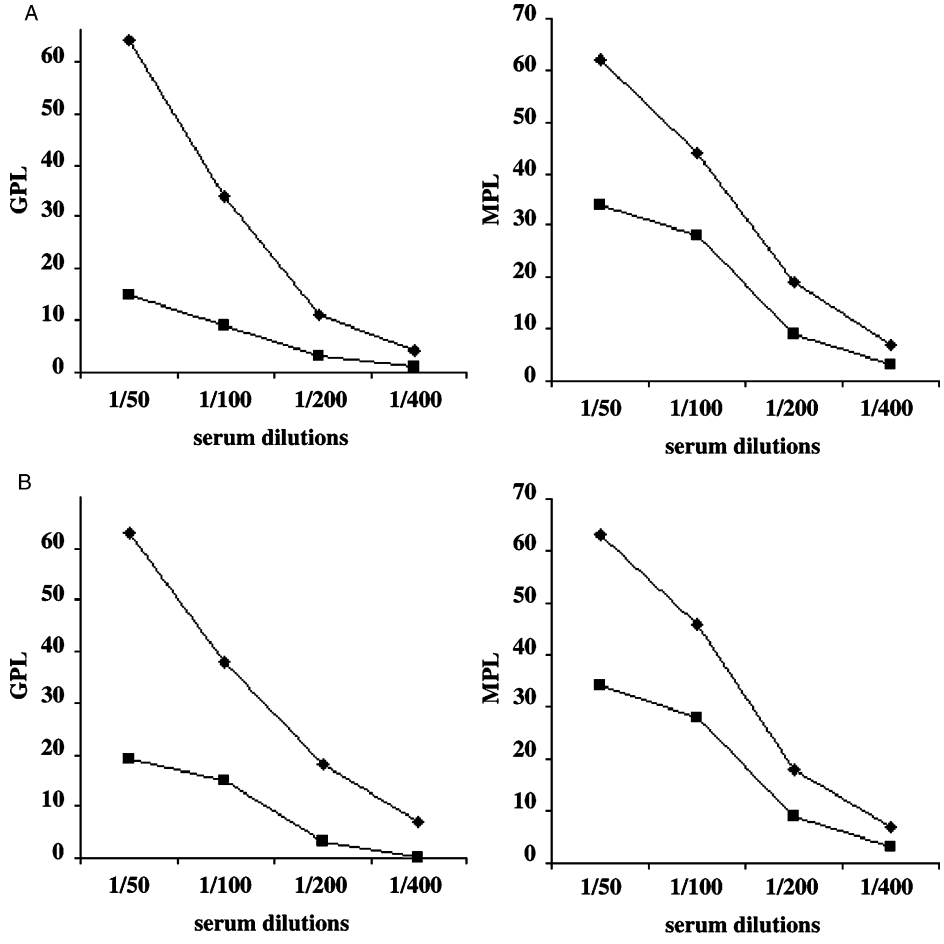

Fig. 3. Beta 2GPI-dependence of IgG and IgM binding to CL-coated plates of representative sera from centenarians (A) and from APS patients (B). Serial dilutions of sera have been tested on serum-free CL-coated plates in the presence (–◆–) (5 μg/ml) or in the absence (–■–) of β2GPI as described in Section 2. The values are expressed as GPL or MPL units.

4. Discussion

Our results report for the first time a high prevalence of anti-human β2GPI antibodies in centenarians in good health without any clinical manifestation of APS.

Anti-β2GPI antibodies have been recently found to be an apparently more specific, although less sensitive, diagnostic tool for the APS, and to represent an antibody population able to mediate potential pathogenic mechanisms in APS (Levine et al., 2002; Meroni and Riboldi, 2001; Tincani et al., 1998). Moreover, some authors reported that a small but consistent number of patients mirroring a full-blown APS can display antibody against the human β2GPI only, without any cross-reactivity with molecules from other species (Cabral et al., 1996). The lack of reactivity against bovine β2GPI was suggested to explain why these patients were negative in the standard assay for aCL antibodies, where bovine β2GPI supplied by FCS is the major target antigen for aPL assay (Levine et al., 2002; Meroni and Riboldi, 2001; de Groote et al., 2002; Roubey, 1999, 2000).

Accordingly, centenarians displayed a high prevalence of antibodies by using human β2GPI-coated plates but lower prevalence and titres of positive results when the same sera were assayed by the standard aCL assay that employs bovine serum as blocking agent. This finding does suggest that centenarians react with a much more specificity for the human molecule.

It has been widely accepted that aPL detectable in APS display a cross-reactivity with anionic PLs and such a reactivity was suggested to be the result of the binding of the cationic β2GPI to the negatively charged PLs (Roubey, 1999, 2000). Once bound, β2GPI expresses new cryptic epitopes specific for the autoantibodies and/or displays an increased antigen density that is required because of the low affinity of the anti-β2GPI autoantibodies (de Groote et al., 2002; Roubey, 1999, 2000). In line with these findings, a strong reactivity with plates coated with anionic, but negligible with cationic and absent with neutral PLs, was found in sera from centenarians, as reported in APS.

To further support the 'autoimmune' nature of the aPL detectable in our subjects, we investigated whether the aCL positive sera bound to CL-coated plates in a β2GPI-dependent manner as reported for APS sera. Our data clearly show that the aCL assay carried out in serum-free buffer displayed decreased binding values, and that the reactivity was restored if human purified β2GPI or FCS, as source of bovine β2GPI, were supplied.

Altogether our data suggest that centenarians react preferentially with human β2GPI but that the presence of a cross-reactivity with bovine β2GPI appears to be also responsible for the less frequent positivities in the standard aCL assay.

In the APS, the breakdown of the tolerance towards the self β2GPI has been suggested to be the result of a molecular mimicry between exogenous and self molecules, at least in part due to the wide aminoacid homology of β2GPI from different species (Matsuura et al., 1991; Tincani et al., 2002; Gharavi et al., 1999; Blank et al., 2002). Still debated is the initial trigger of the response against the β2GPI, although preliminary data suggest that bacterial and/or viral peptides sharing common aminoacid sequences could be responsible (Gharavi et al., 1999; Blank et al., 2002).

It is useful to speculate on the possible mechanisms that could support the appearance of an anti-β2GPI activity in centenarians. Lifelong exposure to self molecules resulting from the continuous apoptosis occurring in the body, an event particularly consistent in centenarians (Aggarwal and Gupta, 1998), may contribute to this phenomenon. In particular, apoptotic blebs were reported to expose anionic PL (mainly PS) that in turn are able to bind circulating β2GPI (Casciola-Rosen et al., 1996). Such an event results in the exposure of epitopes on the bound molecule that are able to induce an anti-β2GPI humoral immune response in naïve mice (Price et al., 1996). So, it could be possible that the increased exposure of β2GPI on apoptotic cells might act as a persistent immunogenic stimulus which could end into an antibody response against the self molecule. Alternatively, it has been also reported that β2GPI bound to oxLDL can be recognized by specific antibodies, suggesting that even in this case, the molecule can display the right immunogenic epitopes (Hasunuma et al., 1997). Furthermore, increased plasma levels of oxLDL have been found in centenarians (Maggi et al., 1993), so offering large amounts of substrates able to bind β2GPI and to make the right immunogenic epitopes available to the immune system.

Whatever the mechanisms by which centenarians elicit an anti-β2GPI response comparable to that found in APS, the clinical records of our subjects do not have any evidence of the manifestations associated with the presence of such autoantibodies.

The lack of clinical manifestations might be related to the absence of LA, and to the fact that most of the aCL positive sera in centenarians were at low titre. Actually, it is widely accepted that LA does represent the strongest risk factor for thrombotic events in APS while medium/high aCL titers are closer associated with clinical events than low titres (Levine et al., 2002; Galli et al., 2003). However, such an explanation does not account for the absence of thrombotic events in centenarians with medium or high titres of β2GPI-dependent aCL.

Anti-phospholipid antibodies are now considered pathogenic autoantibodies rather than a simple serological marker for APS. Several potential mechanisms have been reported to explain the aPL ability to induce thrombosis and/or fetal loss (Meroni and Riboldi, 2001). However, aPL alone apparently are unable to induce thrombotic manifestations per se. In this regard, a *two-hit hypothesis* has been suggested: aPL (*first hit*) increases the risk of thrombotic events that occur in the presence of another thrombophilic condition (*second hit*). In line with such a hypothesis are

the experimental findings in murine models, in which infusion of aPL can increase clotting after mechanical injury to the vessel wall but do not induce thrombus when injected into uninjured vessels (Pierangeli et al., 2000). Moreover, the *two hit hypothesis* might also explain why patients persistently positive for aPL do display thrombotic events only occasionally. In this regard, the positivity for aPL with 'autoimmune' characteristics and the concurrent presence of additional risk factors for thrombosis (i.e. hypercoagulability, factor V mutation, polymorphism 4G4G of PAI-I promoter, G20210A prothrombin mutation, dyslipidemia) (Mari et al., 1995, 1996; Mannucci et al., 1997; Sacchi et al., 1999; Baggio et al., 1998) in centenarians should favour the appearance of the vascular manifestations of the syndrome. Moreover, we found high plasma levels of pro-inflammatory cytokines, such as IL-6, and low levels of anti-inflammatory cytokines, such as IL-10, in centenarians (Bonafè et al., 2001). Pro-inflammatory cytokines might activate monocytes and/or endothelial cells favouring the induction of a pro-coagulant phenotype (Cines et al., 1998; Bouchard and Tracy, 2001) and acting as additional 'second hit' risk factors. Nevertheless, all the subjects of our series escaped major thrombotic diseases. Thus, it is useful to speculate that in centenarians yet unknown mechanisms are active in protecting the thrombophilic state associated to aPL or that in the oldest old, the risk factors could play a different role than in young-adult subjects. Actually, high total cholesterol concentrations have been associated with longevity owing to lower mortality from cancer and infection (Weverling-Rijnsburger et al., 1997).

Further studies aimed to clarify and disentangle such mechanisms could offer new insight to better understand not only the biology of ageing but also the pathophysiology of APS.

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