**THROMBOCYTOPAENIA IN ANTIPHOSPHOLIPID SYNDROME:**

**A FREE RADICAL PERSPECTIVE**

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**Abstract**

Thrombosis associated with thrombocytopaenia is an apparent paradox that is present across a wide spectrum of disorders. While thrombocytopenia has been a controversial clinical classification criterion for antiphospholipid syndrome because initial reports failed to demonstrate a relation between low platelet count with other clinical or laboratory manifestations of the syndrome, recent data highlight the association between mild to moderate thrombocytopaenia and the subsequent risk of thrombosis. Although antiphospholipid antibodies may induce platelet activation *in vitro,* additional stimuli may contribute to their activation *in vivo*, amongst which reactive oxygen and nitrogen species and lipid peroxidation products, that are elevated in patients with the antiphospholipid syndrome: herein we provide a novel plausible framework involving free radicals that could add to the understanding of the thrombocytopaenia/thrombosis paradox in the antiphospholipid syndrome.

**Key words**: thrombocytopenia, antiphospholipid syndrome, reactive oxygen and nitrogen species, lipid peroxidation products.

**Key message**: free radicals may be implicated in the thrombocytopaenia/thrombosis paradox of the antiphospholipid syndrome

**Introduction**

In the first semester of this year two articles published in Rheumatology highlighted the negative impact of thrombocytopaenia in primary antiphospholipid syndrome (PAPS). The first study from Spain reported data from a longitudinal cohort of 114 antiphospholipid patients (APS) followed up for a mean of 19 years, 64% of which with PAPS: this demonstrated that low-moderate and persistent thrombocytopenia (platelet count 50-150 x 109/L), identified PAPS and secondary APS patients at a higher risk of thrombosis-related mortality (hazard ratios 2.8 and 4.4 respectively) (1). The second study from China reported data from a longitudinal cohort of 218 PAPS patients followed up for a median of 25 months, showing that mild thrombocytopenia (platelet count <100 x 109/L) was associated with a higher risk of thrombotic events (hazard ratio 2.9) with a recurrent thrombosis rate of 47.4% at six years (2)

These finding highlight a somewhat unexplored but not unexpected paradox in APS. In 2017, a longitudinal Japanese cohort of 953 consecutive antiphospholipid (aPL) positive patients assessed the predictive effect of platelet number on the risk of thrombosis via the antiphospholipid score (aPL-S) which combines different types and titres of aPL. Having discriminated low from high aPL-S, the authors found that within the low aPL-S group, patients with a platelet count ≤150x109/L were more likely to develop thrombosis than patients with a normal platelet count (HR 3.44), whereas patients with a high aPL-S developed thrombosis independently of the platelet count (3). Likewise, a platelet count ≤150x109/L was identified in 51 patients (26.8%) from another cohort consisting of 85 PAPS, 48 secondary APS patients and 52 asymptomatic aPL carriers: by propensity score analysis, thrombocytopenia (alongside hypertension), independently predicted the compound risk of thrombosis, obstetric morbidity and death (HR 10.95) (4).

The relation between thrombocytopaenia and thrombosis is not unique to APS and is found also in patients with immune thrombocytopaenia (ITP): a systematic review in adults with primary ITP concluded that ITP was associated with an increased risk of arterial thromboembolism (ATE) (RR 1.52) and a greater risk of venous thromboembolism (VTE) in splenectomised patients (RR 2.39) (5). A subsequent bi-national study from France and Sweden showed that the incidence rate of ATE was 15.0 and 14.7 per 1000 person-years respectively, whereas the incidence rate of VTE was 6.9 and 6.5, respectively: older age, male gender and a history of ATE were associated with a recurrent ATE in both countries (6).

In the ITP setting, but in the concomitant presence of aPL, the odds ratio of developing ATE in the presence of the lupus anticoagulant (LA) was 5.52 but it was lower at 2.12 in the presence of anticardiolipin antibodies (aCL) alone. Conversely, the odds ratio of developing VTE in the presence of LA or aCL was 5.13 and 2.00 respectively (7).

**Megakaryocytes, platelets, reactive oxygen and nitrogen species**

Platelets are small a-nucleated fragments deriving from megakaryocytes (MK): the latter originate from a progenitor stem cell that goes through several mitotic self-renewals before undergoing endomitosis into cells with progressive polyploidy; during this process platelet proteins are synthesized and the demarcation membrane system develops (8) giving rise to the long cytoplasmic branches that, once shed into the marrow sinusoids as pro-platelets (9), break up into platelets (10).

Reactive oxygen species (ROS), including superoxide anion (O·̄2) produced from different cell types residing in the bone marrow, are involved in endomitosis, polyploidization, maturation and platelet formation. By activating redox-sensitive transcriptional factors, ROS control the expression of target genes that code for enzymes endowed with antioxidant activity or for the biosynthesis of molecules with antioxidant activity that preserve the intracellular MK oxidant/antioxidant balance (11). An excess of ROS, differentially acting on anti-apoptotic BCL-xl and pro-apoptotic Bax and Bak, (12) decreases MK proliferation, favors cell cycle arrest and promotes senescence (13, 14).

In a simplified schematization, the interaction of O·̄2 with nitric oxide (•NO) deriving from endothelial and monocyte nitric oxide synthase generates reactive nitrogen species (RNS), amongst which peroxynitrite (ONOO-); although **•**NO, in isolation or in combination with thrombopoietin, allows the development of functional platelet-sized particles *in vitro*, the predominance of ONOO- over •NO induces premature apoptosis that limits platelet generation (15). Overproduction of ONOO-, detected as the 3-nitro-tyrosine adduct in plasma proteins, was documented in patients with PAPS (16) and systemic lupus erythematosus (SLE) (17). Interestingly, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) enhanced endogenous RNS and indirectly suppressed MK maturation via the pathway mentioned above (18), whereas interferon- (IFN-) directly suppressed platelet production (19). Serum levels of TNF-α, IFN- and IFN-γ are raised in SLE (20, 21, 22, 23) and APS (24) (Figure 1).

**Megakaryocytes, platelets and lipid peroxidation products**

ROS can cause structural damage to DNA, proteins and lipids both directly and indirectly by fuelling the peroxidation of polyunsaturated fatty acids, such as arachidonic acid. Lipid peroxidation products, such as the reactive aldehydes acrolein, malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE), can form covalent electrophilic addition products with DNA and proteins (25); MDA and HNE adducts are present in SLE (17) and it is of particular interest that acrolein and 4-HNE can react with DNA via Michael addition to the *N2*-amino group of deoxyguanosine, resulting in the formation of 1,*N2*-exocyclic propane adducts (reviewed in 26). These can form inter-strand cross-links which impede DNA replication and transcription. Notably, in a murine model of Fanconi anaemia (FA), the knockout of the *Fanca* gene, encoding Fanconi anaemia group A protein (FANCA) which is required for DNA incision at sites of psoralen-mediated inter-strand cross links (27), caused defective megakaryopoiesis, accumulation of MKs with low ploidy, decreased platelet production, formation of nucleoplasmic bridges and chromosomal instability (28).

Lipid hydroxyperoxyl radicals also undergo, in addition to aldehyde formation, endocyclization to F2-isoprostanes (F2-IPs); in particular 8-*epi*-prostaglandin-F2 (8-*epi*-PG-F2), originating from arachidonic acid by the effect of O·̄2, independently of cyclo-oxygenase, caused a dose-dependent irreversible platelet aggregation in the presence of sub-threshold concentrations of collagen, adenosine diphosphate, arachidonic acid and analogues of thromboxane A2(29).

F2-IPs are increased in SLE and primary APS (reviewed in 30): urinary concentrations of 8-*ep*i-PGF2and IPF2-I were higher than normal in patients with SLE and correlated with serum levels of aCL (31) and of prothrombin fragment F1+2 (32).

**Megakaryocytes, platelets and thrombin**

It was known since the 1990s that thrombin could inhibit MK growth, once bound to its specific receptor on MK progenitors (33); indeed, thrombin causes G0/G1 arrest and down- regulation of BCL-2, inducing MK apoptosis and blocking platelet generation (34). Moreover, as little as 30 min of thrombin exposure *in vitro* may induce platelet fragmentation and exhaustion, accompanied by loss of platelet contractility, mitochondria depolarization and an increase in ROS; the resulting platelet-derived fragments have been defined “apoptotic cellular bodies” (35), whereas apoptotic platelets in plasma become smaller after shedding microparticles (Luff) and in both instances the membrane exposure of phosphatidylserine acts as an “eat me” signal for macrophage clearance (36).

**Free radicals, impaired megakaryocyte production and increased platelets destruction**

As mentioned earlier, *in vitro* experiments showed that free radicals reduce MK proliferation, favor cell cycle arrest and promote senescence (13,14). Similar *in vitro* experiments demonstrated that plasmas from SLE patients (but without APS), increased megakaryopoiesis but inhibited proplatelet formation; this effect was reverted by the same plasmas depleted of total antibodies containing the anti-platelet antibody fraction; additionally, platelet apoptosis, assessed by measuring the loss of mitochondrial membrane potential, the exposure of active caspase 3 and of phosphatidylserine, was increases in thrombocytopenic patients compared to patients with normal platelet counts. Both effects were maximal in patients with higher disease activity (Baroni Pietto), who were not necessarily harboring higher anti-platelet autoantibodies titers: it cannot be excluded that plasma free radicals mediate these effects, given that oxidative stress, measured as 8-*epi* PGF2, is greater in plasma of SLE patients with higher disease activity (Ames). Nevertheless, these data confirm premature MK apoptosis (15) that limits platelet production as well as increased platelet consumption that complements the effect of thrombin described in the previous paragraph. Although we have no data regarding *in vivo* survival of 111In-labeled or 51Cr-labelled autologous platelets in APS, there is indirect evidence for increased platelet turnover in some patients with APS, indicated by increased median CD63 expression compared to healthy controls (Joseph) and by reduced platelet size and increased platelet microparticles resulting from apoptosis in relation to APS (Lood).

**Coagulation and complement, reactive oxygen and nitrogen species**

While the occurrence of oxidative (30) and nitrative stress (16,17) in APS has been known for more than two decades, recent *in vitro* studies confirmed that aPL may induce ROS production from mitochondria (37) and from endothelial cells (38) and that aPL, either monoclonal or purified from APS patients, may activate endosomal NADPH-oxidase-2 that in turn releases O·̄2 and prompts inflammasome activation (39); the subsequent release of IL-1β, in a positive feedback loop, favors the uncoupling of superoxide dismutase, glutathione peroxidase and catalase that perpetuates unhindered intracellular ROS generation (40).

This oxidative and nitrative stress background promotes a procoagulant state characterized by enhanced coagulation (41,42,43) and complement activation (44, 45) processes known to be interlinked in APS (46) as well as fibrinolysis inhibition (47).

**Reversal of thrombocytopaenia and paradoxical thrombosis or re-thrombosis**

There have been reports where reduction of thrombin generation and/or quenching of oxidative stress led to reversal of thrombocytopenia; few cases occurred after the administration of acetylsalicylic acid (ASA) in PAPS (48-51): ASA may directly (52) or indirectly (53,54) increase the effect of antithrombin against thrombin (55) both at low (53,55) and at high dose (54); other cases occurred after the initiation of warfarin in some PAPS and SLE-related APS patients (56, 57), and by its very mechanism of action (58) warfarin prevents thrombin generation (59). Cloroquine led to the normalization of thrombocytopaenia in PAPS (60) and, interestingly, hydroxychloroquine improved platelet counts in paediatric (61) and adult (62) immune thrombocytopaenia. Hydroxychloroquine inhibits the NADPH oxidase system and dampens the generation of O·̄2 (63,64) and of isoprostane (65). Altogether, releasing the inhibitory effect of thrombin and ROS on MK and platelet apoptosis might restore megakaropoiesis and decrease platelet consumption.

**Concluding remarks**

In the light of the above information, persistent thrombocytopaenia in PAPS may be seen as a consequence of ongoing oxidative stress (30), nitrative stress (16, 17) and thrombin generation (66), key players in atherosclerosis (67, 68), that may exert different degrees of inhibition of megakaryocyte maturation; mechanistically, the ensuing thrombocytopaenia attempts to minimize the chance of platelet clot formation within atherosclerotic arterial vessels (69).

Of note, while the degree of thrombocytopenia reported by the previous authors does not need intervention, reversal of severe thrombocytopaenia, with a sudden rebound in the production of fully functional platelets, may prompt re-thrombosis or ex novo thrombosis in APS, mostly arterial; in particular, one myocardial infarction re-developed after commencement of warfarin (56), one after immune suppression (70) and two after thrombopoietic agents (Guitton), a recurrent ischemic stroke developed after starting warfarin (56) and another after starting a thrombopoietic agent (Guitton); the administration of thrombopoietic agents was followed by two ex-novo catastrophic APS (71, Guitton), one ex-novo pulmonary embolism and deep vein thrombosis each (Borrell, Guitton). It should be remembered that platelets constitute 31.3% of volume of arterial thrombi and 0.4% and 0.8% of venous thrombi and pulmonary embolism, respectively (Chernysh).

While the mechanisms leading to acute or chronic autoimmune thrombocytopaenia are not the scope of this article (73) the pathogenesis of thrombocytopaenia in APS may be more complex than anticipated: oxidative, nitrative and sulfhydryl stress, by inducing post-translational modifications of proteins and/or lipids, may conduce to the emergence of neoepitopes or favor epitope spreading in known and unknown platelet and MK targets, eliciting novel autoantibodies that add to those already known in autoimmune thrombocytopenia (74, 75).

The observation that different agents with antioxidant properties dampen oxidative stress (76, 77) inflammation (77), urinary thromboxane excretion (76), coagulation activation (76,77) and restore fibrinolysis (76) suggests that antioxidant administration represents an attractive therapeutic modality that could benefit patients with APS and should be explored in a randomized controlled trial.

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**Conflict of interest**

None of the authors have any conflicts of interest to declare.

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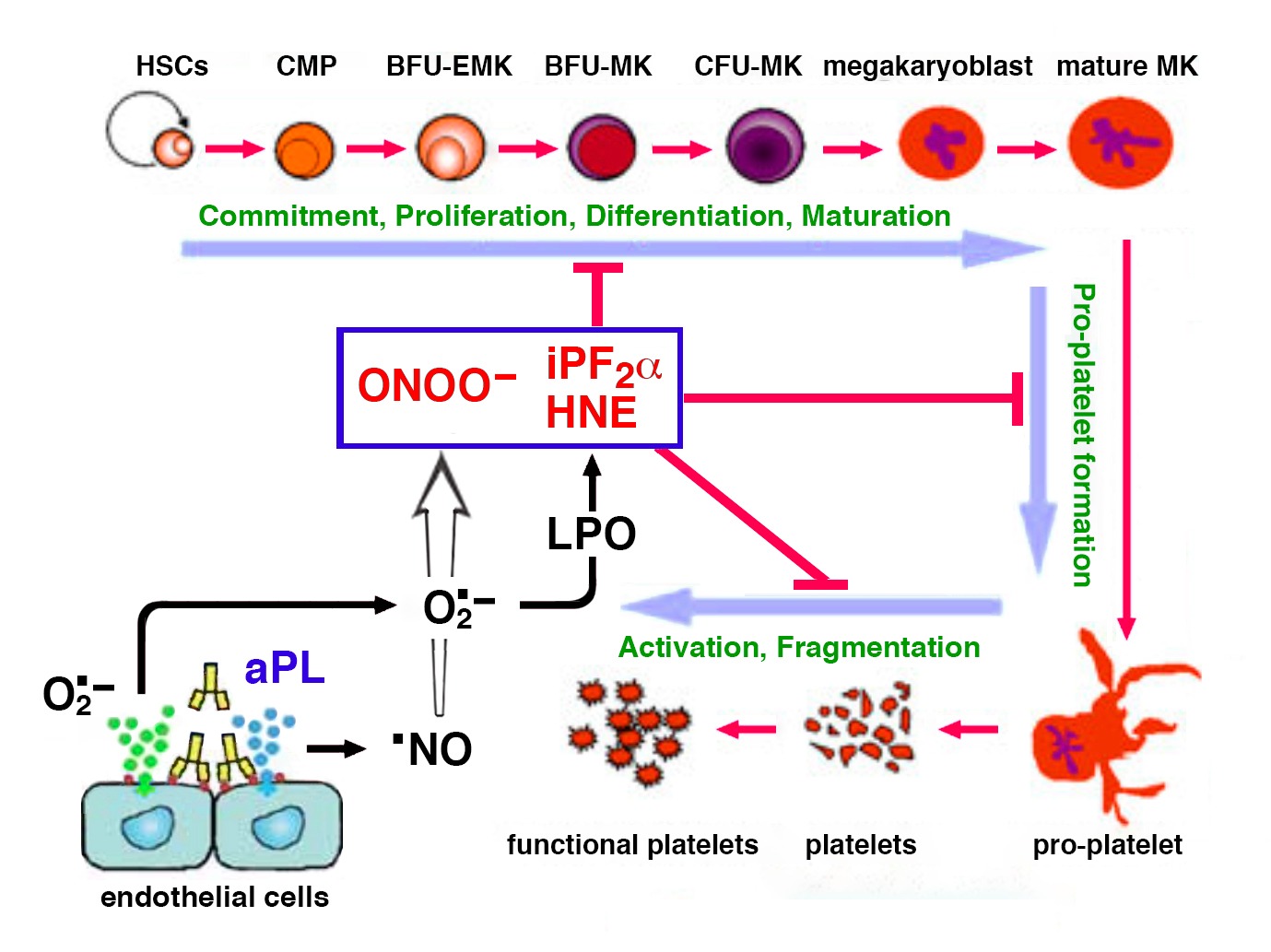


Figure 1.

**Effects of oxidative and nitrative stress molecules on megakaryocyte and platelet production.**

Antiphospholipid antibodies stimulate the release of superoxide anion (O·̄2) and nitric oxide (•NO) from endothelial cells giving rise to peroxynitrite (ONOO-) and lipid peroxidation products (LPO) such as 4-hydroxynonenal (HNE) and isoprostanes; these may induce premature megakaryocyte (MK) and platelet apoptosis leading to thrombocytopaenia and platelet exhaustion. Image adapted from Chen et al. Cell Death and Disease 2013; 4: e722