PREDICTORS OF MORTALITY IN PRIMARY ANTIPHOSPHOLIPID SYNDROME: A SINGLE CENTRE COHORT STUDY

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Short running head: mortality in primary antiphospholipid syndrome

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Abstract

Background. The vascular mortality of antiphospholipid syndrome (APS) ranges from 1.4% to 5.5% but its predictors are poorly known. **Objectives**. To evaluate the impact of baseline lupus anticoagulant assays, IgG anticardiolipin (aCL), plasma fibrinogen (FNG) and von Willebrand factor (VWF), platelets (PLT) and of genetic polymorphisms of methylenetetrahydrofolate reductase C677T, of prothrombin G20210A and of paraoxonase-1 Q192R on mortality in primary APS (PAPS). **Patients.** Cohort study on 77 thrombotic PAPS and 33 asymptomatic carriers of aPL (PCaPL) seen from 1989 to 2015 and persistently positive for aPL as per annual review. At baseline all participants were tested twice for the ratios of kaolin clotting time (KCTr), activated partial thromboplastin time (aPTTr), dilute Russell viper venom time (DRVVTr), IgG aCL, FNG, VWF and once for PLT. All thrombotic PAPS were on warfarin with regular INR monitoring. **Results.** During follow-up 11 PAPS deceased (D-PAPS) of recurrent thrombosis despite adequate anticoagulation yielding an overall vascular mortality of 10%. D-PAPS had the strongest baseline aPTTr and DRVVTr and the highest mean baseline IgG aCL, FNG, VWF and PLT. Cox proportional hazards model identified baseline DRVVTr and FNG as main predictors of mortality with adjusted hazard ratios of 5.75 (95%CI: 1.5, 22.4) and of 1.03 (95%CI: 1.01, 1.04) respectively. **Conclusions.** Plasma DRVVTr and FNG are strong predictors of vascular mortality in PAPS; while FNG lowering agents exist further research should be directed at therapeutic strategies able to dampen aPL production.

Key words: antiphospholipid syndrome, DRVVT, fibrinogen, mortality.

Introduction

The primary antiphospholipid syndrome (PAPS) encompasses the occurrence of arterial and venous thrombosis in the presence and persistence of antiphospholipid antibodies (aPL) measured by immune and clotting assays and in the absence of any underlying autoimmune or chronic inflammatory disorder (1, 2). Unless adequately anticoagulated APS patients may undergo recurrent vascular occlusions but even when adequately anticoagulated some of them may suffer thrombotic recurrences that may be fatal (3, 4). In this respect a European multicentre survey has recently derived an all-cause mortality of 7.1% for PAPS but did not identify factors associated with mortality probably due to the multicentre nature that creates some difficulties when employing different reagents and methods across the participating centres (5). We have been following up patients with PAPS since 1989 and we evaluated the impact of several coagulation assays, haemostatic variables, thrombophilic and antioxidant genotypes on the mortality of this cohort.

Patients and methods

Patients

This cohort includes 110 patients followed up since 1989 who were found positive for aPL on two separate occasions six weeks or three months apart according to evolving guidelines (1, 2). They were evaluated for a history of thrombosis and for a coagulation abnormality suggesting the presence of a lupus anticoagulant (LA). Since the inception of the cohort our focus was on vascular involvement (arterial and venous occlusions) hence women in whom aPL was sought as part of their obstetric assessment and women with miscarriages (with or without thrombosis) were not included and this reflects in our entry criteria. Inclusion criteria for this study were: regular follow-up for INR monitoring for thrombotic PAPS patients on oral anticoagulation and at least one yearly clinical and laboratory follow-up for persistent carriers of aPL (PCaPL) without underlying autoimmune or non-autoimmune diseases. Our exclusion criteria were: 1) history of miscarriages (with or without thrombosis) and/or positivity for aPL in women whose aPL was sought as part of their obstetric assessment, 2) any positive aPL in relation to autoimmune and/or inflammatory disorders, 3) APS related to systemic lupus erythematosus and secondary APS. The study was approved by the ethics committee of the Cardarelli

Hospital (Naples, Italy) and all participants gave written informed consent at the time of enrolment. None of the authors declare any conflict of interest.

Patients were followed up at the Haemostasis Unit of the Cardarelli Hospital in Naples (Italy) until its phased closure between June/October 2007 although the Haemostasis Unit had stopped seeing new patients by mid-2006. After June 2007 patients were followed-up under the umbrella of a patient association in a Clinical Pathology Laboratory (Multimedica, Naples, Italy), partially funded by the Regional Health Authority. During the observational period new patients accrued but those not having their INR monitored at our centre (n=16) and at least one annual follow-up (n=3) with the centre were not included in the current evaluation. The demographics of the cohort are depicted in Table 1.

Blood sampling

Blood samples for clotting assays were collected by venepuncture in 1/10 volume of 0.129 M trisodium citrate. After centrifuging at 2000 x g for 15 min at 4C, aliquots of platelet poor plasma (PPP) were stored at -70ºC for the measurement of fibrinogen (FNG) and von Willebrand factor (VWF) and the remaining were spun twice at 10.000 x g for 5 minutes to obtain platelet free plasma (PFP). Control platelet free plasma for clotting assays was obtained by 60 healthy hospital personnel (34F, 26M, mean age 33±19), aliquoted then frozen at -70ºC. Blood for serum preparation was collected into glass tubes, allowed to clot for 2 hours at room temperature, spun at 1000 x g for 10 minutes, aliquoted and frozen at -70ºC until use.

Measurement of antiphospholipid antibodies

PFP for LA assays were processed immediately; LA was detected by 1) activated partial thromboplastin time (aPTT) using rabbit brain kaolin (Diamed, Switzerland); 2) kaolin clotting time (KCT) according to the method of Exner (6); 3) dilute Russell's viper venom time (DRVVT) according to the method of Thiagarajan (7). A clotting time ratio between patient and control sample greater than 1.2 for the aPTT, 1.18 for the DRVVT and 1.3 for the KCT indicated an abnormal result; the upper cut-offs for each assay was set at the 99th percentile from testing 60 plasmas from 38 females and 22 males who were healthy hospital personnel. In any of the assays, a clotting time of a 1:1 mixture of sample and pooled control plasma greater than that of pooled plasma alone suggested the presence of a lupus inhibitor. This was

confirmed by the platelet neutralisation procedure (PNP) according to Triplett (8), and by high phospholipid concentration according to Rosove (9). IgG and IgM anticardiolipin antibodies were measured by a commercially available ELISA (Melisa System, Cambridge Life Sciences, Ely, UK changed after June 2007 to Autozyme by the same company). A normal range was established using the same 60 healthy hospital personnel as mentioned above, with a cut-off of 5GPL U/ml being 5 standard errors above the geometrical mean.

During follow-up all patients of the cohort had their aPL status checked annually: those patients on warfarin whose LA was initially expressed in the aPTT (with or without DRVVT positivity) had its presence reconfirmed by comparing a sensitive and insensitive aPTT reagent to LA that allows the detection of LA whilst on warfarin (10); those patients whose LA was expressed only in the DRVVT had this rechecked after mixing with normal plasma if the INR was greater than 1.5 (reviewed in Moore (11).

Measurement of plasma fibrinogen and von Willebrand factor

Plasma concentrations of clottable FNG was measured in duplicate (Mascia Bruelli, Italy) according to Clauss (12); the coefficient of variation (CV) was 4.2%. Plasma VWF was measured in duplicate by Elisa (Boehringer Mannheim, Germany). Inter-and intrassay CV were 3.2% and 4.2% respectively. Full blood counts were processed by an automated analyzer (Beckman Coulter, Italy). All aPL and haemostatic variables assays were measured the first time between 3 and 6 months after the occlusive event to account for a possible transient acute phase reaction then re-tested the second time after six then twelve weeks according to guidelines (1, 2). None of the patients reported acute illnesses in the intervening period between aPL measurements. All data in the present study represent the mean of the values obtained on those two separate occasions but for the platelet count that was measured only once, at the time of the first aPL sampling.

Determination of genetic thrombophilia

The following gene polymorphisms were determined by polymerase chain reaction as described previously: C677T of the methylenetetrahydrofolate reductase (MTHFR), G20210A of prothrombin, A1691G of factor V Leiden (13) and Q192R of paraoxonase-1 (14).

Statistics

Continuous variables are summarised by their mean, standard deviations, medians and interquartile ranges; transformation have been considered for non-symmetrical distributions. For the purpose of modelling time since diagnosis to death and for the calculation of the hazard ratio (HR) of death censoring time was considered from time of diagnosis to 26th May 2015. Appropriate statistical tests (parametric or non-parametric) have been implemented to compare crude differences between baseline variables across groups. Variables were log transformed when necessary to meet the assumptions required by the statistical models.

The Cox Proportional Hazard (PH) model was applied to investigate the HR ratio of death since the baseline assessment in association with a series of demographic and clinical factors. This semi-parametric technique allows no assumption to be made on the baseline hazard though the proportionality of the hazards should hold. The latter assumption has been tested on the basis of Shoenfeld's residuals after fitting the model to the data (15). Given the presence of missing data, multiple imputation techniques tailored to interval censored data have been implemented under missing at random assumption (16).

Results

Comparison of baseline variables across groups

A comparison of baseline variables reveals that average IgG aCL (log transformed)(Figure 1A), DRVVTr (Figure 1D), platelet counts (Figure 1E), VWF Ag (Figure 1F) and FNG (Figure 1G) were highest in the D-PAPS than in the other groups.

Follow-up of baseline variables

During follow up none of the participants became fully negative to an aPL test but there were fluctuations and some negative tests became positive. Of 13 positive aPTT at baseline with a ratio between 1.2 and 1.5, 8 were still positive at last FUP; of 18 aPTT that were negative at baseline with a ratio <1.2, 4 had become positive with a ratio between 1.2-1.4. Of 17 positive DRVVT at baseline with a ratio between 1.19-1.30, 12 were still positive: 7 within the same baseline ratio, four with a ratio between 1.3-1.4, one with a ratio of 1.6. Of 16 negative DRVVT at baseline, 6 had become positive, 4 with a ratio between 1.18 and 1.3, one with a ratio of 1.42 and one with a ratio of 1.48). Of the 28 IgG aCL with a baseline value less than

20GPL, 12 were still negative under the same threshold, 4 became positive above 40 GPL rand the remainder fluctuated between 20-40 GPL. All other aPTT and DRVVT ratios as well as IgG aCL titres above those indicated remained positive at last follow up with variations that did not alter the aPL status of the individuals of the cohort.

KCT

Proportion of morbidity

During follow-up 0.9% (1/110) of participants developed a hypernephroma that was resected without complications, 1.8% (2/110) developed a cerebral vasculopathy and 6.3% (7/110) developed essential hypertension; the latter two conditions were mutually exclusive and occurred all in the S-PAPS group and not in the D-PAPS group. With regards to non-fatal thrombotic recurrences 0.9% (1/110) patients developed bilateral adrenal infarction and 3.6% (4/110) recurrent deep vein thrombosis. These recurrences occurred despite patients being in the target range (INR 2.0-3.0); our time in target range was 80%; the INRs of these patients was upped by 0.5 INR units compared to the average of their INRs pre-recurrent event. With regards to bleeding episodes, 3.6% (4/110) participants suffered major bleeding episodes, all in the S-PAPS group: two metrorrhagias, one spontaneous and one due to fibroids (at an INR 3.2 and 3.0 respectively); one gastrointestinal haemorrhage due to bleeding polyps (at an INR at 3.2) in one male; one post traumatic subdural hematoma (at an INR 2.0–3.0) in a woman.

Proportion of mortality

The percentage of deaths of the whole cohort, including D-PAPS + surviving PAPS (S-PAPS) and PCaPL groups was 10% (11/110); by considering only the thrombotic groups (D-PAPS and S-PAPS) it was 11%. Most patients died of recurrent arterial occlusions: in this group one patient had nephrotic syndrome, one had slightly elevated cholesterol and two smoked (10 and 15 cigarettes per day); the remaining deceased patients did not present other cardiovascular risk factors. Table 2 shows some laboratory and clinical features of the D-PAPS; the fatal recurrent events are split into unprovoked and provoked to highlight that the latter occurred in patients with medium titre IgG aCL and the lowest DRVVTr of this group.

Predictors of mortality by Cox proportional hazard

Cox PH model explored the possible crude effect of demographic (age, gender), clinical (age at 1st thrombosis, number of thrombosis), laboratory (baseline log IgG aCL, aPTTr, DRVVTr, KCTr, FNG, VWF, PLT) and genetic variables (MTHFR, PT, PON) on survival. Factor V Leiden was not included in the analysis as too few patients carried the mutation. Log IgG aCL, DRVVTr, FNG and PLT emerged as mortality predictors by univariate analysis. These factors underwent a multivariable analysis employing multiple chained imputation techniques that identified DRVVTr, FNG and PLT as independent predictors of mortality (Table 4). Figure 2 also shows the effect of baseline DRVVTr, FNG and PLT on survival based on the adjusted multivariable model.

Discussion

The mortality of our cohort, all vascular related, was 10% (11% considering only the thrombotic patients) a figure almost 10 fold higher than the 1.4% vascular mortality derived from the PAPS group of the Euro-Phospholipid-Project (EPP)(5). Other surveys reported a vascular mortality of 5.5% in PAPS (17), and of 1.3% and 2.9% in a mix of secondary and primary APS (4, 18). Other cohorts presented with a non-vascular mortality ranging from 1.3% to 26.9% (4, 5, 17) but they included patients with systemic lupus erythematosus (SLE) and various autoimmune diseases (4, 5, 17) that were not present in our cohort hence none of our patients required immune suppression that could increase the risk of fatal infections (5, 17). Moreover all of our participants are from the same geographical area and on a Mediterranean diet that may account for a higher intake of anti-oxidants and a lesser intake of carcinogens: this may explain the lack of cancer related mortality and the low prevalence of cardiovascular traditional risk factors in the patients deceased for arterial occlusions (19, 20).

The previous studies did not evaluate predictors of mortality though it was known that APS adversely affected survival of patients with SLE (21). We had the opportunity to investigate predictors of mortality across three groups of patient: D-PAPS, S-PAPS and PCaPL, the latter a group of persistent carriers of aPL who never suffered thrombosis throughout the follow-up: amongst aPL tests at baseline our D-PAPS patients showed the highest average IgG

aCL titres and the strongest average DRVVTr whereas amongst baseline haemostatic variables our D-PAPS presented with the highest average FNG, PLT and VWF levels.

The baseline impact of aPL tests, haemostatic variables and some clinical and genetic factors on survival was approached by the Cox PH model: baseline DRVVTr independently and adversely affected survival with a HR of almost 6, that is, for each unit increase in DRVVTr the risk of death increased almost six times and the same applied for plasma FNG and PLT counts but with lower HRs. Amongst our D-PAPS, those with the highest DRVVTr all passed away for recurrent spontaneous thrombosis, whereas two passed away for provoked events (post-partum and post heart valve surgery) that may have contributed to catastrophic APS despite having IgG aCL titres and DRVVT ratios amongst the lowest of the group. We had previously demonstrated that DRVVT was the only LA assay that correlated with a history of arterial occlusions, prior to the diagnosis of PAPS (22), but this is the first report of a graded adverse effect for the DRVVT, where the stronger its ratio, the worse the survival. A Finnish survey found that LA detected by a re-calcification time (23) and by KCT (6) was related to vascular mortality in a small cohort of SLE patients: the mortality was explained in categorical fashion on the basis of the presence or absence of the LA (24). A decade later the LA resulted as an independent predictor of venous thromboembolism but not of mortality in a much larger cohort of SLE patient but the authors did not mention which LA assay was employed (25). Despite the obvious poor comparability, the predictive pattern of our aPL panel is similar to that of a study on obstetric APS, where the LA was superior to aCL and aβ_2GPI of either IgM/IgG isotype in predicting adverse pregnancy outcome, though DRVVT and aPTT were equally predictive (26). Very recently an Austrian group sought predictors of mortality in a cohort of 151 participants positive for LA detected as aPTT and DRVVT followed up for a median of just over 8 years: after adjustment for age and hypertension they identified occurrence of new thrombosis as the strongest predictor of mortality but not LA (27).

The role of other haemostatic markers as possible predictors of survival has never been explored in APS; with regards to plasma FNG we had described its association with a history of arterial thrombosis (22) and its relation to the intima media thickness of carotid arteries (in univariate analysis) in patients from this same cohort (28). A meta-analysis from 1999, including thirteen prospective studies, estimated that apparently healthy subjects in the higher baseline tertile of plasma FNG had twice the odds of developing cardiovascular disease over

time than subjects in the lower baseline tertile (29). Moreover, elevated FNG emerged as an independent predictor of mortality not only in patients with a previous stroke with a HR of 1.71 (30) but also in normal subjects with an adjusted HR of 1.05 (31). Similar data derived from the Thrombosis Prevention Trial showed that FNG conferred a HR of 1.59 for fatal coronary heart disease and 1.56 for fatal ischaemic stroke (32). The predictive value of elevated PLT counts on short and medium term all-cause mortality has been established in the general elderly population (33), in chronic obstructive pulmonary disease (34) as well as in solid tumours (35). A relationship between thrombocytosis and vascular mortality in APS has never been noted before.

Amongst our demographic and clinical factors, age, sex, age at 1st event and number of thrombosis were not associated with mortality, contrary to other reports (27); we also explored the possible contribution of MTHFR and PON genotypes. With regards to the homozygous MTHFR mutation we had shown that homozygous MTHFR PAPS patients suffered their first thrombotic event at an earlier age compared to heterozygous and homozygous normal PAPS patients (36). With regards to PON we had noted that antibodies against high density lipoprotein (HDL) were inhibitory against PON activity favouring oxidative stress (37). Because anti-HDL was not measured at baseline in this cohort we considered PON genotypes instead, determined on 75 participants only, as homozygous and heterozygous PON mutations code for enzymes with reduced antioxidant activity that favour oxidative stress: the latter occurs in PAPS (38) and is involved in platelet activation and atherosclerosis (39). Neither homozygous MTHFR nor PON mutations had any impact on survival even if the combined frequency of homozygous and heterozygous PON mutations was more common in D-PAPS.

Our studies has several limitations: 1) although FNG and VWF were measured between three to six months after the vascular occlusion, we did not check C-reactive protein to exclude the possibility of a persisting low grade acute phase reaction, though we minimized this possibility by averaging the measurement of plasma FNG and VWF at the time the two aPL tests were checked; 3) the PLT counts was measured only once at the time of the first aPL check and we did not pursue any genetic test to ascertain the possibility of a myeloproliferative disorder during follow-up as only three patients had a PLT count above $4x19^{9}$/L. We retrospectively checked the Jak2 mutation on a frozen DNA sample from the patient who passed away for Budd-Chiari syndrome and it yielded a homozygous normal result; 4) we did

not evaluate the predictive role of aβ_2GPI either on its own or as part of the triple positivity concept (40) because we did not have frozen baseline aliquots available for all patients; 5) we did not include data on smoking status because we had cross-sectional information only for 50% of the cohort and we argued that recall based on a questionnaire would not effectively capture the annual average consumption.

Notwithstanding, we report a 10% vascular mortality for our PAPS cohort that excluded obstetric, secondary of systemic lupus associated APS; more importantly amongst the baseline variables we highlighted for the first time the independent predictive role of DRVVT and FNG on mortality. Moreover, warfarin was unable to prevent spontaneous ischaemic stroke in 66% (6/9) and myocardial infarction in 11% (1/9) of the D-PAPS group, challenging the preventative efficacy of antivitamin K anticoagulants against recurrent arterial occlusion in PAPS. Likewise unfractionated heparin was ineffective in preventing the death of the two patients whose fatal events were provoked. Targeting modifiable risk factors such as sedentary behaviour, diet, smoking, homocysteine and plasma FNG are intuitive strategies to reduce the vascular risk of PAPS but therapeutic measures to dampen the production of aPL are urgently required.

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Table 1. Demographics of the cohort

	PcaPL		S-PAPS		D-PAPS	
No	33		66		11	
M/F	5/26		23/43		5/6	
Age	48.5±15.8		47.3±14.3		55.5±8.9	
Age 1st event	Na		33.9±12.5		40.1±16.3	
Time 1st event to PAPS diagnosis, years			1.08±0.38		1.09±0.33	
FUP, years	12±3.7		10.3±3.7		7.9±2.7	
Total thrombosis	Na		66		11	
	No	%	No	%	No	%
No 1			44	66.6	4	36.3
No 2			15	22.8	4	36.3
No 3			7	10.6	1	9.1
No 4			0	0	2	18.1
Thrombosis type						
Arterial			20	30.3	6	54.5
Venous			45	68.1	5	45.5
Arterial + venous			1	1.5	0	
IgG aCL <20 (GPL)	15	45	13	20	0	0
IgG aCL 21-40 (GPL)	7	21	15	22	2	18
IgG aCL 41-80 (GPL)	4	12	10	15	2	18
IgG aCL >80 (GPL)	7	22	28	28	7	64
FVL +/-	0	0	2	3	0	0
PT +/-	2	6	5	7.5	0	0
MTHFR +/+	8	24.2	14	21.2	2	18.1
PON +/+	2	6	4	6.2*	2	18.1
PON +/-	17	51.5	17	26.7*	5	45.4
PON +/+ & +/-	19	57.5	21	32.8*	7	63.6#
Antithrombotic agents						
Warfarin	0	0	66	100	11	100
Aspirin	4	12	0	0	1	9

Table 2. Laboratory and clinical features of deceased primary antiphospholipid patients

	INR		**IgG aCL**		**DRVVTr**		1st event	Fatal event
Age/Sex	Pre-event	At event	Baseline	Year of fatality	Baseline	Year of fatality	**Unprovoked**	
52/F	3.6	3.8	999	1180	3.05	3.34	IS	IS
57/M	2.8	2.4	1000	858	3.00	2.88	IS, AI	IS, necrotic toes
41/M	2.5	2.7	400	540	2.62	2.79	DVT	Saddle PE +PTA
59/M	2.4	2.7	380	359	2.58	3.02	IS	IS●
49/M	3.3	3.9	321	353	2.52	2.84	RAT	IS
45/F	2.7	2.9	160	188	2.20	2.33	RVT*	MI
55/M	2.8	2.5	130	101	2.11	2.23	DVT	IS
47/F	2.7	na#	55	68	1.74	1.92	DVT	BCS
50/F	2.6	2.9	34	52	1.53	1.68	DVT	DVT + IS●
							Provoked	
50/F	2.7	UFH	60	75	1.77	1.58	IS	MI/IS post AVR@
38/F	2.6	UFH	37	55	1.43	1.55	DVT	CAPS post-partum

Table 3. Univariate survival analysis

Variable	HR	p-value	95%CI		Observations
			Low	High	No
Age	1.030	0.10	0.993	1.069	110
Gender	2.104	0.22	0.641	6.904	110
Age 1st event	1.031	0.16	0.987	1.076	77
Event No 2	2.442	0.20	0.609	9.793	77
Event No 3	4.173	0.06	0.932	18.687	77
Log IgG aCL	8.288	0.001	2.362	29.073	110
KCTr	0.754	0.47	0.348	1.633	110
APTTr	1.502	0.23	0.766	2.945	110
DRVVTr	8.582	<0.0001	3.789	19.438	110
PLT	1.015	<0.0001	1.007	1.022	94
VWF Ag	1.011	0.21	0.993	1.030	96
FNG	1.027	<0.0001	1.016	1.038	100
PT +/-	1.326	0.78	0.169	10.366	109
MTHFR +/-	1.278	0.71	0.342	4.764	109
MTHFR +/+	0.833	0.83	0.152	4.558	109
PON +/-	1.919	0.33	0.514	7.158	75
PON +/+	4.001	0.11	0.731	21.889	75

Table 4. Multivariable survival analysis: Cox proportional hazard most parsimonious model

Adjusted multiple imputation parameter estimates			95%CI	
	HR	**p-value**	**Low**	**High**
DRVVTr	5.751	0.012	1.472	22.459
FNG	1.029	<0.0001	1.014	1.044
PLT	1.017	0.003	1.006	1.026