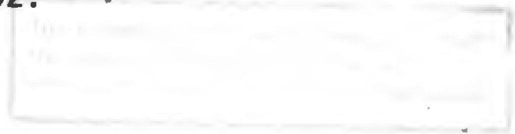
AN INVESTIGATION OF THE LUPUS ANTICOAGULANT AND ANTICARDIOLIPIN ANTIBODIES IN SYSTEMIC LUPUS ERYTHEMATOSUS

by

GARY ARTHUR CULLIGAN

Submitted in partial fulfillment
of the requirements for the degree
Master of Medicine in Haematological Pathology
in the Faculty of Medicine at the
University of Cape Town

September 1992.



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ACKNOWLEDGEMENTS

I would like to thank the following people:-

Dr Arthur Bird whose idea it was that I choose to investigate the lupus anticoagulant enigma for this M Med dissertation. He was most supportive during the planning and data collection phases of the project.

Barry Kossow, Senior Technologist at Groote Schuur Haematology Laboratory, who diligently performed the lupus anticoagulant assays.

Dr A Kalla of the Department of Rheumatology, Groote Schuur Hospital, for permitting me to use his patients and to the staff of the Lupus Outpatients Clinic for their patience and help in tracing departmental folders. Thanks too to the patients attending the Clinic, who participated in this study.

Staff members of the Haematology Department who agreed to give blood samples for the normal control group.

Staff of the Haematology Outpatients Department, Groote Schuur Hospital, who traced and found numerous Hospital folders for this project and took blood samples on the control group.

Rosemary Cooper of the Department of Medical Microbiology, University of Stellenbosch, for performing the anticardiolipin antibody assays on this study group. Her attention to detail is particularly appreciated.

Liesel Wolmarans and Professor C A van der Merwe of the Institute for Biostatistics of the Medical Research Council, Pretoria, for analysing data and discussing the implications with me.

To my parents for much needed motivation and encouragement to see this project through to its completion.

Thanks particularly to my supervisor Professor Peter Jacobs for his ongoing support and encouragement over the years.

ABBREVATIONS

ACA	Anticardiolipin antibody
APA	Antiphospholipid antibody
APS	Antiphospholipid Syndrome
APTT	Activated Partial Thromboplastin Time
BCSH	British Committee for Standardization in Haem
DRVVT	Dilute Russell's Viper venom time
ELISA	Enzyme Linked Immunosorbant Assay
FTA	Fluorscent Treponema Antibody [test]
F-	Filtered-
ICTH	International Committee on Thrombosis and Hae
INR	International Normalised Ratio
KCT	Kaolin clotting time
LA	Lupus anticoagulant
PA	Phosphatidic acid
PC	Phosphatidylcholine
PCT	Plasma clotting time
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PNP	Platelet neutralization procedure
PPP	Platelet poor plasma
PS	Phosphatidylserine
PT	Prothrombin time
SLE	Systemic lupus erythematosus
SSC	Scientific and Standardization Committee
TTI	Tissue thromboplastin inhibition [test]
VDRL	Venereal Disease Reference Laboratory [test]

SUMMARY

Purpose: Antiphospholipid antibodies cause a prolongation of phospholipid dependent coagulation tests and a predisposition to thrombosis, recurrent fetal loss and other clinical manifestations. Lupus anticoagulants (LA) and anticardiolipin antibodies (ACA) are two forms of antiphospholipids present in high frequency in subjects with SLE. A plethora of different tests for LA have been described with few comparative studies available at the time of this study. The aims were to determine the prevalence of LA in a high risk group consisting of patients with SLE using a variety of tests and to determine the most appropriate procedure for routine laboratory use; to establish the frequency of ACA in this group and to analyse the association between antibodies to phospholipids and clinical complications.

Patients and Methods: A normal control group of 40 healthy staff members (20 males and 20 females) were tested using a full range of tests to determine normal ranges for platelet poor and filtered plasma. Seven positive control patients with SLE and previously confirmed LA were retested to evaluate the test methodology. A study group of 50 consecutive untreated patients with confirmed SLE attending the Lupus Clinic at Groote Schuur Hospital (48 female and 2 males; mean age 36.9 years) were tested for LA using centrifuged platelet poor plasma and filtered plasma by APTT, KCT and DRVVT tests and for IgG and IgM ACA by ELISA. Abnormal APTTs were subjected to 1:1 mixture tests for detection of inhibitors and confirmatory procedures PNP and TTI.

Results: Abnormal tests were defined as those values exceeding the 97.5% confidence interval of the normal range. Six (12%) of the SLE group exhibited LA activity. Moderate levels of IgG and IgM ACA antibodies were detected in 10 (20%) and 26 (52%) respectively. Filtering of plasma produced a statistically significant prolongation of the KCT only without improving sensitivity. A history of clinical complications were recorded in 11 (22%) with 13 evaluable incidents. Six cases of recurrent fetal loss, two venous thromboses, one myocardial infarction, two neuropsychiatric disorders and one each of thrombophlebitis and pulmonary hypertension.

Conclusions: The frequency of LA in patients with SLE was found to be quite low by current estimates. The APTT test and KCT were found to be equally sensitive in detecting the LA positive cases with the DRVVT positive in 66%. Filtering produced no improvement in test sensitivity. Confirmatory procedures (PNP and TTI) appear equally effective in confirming suspected cases. The ACA assay appeared to produce many spurious low titre positive results and thus only moderately elevated levels were taken into consideration. ACA of the IgM isotype showed a strong correlation with LA positivity (83%) and occurred in 38% of patients with a history of clinical complications.

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Circulating anticoagulants or inhibitors of clotting are defined as endogenously produced substances that interfere with various *in vitro* tests of coagulation.[1] Specific factor inhibitors are usually immunoglobulins with epitope specificity for a single coagulation protein, the most frequent of which are auto or alloantibodies to factor VIII. Nonspecific inhibitors are antibodies which are not directed at any single coagulation factor but which interfere with *in vitro* coagulation tests and are not generally associated with bleeding.

The lupus anticoagulant (LA) is a spontaneously acquired anticoagulant initially described in patients with systemic lupus erythematosus.[2] The term "lupus anticoagulant" was proposed by Feinstein and Rapaport in 1972 [3] but is unfortunately a misnomer since the majority of patients do not suffer from systemic lupus erythematosus (SLE) and, in the absence of other haemostatic abnormalities, do not bleed. The LA was regarded chiefly as a nuisance by clinicians and laboratory researchers causing an unexplained prolongation of the partial thromboplastin time. Paradoxically, an increase in thrombotic events and/or a high rate of spontaneous pregnancy loss has been observed in patients in whom this laboratory abnormality appears.[4-14]

1.2 PATHOPHYSIOLOGY OF LUPUS ANTICOAGULANTS

The lupus anticoagulant is an immunoglobulin of the IgG or IgM class (or a mixture of both) which may act by inhibiting the activity of phospholipid in clotting reactions. Characteristically, the antibody prolongs the partial thromboplastin time and fails to correct to normal upon mixing with equal volumes of normal plasma.

Early studies of lupus anticoagulants emphasised the frequent association with false positive tests for syphilis, a reaction which depends on the presence of cardiolipin.[15] and that the LA could be adsorbed by cardiolipin and Kahn antigen.[16] This led to the understanding that lupus anticoagulants react with phospholipid but the precise epitope(s) are at present poorly defined.

Pengo *et al* used affinity purification on cardiolipin liposomes followed by protein A-Sepharose chromatography to prepare phospholipid-free purified IgG LA from five patients to evaluate antibody specificity.[17] The purified lupus anticoagulant reacted with anionic phospholipids like phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidic acid (PA) but not with neutral phospholipids, phosphatidylethanolamine (PE) or phosphatidylcholine (PC). The lupus anticoagulants inhibited the calcium dependent binding of prothrombin and of factor X to cardiolipin and phosphatidylserine coated surfaces. These data support a common *in vitro* mechanism by which lupus anticoagulants bind to anionic phospholipids, thereby blocking the calcium-

mediated assembly of vitamin K-dependent proteins to these coagulation-active surfaces.

Recent research by Beavers *et al* has shown that expression of lupus anticoagulant activity requires the presence of anionic phospholipids, ***human*** prothrombin and calcium ions.[18] The IgG antibodies showed a dose dependent inhibition of the prothrombinase activity in a system with human coagulation factors Xa, Va and prothrombin in the presence of lipid vesicles (20% PS and 80% PC). In contrast, no inhibitory effect was observed when human prothrombin was replaced by bovine prothrombin. The findings indicate that LA recognize an epitope which becomes exposed upon Ca-mediated binding of prothrombin to anionic phospholipids only in the presence of human prothrombin. LA should, therefore, be considered as a class of anti-prothrombin antibodies. Indeed, a high prevalence of anti-prothrombin antibodies has been reported for LA-positive plasmas, sometimes associated with low levels of prothrombin.[19] Our current understanding of the clinical significance of anti-prothrombin antibodies in patients with lupus anticoagulants confirms that they are non-neutralizing in nature and will not cause abnormal bleeding even when present in high titre. Clinical bleeding will only be encountered when the plasma is depleted of prothrombin as a result of accelerated clearance of prothrombin/prothrombin antibody complexes.[19]

Watson & Schorer have demonstrated that lupus anticoagulants inhibit the *in vitro* release of prostacyclin in patients with a history of thrombosis.[20] Using cultured human

endothelial cells exposed to sera from patients with laboratory proven LA and antiphospholipid antibody activity, they showed that the release of prostacyclin was markedly inhibited in those patients with a recent history of arterial thrombosis. The effect was less in those with venous thrombosis and absent in patients with APA who had not experienced thrombosis. In this experimental model, thrombin is used as a secretologue, causing a 2.5 fold increase in prostacyclin secretion compared to basal production. Schorer *et al* have recently shown that LAs induce a selective defect in the thrombin mediated endothelial prostacyclin release without affecting thrombin-mediated membrane inositol metabolism and total thrombin binding to endothelium.[21] It must be noted that Watson & Schorer's results appear to be at variance with reports which found no inhibition of prostacyclin release in patients with SLE.[22] In addition, lupus anticoagulants selectively blocked platelet aggregation with thrombin but not in response to arachidonate, ADP or epinephrine.[21]

The protein C/protein S/thrombomodulin system is an important anticoagulant system dependent on the endothelial membrane. Endothelial bound thrombomodulin is essential for thrombin-mediated activation of protein C *in vitro*.[1] Malia *et al* demonstrated that IgG antiphospholipid antibodies (LA/ACA) have an inhibitory effect on the activated protein C/protein S complex and provide some explanation for a relationship between APA and thrombosis.[23]

Thrombocytopenia is a common finding in patients with LA and thrombosis. One explanation is the activity of LA against platelet phospholipids which may damage blood platelets and increase their adhesiveness, thereby initiating thrombosis.[7]

1.3 CLINICAL ASSOCIATIONS

The estimated incidence of lupus anticoagulants in patients with SLE ranges from 6 to 16% in earlier reports, [24] and 6 to 73% (average prevalence: 34%) in a current review.[90] It may be found in a variety of other disorders, as a result of drug therapy and in apparently normal persons.

Jude and colleagues published the clinical features in 100 cases positive for lupus anticoagulant.[25] Their findings are listed in the tables 1 and 2.

Table 1. AUTOIMMUNE DISEASES ASSOCIATED WITH LA

Systemic lupus erythematosus
SLE-like disease
Rheumatoid arthritis
Still's disease
Scleroderma
Primary amyloidosis
Chronic active hepatitis
Goujerot-Sjögren's syndrome
Drug-related lupus syndrome associated with
hydantoins
acebutolol
chlorpromazine
(procainamide [26,27])

Table 2. NON-AUTOIMMUNE PATHOLOGY ASSOCIATED WITH LA

Category	Conditions
Neoplastic	
	Uterus, breast, naevus, ovary, tongue, lip
Haematological	
	Waldenström's disease, non-Hodgkin's lymphoma
	Chronic lymphocytic leukaemia
	Polycythaemia
	Myelodysplasia
Gastroenterological	
	Angiocholitis, cirrhosis
	Pancreatitis, colic diverticulosis
Cardiovascular	
	Hypertension, coronaritis
	Arteritis, cardiac failure
Psychiatric	
	Dementia
	Depressive syndrome
Endocrinological	
	Hyperthyroidism
	Adrenal failure
	Diabetes
Pulmonary	
	Viral pneumopathy
	Asthma
	Tuberculosis
Renal	
	Chronic renal failure
Rheumatic	
	Sciatica, cervicoarthrosis
Neurological	
	Parkinsonism
	Epilepsy
Alcoholism	

Other examples of conditions giving rise to LA abound in the literature. For example, Yamazaki *et al* attributed transient induction of LA in a young woman to Epstein-Barr virus infection; [28] LA associated with primary malignant lymphoplasmacytic lymphoma of the spleen; [29] and induction by hydatidosis.[30] It remains to be seen if these findings can be substantiated by independent reporting. In some instances, no apparent underlying disease can be detected.[13]

Drug-induced LA has been reported with hydralazine, chlorpromazine, quinidine, phenytoin, penicillin and most commonly with the use of procainamide.[26 27 31 32] Long term use of chlorpromazine may be associated with the development of an IgM type LA with no apparent thrombotic predisposition.[1]

Upper respiratory tract infections are frequently the cause of LA in children and detected as a result of preoperative coagulation screening performed prior to tonsillectomy or adenoidectomy. LA have been reported in 70% of patients with AIDS,[33] without clinical evidence of bleeding. There appears to be an association between infectious agents (eg *Pneumocystis carinii*) and the appearance of LA which disappear when the infection was satisfactorily treated.[34] The finding of a LA is clinically noteworthy, because a prolonged APTT should not prevent a patient with AIDS from undergoing invasive procedures if no other coagulation or platelet abnormality is present.[33]

1.4 COMPLICATIONS ASSOCIATED WITH LUPUS ANTICOAGULANTS.

The clinical manifestations and complications seen in patients with lupus anticoagulants fall into two main categories namely venous or arterial thrombotic episodes and obstetric complications including spontaneous abortions and intrauterine fetal deaths.

Thrombosis has long been recognized as a complication of SLE with the occurrence of leg vein thrombosis in up to 15% of

patients.[2] Boey *et al* extended this observation in 1983 by reporting a striking association of thrombosis in patients with SLE and LA activity.[8] Testing a group of 49 patients with SLE and 11 other connective disorders, 31/60 were found to have LA activity; 25 of these had SLE. Thrombotic events were recorded in 18 of the 31 LA positive patients but only 3/29 of the patients without LA gave a history of thrombosis. The wide ranging variation in frequency of LA reported in SLE (6 to 73%) [24, 35, 90] may reflect fluctuation in the LA level with disease activity or treatment, but is more likely to reflect differing sensitivity of the various assays to detect lupus anticoagulants and lack of standardization.[35]

The risk of a thrombotic event appears to be about 30% in patients with SLE [1, 24, 5, 35] but may be as high as 68% in individuals with active disease and renal involvement.[36] Wong and coworkers were unable to find any association with thrombosis in a recent study of 91 Chinese patients with SLE.[37]

The lower extremities are the most common site of venous thrombosis, while cerebral arteries are the most frequent site of arterial thrombosis. Manifestations of arterial thrombosis include stroke and transient ischaemic attacks,[38, 39] late-onset seizures,[40] cerebellar ataxia,[41] and ocular complications such as retinal artery occlusion, ischaemic optic neuropathy, transient visual loss or diplopia.[42] Myocardial infarction [12, 43, 44] is a recognized complication sometimes occurring in childhood.[45]

Skin manifestations described in patients with SLE and lupus anticoagulants are necrotic ulcers, livedo reticularis and pyoderma gangrenosum.[46] Histologically the lesions are characterized by capillary angiogenesis with extravasated red blood cells, sparse inflammatory cell infiltrate and microthromboses, without evidence of vasculitis. In some cases the ulcers may resemble those seen in Degos' disease.

Nilsson *et al* were the first to report the association between LA and intrauterine death.[47] This finding has been confirmed by a number of other investigators.[6 9 10 14 48 49 50] Fetal growth retardation, first trimester abortion and unexplained death in the second and third trimesters are more frequently seen in women with LA and/or anticardiolipin antibodies. The placentae in some cases may be found to have extensive infarction but the degree of impairment due to infarction in other cases does not appear to be sufficient to cause fetal demise.[6 9] Therefore, the relationship between placental thrombosis and fetal death remains unproven.

The incidence of LA in patients with fetal loss varies considerably from 5%[49] to 48%.[14] These women may represent a subset of patients who are risk of thrombosis as they may also frequently give a history of thromboembolic events.[1]

1.5 ANTIPHOSPHOLIPID ANTIBODIES (APA)

Antibodies to cardiolipin are among various circulating autoantibodies found in SLE directed against complex lipid antigens. These antibodies are of the IgG, IgM or the IgA isotype. The association of a biological false positive test for syphilis in LA positive sera, is presumably based on the presence of antibodies cross-reacting against cardiolipin (the phospholipid antigen directed in standard test for syphilis), phospholipids in platelet membrane, and a phospholipid in the prothrombin activator complex of the coagulation cascade.[51] Studies of this group of cross-reacting antibodies had been limited by the relative insensitivity of precipitation tests.

Harris *et al* in 1983 reported on a new solid-phase radioimmunoassay for the detection of anticardiolipin antibodies which was 200 - 400 times more sensitive than the precipitation method used in Venereal Disease Reference Laboratory test (VDRL).[51] As the assay system has been shown to react with other negatively charged phospholipids (PS, PI, PA), the more generic term antiphospholipid antibodies was proposed.[52] In recent years, the enzyme-linked immunosorbant assay (ELISA) has replaced RIA as the preferred technique in the detection of APA.[50 91] Other potential advantages were that the tests could be done on sera rather than plasma, and results would be less affected by methods of preparation, transportation, and storage of specimens than the lupus anticoagulant test. In addition,

the anticardiolipin antibody test is not affected by concomitant anticoagulation treatment.[55]

Antiphospholipid antibodies are associated with the same spectrum of clinical complications seen in LA positive patients namely arterial and venous thrombosis, recurrent fetal loss, thrombocytopenia and dermatological and neurological sequelae.[48 53] They are also found in patients with AIDS.[34 54]

The term 'Anti-Phospholipid (Anti-Cardiolipin) Syndrome' (APS),[53 85] has been introduced to identify patients with persistently elevated levels of anti-cardiolipin antibodies or positive lupus anticoagulant tests, who are prone to complications. In an editorial in the Journal of Rheumatology, Hughes *et al* [53] summarized the clinical associations of the Anti-Phospholipid Syndrome as shown is Table 3.

The first three clinical features are most frequently seen in association with the syndrome, but it is not always clear whether the other features are part of another underlying disorder, or whether they are related to the presence of anti-phospholipid antibodies.[55]

The 'Primary' Antiphospholipid Syndrome [56] describes a subset of the above group, in which the patients have no features of lupus or who are classified as "lupus-like", exhibiting less than 4 of the American Rheumatology Association Criteria for the diagnosis of SLE.[57]

Table 3. *CLINICAL ASSOCIATIONS OF THE APS*

1. ***Thrombosis***

Venous	Recurrent DVT (also axillary, IVC, and retinal vein thrombosis)
Arterial	Cerebrovascular accidents Peripheral arterial gangrene Coronary thrombosis Retinal artery thrombosis
Other	Pulmonary hypertension ? Avascular necrosis

2. ***Abortion*** Recurrent IUD, placental thrombosis, and infarction

3. ***Thrombocytopenia***
   Intermittent, often severe

4. ***Other Occasional Features***
   Coombs' positivity
   Livedo reticularis
   Migraine
   Chorea
   Epilepsy
   Chronic leg ulcers
   ? Endocardial disease
   ? Progressive dementia due to repeated cerebrovascular thromboses

The reported incidence of ACA in patients with SLE has våried widely from 21 to 81 percent.[58] The simultaneous presence of ACA and LA in SLE has been reported as being positive in 91% of patients [51] while Triplett *et al* found 71% of medical patients who have a prolonged APTT and LA to have ACA. In the latter group, the presence of ACA was not associated with an increased risk of thrombosis.[59] Recently Barbara Alving and coworkers published results of a study on 70 medical patient with prolonged APTTs. The ACA assay gave positive results in 47 patients, 44 of whom also tested positive for LA. Thus the sensitivity of the LA assay for detecting APA was 94%. Twelve of the 47 patients (26%) had

a history of venous or arterial thrombosis. Of these, 75% tested in the high-positive range for IgG or IgM ACA, or both. Of the 35 patients without thrombosis, only 14% were in this range. Patients with thrombosis have either underlying SLE, lymphoma or no apparent aetiology for LA. Thus determination of ACA levels in patients who have LA that is not induced by medication or infection may define those patients at increased risk for thrombosis.[60]

The lack of concordance between the results of the various tests (which cannot be explained by differences in test sensitivity), has led most investigators to agree that anti-phospholipid antibodies detected by one test need not be the same as those detected by another.[1 55 61] Therefore, positive results for either the LA or ACA should be regarded as independent risk factors for thrombosis and both assays should be performed to maximize the likelihood of detecting antiphospholipids that may have clinical relevance.[62]

1.6 LABORATORY TESTS FOR LUPUS ANTICOAGULANTS

1.6.1 DIAGNOSTIC CRITERIA

Laboratory criteria for the diagnosis of lupus anticoagulants remain a contentious issue, four decades after their initial description. The difficulty of evaluating patient samples for LA include the collection, transportation and processing of the blood sample which are very important in optimising laboratory detection of any coagulation abnormality. Care should be taken to avoid haemolysis or contamination by tissue fluid during collection of the

sample and the use of plastic or siliconised glass containers is recommended. The centrifugation step is critical, as the ability of coagulation assays to detect LA is inversely proportional to the number of platelets in the platelet poor plasma (PPP). The more platelet free the sample, the greater the sensitivity of most tests to the presence of LA.[63] It is particularly important to ensure the absence of platelets in PPP which is to be frozen for later testing, as freezing and subsequent thawing results in disruption of platelet with a release of phospholipids and a significant shortening of prolonged APTT values. Exner used Millipore [R] filters to prepare PPP and found enhanced sensitivity of both APTT and kaolin clotting time to LA.[65] Alternatively, the commercial serum-plasma separators allow preparation of excellent PPP (Sure-Sep,[R] Organon Teknika).[1]

The Working Party on Acquired Inhibitors of Coagulation of the International Committee on Thrombosis and Haemostasis proposed a set of criteria for the diagnosis of LA in 1983.[66] They suggested the following:

1. Prolongation of the APTT.
2. The APTT of a 1:1 mixture with normal plasma should be greater than 4 seconds over the control.
3. A decrease in at least two factors (VIII, IX, XI,or XII) by one-stage assay, with normal values by two-stage assay.
4. A sensitivity to dilution, so that the apparent activity of the factor increases with dilution.

These criteria have proved to be too stringent, especially mixing studies which are difficult to interpret with mildly prolonged clotting times and factor assays which do not always show nonparallel curves.[1] Recent evidence suggests that a significant number of patients will be misdiagnosed using these criteria.[66]

It is generally accepted that the minimum criteria for diagnosis should demonstrate:

1. An abnormality of phospholipid-dependent coagulation reaction(s)
2. The abnormality is due to an inhibitor.
3. Inhibitor activity is directed at phospholipid (not specific factors).

Based on these criteria, the test system can thus be divided into (1) screening procedures to identify the coagulation abnormality; (2) procedures to demonstrate the inhibitory nature; and (3) a confirmatory procedure to identify phospholipid dependence.

1.6.2 SCREENING PROCEDURES

Lupus anticoagulants are most frequently detected in the laboratory by an unexplained prolongation of the activated partial thromboplastin time (APTT). This test has, therefore, become the preferred screening procedure. Until recently, reagent manufacturers did not consider the sensitivity of their APTT reagent to LA when marketing their

products. Considerable variation in the sensitivity of APTT reagents to LA have been documented [67 68 69] and it is important to select a reagent whose ability to detect weak LA has been proven. These differences are due to the choice of activator and to the nature and quantity of the phospholipids used as platelet substitute. In general, reagents with relatively low levels of phospholipids appear more sensitive to LA.[1 70]

It has recently become apparent that two additional variables encountered in the detections of LAs need to be considered. They are the source and preparation of the reagents used in mixing studies and the dilution effect. Kaczor *et al* compared three commercially prepared reagents, Verify Normal citrate (Organon Teknika), Thromboscreen UCRP (Pacific Hemostasis) and Assayed Reference Plasma ARP (Helena) with an in-house reagent. The results showed Helena ARP to be the most sensitive commercial reagent producing the most prolonged APTT in mixing studies.[71] To improve the sensitivity of the tests, it is important that the platelet-phospholipid content of the patient plasma and mixing reagent should both be as platelet-free as possible to enhance the LA effect. The dilutional effects will be explained in the next section.

The Kaolin Clotting Time (KCT) is believed to be more sensitive to LA than a variety of other tests. [63 64 65] Gastineau *et al* [24] found the plasma clot time (PCT) to be the most sensitive test for LA detection with a sharper, more reproducible end point. The disadvantage of the KCT

and PCT is that they are not readily automated or currently widely available.[1] These tests should be used to evaluate patients with a minimially prolonged APTT, or in patients with the antiphospholipid syndrome or with autoimmune diseases in whom the APTT is normal.

A recent report by O'Neill *et al*, describes an automated KCT developed to simplify screening for LA. The assay is performed on an Automated Coagulation Laboratory ACL 3000 Research coagulation analyser, but may be modified for other centrifugal analysers. It has been difficult to automate the KCT as the opacity of the kaolin suspension interferes with a photo-optical determination of the clot end-point. By using a centrifugal analyser and modifying the strength of the kaolin suspension, it is possible to achieve an automated KCT.[72]

Prothrombin time (PT) is relatively insensitive to LA [73] and not suitable as a screening test. This insensitivity to the inhibitor may reflect the amount of phospholipid in the reagent or its presence as a lipoprotein.[1] When significantly prolonged, however, prothrombin deficiency should be suspected.

1.6.3 IDENTIFICATION OF INHIBITOR ACTIVITY

The use of mixing studies are the first step to identify the presence of an inhibitor in the event of a prolonged APTT. A 1:1 ratio of patient plasma to normal pooled plasma is recommended [66] and the APTT is repeated. If the prolonged

time fails to correct, the diagnosis of an inhibitor is confirmed. The definition of "failure to correct" is not clearly defined and may vary depending on the degree of prolongation of the initial APTT.

Mixing studies in a minimally prolonged APTT (<7 seconds longer than the control time) are difficult to interpret as distinction of a *correction* from a *dilution* effect may be problematic.[1,68] In these instances, the use of a 4:1 ratio of patient plasma to mixing reagent is more sensitive than the commonly used 1:1 ratio.[71]

Occasionally mixing studies on a patient with LA may yield a time which is much longer than the initial APTT. This paradoxical finding is seen is test systems with low phospholipid concentrations and is due to the lupus cofactor.[1,24,71,74] Laboratories which utilize a 4:1 mixture will observe the cofactor effect more often than those employing a 1:1 mix. The substance present in normal plasma which accounts for the cofactor effect is unknown.

An alternative procedure for the detection of inhibitors is the use of plasma agarose gels. A modified method which is particularly suited to detect lower titre inhibitors, was described by Cassidy *et al.*[75] The results are reproducible and precise and interpretation is unambiguous. The test system is positive for any type of circulating anticoagulant (heparin, factor VIII inhibitor, LA) and thus further evaluation is necessary to differentiate the nature of the inhibitor.

With the exception of a rare dissenting opinion [76] there is general agreement that LA are immediate acting, that is, they do not require incubation to demonstrate maximal anticoagulant activity. This characteristic is often contrasted with factor VIII inhibitors, especially those of low avidity, which frequently require incubation to be detected.[77] In a retrospective study, Clyne and White showed that in 60% of cases the theory was indeed vindicated. In the remaining 40% of cases, however, a time dependent pattern was evident where the 60-minute ratio exceeded the increment predicted to occur through variability. The authors conclude that many potent LA require incubation to maximimally express anticoagulant activity and this activity might not be detected if mixing studies are restricted to the preincubation phase.[77]

1.6.4 CONFIRMATORY PROCEDURES: IDENTIFICATION OF PHOSPHOLIPID DEPENDENCE

Two approaches may be use to document phospholipid dependence. The first utilizes the inverse relationship between the phospholipid content and the degree of prolongation. Tests in this group include tissue thromboplastin inhibition test (TTP), dilute Russell Viper Venom Test (DRVVT), the dilute phospholipid APTT (DL-APTT) and the KCT. The reduction of phospholipid in these tests accentuates the LA induced prolongation. The second approach is through neutralization of the inhibitor effect

by increasing the phospholipid content ie the platelet neutralization procedure (PNP).

1.6.4.1 TISSUE THROMBOPLASTIN INHIBITION TEST (TTI)

This test was first described by Schleider *et al* [78] and is based on a dilute prothrombin time using Simplastin [R] reagent in a dilution of 1:50 and 1:500. The results are expressed as a ratio of patient to normal with a value of >1.3 being abnormal. As the test is sensitive but not specific for LA [79,80] it should not be used alone to confirm the diagnosis. False positives results have been seen in normal subjects (30%), specific factor inhibitors, hereditary coagulation deficiencies and in patients receiving warfarin or heparin.[1]

1.6.4.2 DILUTE RUSSELL VIPER VENOM TIME (DRVVT)

This test can be used both as a sensitive and specific screening procedure and as a confirmatory test for LA. Originally described by Thiagarajan *et al*, this test uses a dilute source of phospholipid with a predetermined dilution of Russell's viper venom to give a normal range of 25 to 28 seconds.[81] This test has found wide favour [74,82] and was found to be significantly associated with thrombotic events in one study.[35] A disadvantage is that the DRVVT is not easily automated and, therefore, the DL-APTT is more widely used.

1.6.4.3 DILUTE PHOSPHOLIPID APTT (DL-APTT)

This simple and sensitive method for the verification of LA utilizes dilution of phospholipid in the activated partial thromboplastin time system. Patient plasma, mixed with an equal volume of normal plasma, is activated with micronized silica. To this mixture are added different dilutions of Thrombofax R and then calcium chloride. Clotting times are plotted linearly against the logarithm of the phospholipid dilution and slopes are calculated by regression analysis. The advantage of this assay is that LA can be monitored in patients on warfarin or heparin therapy.[83]

Alving and co-workers have modified and simplified their original method utilizing a one-point dilute phospholipid-APTT expressed as a ratio of the a mixture of normal and patient plasma to that of normal plasma alone. Standardized preparations of phosphatidylcholine/phosphatidylserine (PC/PS) was substituted for bovine thromboplastin (Thrombofax).[84] A positive test for LA was defined as a ratio of ≥1.3 with liposomes and ≥1.2 with Thrombofax. In a comparison between their modified DL-APTT and a similarly modified RVVT, they demonstrated higher sensitivity of the DL-APTT and greater specificity of the RVVT in detecting LA.

1.6.4.4 PLATELET NEUTRALIZATION PROCEDURE (PNP)

The PNP is a useful test to confirm the diagnosis of LA. It was first described by Triplett *et al* in 1983 [79] and is based on earlier observations by Exner [63] of the ability of

platelets to bypass the LA or significantly correct prolonged clotting times. Outdated frozen and thawed platelets are added to patient plasma and APTT reagent. There is a significant shortening of the APTT if LA are present, whereas a factor specific inhibitor plasma is not shortened. The test is highly specific in this regard as alluded to in several reports.[24 79] A commercial source of platelets is now available (Platelet Extract Reagent, [R] Bio-Data Corporation).

1.6.4.5 OTHER CONFIRMATORY TESTS: FACTOR ASSAYS

The ICTH Working Party on Acquired Inhibitors of Coagulation indicated that assays of factors of the proximal intrinsic pathway may need to determined to confirm the diagnosis of LA. Typically, two or more will show diminished activity, with greater dilutions of patient plasma showing a higher apparent factor activity giving rise to non-parallel curves. Factor activity using two-stage assays will be normal.[66]

1.6.5 STANDARDIZATION OF CRITERIA FOR THE DIAGNOSIS OF LA

The clinical diversity of the primary antiphospholipid syndrome has recently become widely recognised [85] and the identification of the LA is important in the diagnosis and management of this condition. Consequently lupus anticoagulant testing has become an essential routine procedure for haemostasis laboratories. Nevertheless, there is considerable controversy about the most appropriate methods for detecting LA.[67]

Dissatisfaction and frustration with the criteria for the diagnosis of LA has led to the appearance in 1991, of guidelines for standardization of lupus anticoagulants from parties on opposite sides of the world.

1.6.5.1 BCSH LA GUIDELINES

The Lupus Anticoagulant Working Party, of behalf of the BCSH Haemostasis and Thrombosis Task Force of the British Society for Haematology, published guidelines on testing for the lupus anticoagulant in November, 1991.[67] The report is based on the findings of the first and second United Kingdom Lupus quality control survey conducted in 1987 and 1988. As a result of these observations, standardised methodological guidelines for laboratories wishing to test for the presence of LA were formulated. The recommendations are given briefly below.

> Any definition of the lupus anticoagulant must include the phospholipid dependency of the inhibitory activity in clotting testing and the relative correction by lysed platelets or increased phospholipid concentration. (Table 4)

Table 4. CRITERIA FOR LUPUS ANTICOAGULANT

1. Prolongation of a phospholipid dependent clotting test
2. Clotting time of a mixture of test and normal plasma should be longer than the clotting time of normal plasma
3. There should be a relative correction of the defect by the addition of lysed platelets or phospholipids

The preanalytical conditions with regard to sample collection and handling were specified. Minimal stasis to avoid platelet activation; processing of sample ideally within one hour of collection; and double centrifugation or filtration to obtain platelet depleted plasma (<10 x $10^9/l$) were the main recommendations. Control plasma must be prepared in a similar way to test plasma (or check manufacturers specifications).

It is recognised that the inhibitors are heterogeneous in their behaviour in phospholipid dependent coagulation tests and no single test is sufficient. At least two are advisable, one of which could be the screening test (APTT).

A coagulation screen including PT, APTT, with TT or fibrinogen estimation is required to exclude abnormalities unrelated to LA. A flow diagram for the laboratory investigation of LA is given in Figure 1.

The APTT should be performed on freshly prepared PPP, on pooled normal PPP and on a mixture of four parts patient PPP and one part normal PPP (4:1 mixture).

It is important that consideration be made of the clinical situations in which screening for LA may be indicated (thrombosis, fetal loss, SLE, ITP, livedo reticularis and false positive test for syphilis and

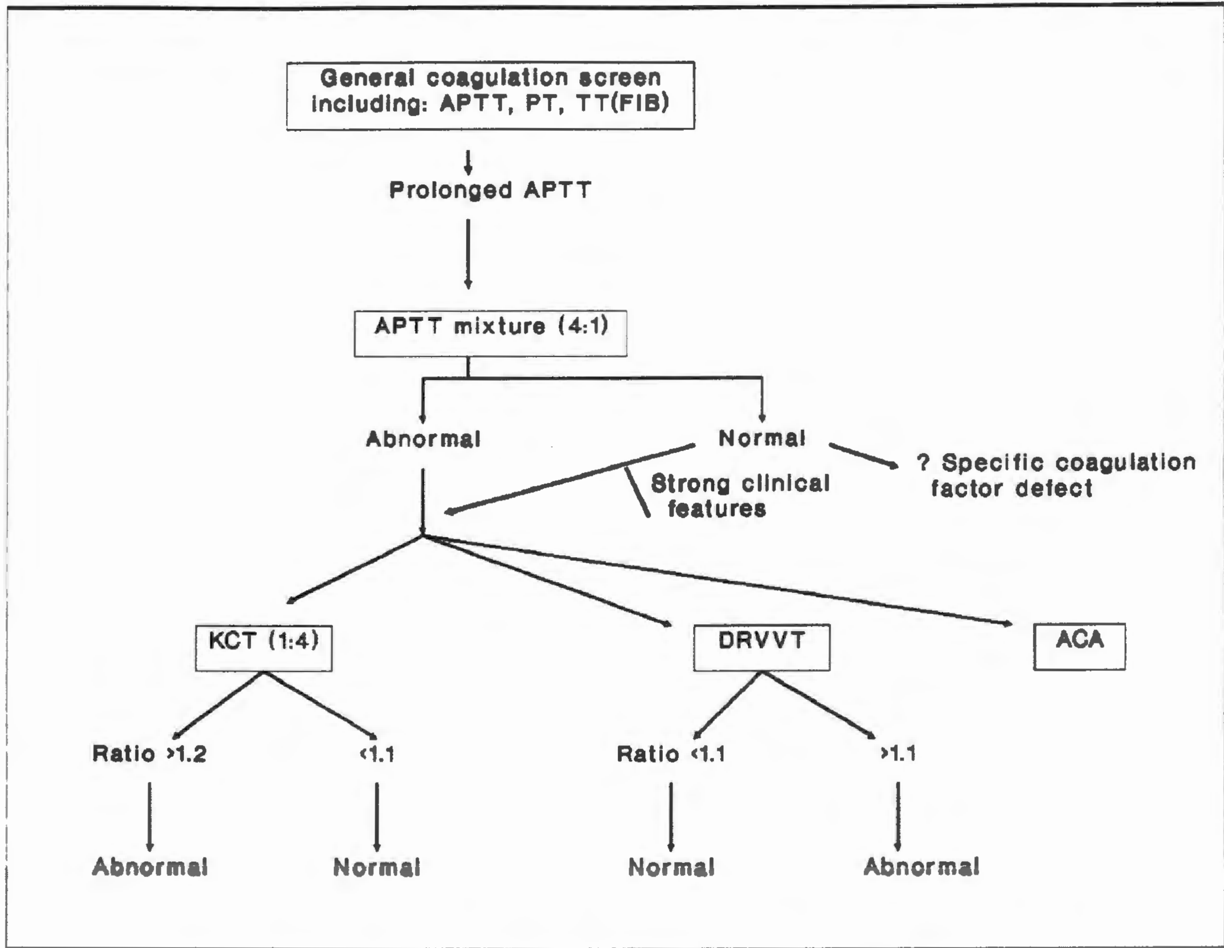

Figure 1. FLOW DIAGRAM FOR LABORATORY INVESTIGATION WHEN LUPUS ANTICOAGULANT IS SUSPECTED.

To confirm the presence of LA, two positive results are advisable, of which one may be the screening APTT. (After Machin et al [67]).

undue sensitivity of APTT to heparin therapy), as even the most sensitive APTT method will not detect all inhibitors and so an additional specific test should be preformed in suspected cases of LA, even if the APTT is normal.

Confirmatory tests demonstrate that the inhibitor activity of LA are directed against procoagulant phospholipids. Standardised methodologies for the recommended tests, KCT and DRVVT, are supplied in the text.[67]

1.6.5.2 SSC LA GUIDELINES

The Scientific and Standardization Committee (SSC) Subcommittee for the Standardization of Lupus Anticoagulants published Guidelines of Testing and Revised Criteria for Lupus Anticoagulants in March, 1991.[86] (Table 5)

Table 5. REVISED SSC CRITERIA FOR LUPUS ANTICOAGULANTS

1. Prolongation of phospholipid-dependent clotting tests i.e. kaolin clotting time (KCT), dilute Russell's viper venom time (DRVVT), tissue thromboplastin inhibition test (TTI), plasma recalcification time (RCT) or a sensitive APTT.

2. Clotting time of a mixture of test and normal plasmas should be significantly longer (i.e. more than 3 standard deviations) than that of the normal mixed and various non-LA patient plasmas.

3. There should be a relative correction of the defect by the addition of lysed, washed platelets or preferably phospholipid liposomes containing phosphatidylserine or hexagonal phase phospholipids.

4. Other criteria for LA include: Non-specific for any individual clotting factor, rapidly losing apparent activity on dilution of test plasma with saline (i.e. giving non-parallel lines in factor assays), usually fast acting and associated with positive antiphospholipid antibody ELISA's. The anticoagulant should be identified as an immunoglobulin whenever possible (stable at 56°C for 20 minutes, protein G extractable, etc).

CHAPTER 2

AIMS OF THE PROJECT

The idea for this project was conceived in 1987 when the guidelines for the investigation of lupus anticoagulants required further definition and refinement. A plethora of different tests were being reported in the medical literature with very few comparative studies available. It was imperative that the various methods of investigating lupus anticoagulants be examined and compared, so that recommendations could be made regarding the most suitable tests for confirming this laboratory phenomenon and applying them to routine testing in a haematology laboratory.

In a attempt to clarify understanding of the lupus anticoagulant, it was necessary to study a uniform well managed population in which there was a good statistical chance of detecting this inhibitor in high frequency. Patients with clinically proven systemic lupus erythematosus were selected as test group with the aim of establishing the incidence of lupus anticoagulants and anticardiolipin antibodies. In view of the suggested heterogeneity of the antibody, a variety of different tests for their detection were used to determine the sensitivity and specificity of each procedure.

A retrospective study of the correlation between antibodies to phospholipids and the occurrence of clinical manifest-ations such as arterial and venous thrombosis and

spontaneous fetal loss occurring and other related problems, was undertaken in this study group.

A group of normal controls were tested using the same test procedures to establish the normal range for each variable.

CHAPTER 3

MATERIALS AND METHODS

3.1 STUDY GROUPS

The SLE study group was drawn from the Lupus Clinic at Groote Schuur Hospital in Cape Town. It comprised fifty consecutive adult patients attending the out-patients clinic over a six month period between April and November 1988. There were 48 females and 2 males in the group and all fulfilled at least four the 1982 American Rheumatism Association (ARA) revised criteria for the diagnosis of systemic lupus erythematosus.[57] None of the patients was receiving immunosuppressive treatment for active SLE at the time of testing or during the preceding four weeks and none were on any anticoagulant therapy.

The control group was made up of forty healthy members of staff of the Haematology Department, Groote Schuur Hospital, consisting of an equal number of males and females from both white and mixed racial groups.

A positive control group, consisting of patients with previously diagnosed lupus anticoagulants, were retested using the proposed laboratory procedures to establish the methodology. Laboratory records were examined and all available LA positive patients were contacted. Seven patients agreed to retesting.

Informed consent was obtained from patients and controls, in keeping with the policies of the Ethics and Research Committee of the Groote Schuur Hospital and the Faculty of Medicine, University of Cape Town.

3.2 BLOOD COLLECTION AND SAMPLE HANDLING

Fifteen millilitres of whole blood was collected by venepuncture using a 19 gauge butterfly and two syringe technique. Meticulous attention was taken to ensure adequate blood flow and to avoid aspiration of any tissue fluid. Blood was anticoagulated with 3.8% trisodium citrate in the ratio of one part anticoagulant to 9 parts of blood and placed in plastic containers.

Platelet poor plasma was prepared following centrifugation at 2500g for 15 minutes at room temperature. Plasma samples were tested immediately and aliquots were also stored in polystyrene tubes, quick frozen and kept at -70°C. A 5ml sample of clotted blood was taken at the time of venepuncture and following centrifugation, aliquots of serum were stored in similar containers and storage conditions until tests for anticardiolipin antibodies were performed.

Filtered plasma was prepared on test and normal groups by passing aliquots of platelet poor plasma through a 0,22 µm Millipore [R] type GS cellulose acetate filter in stainless steel screw-together assemblies.

3.3 CLOTTING TESTS

Prothrombin time (PT)

The one-stage prothrombin time was determined using acetone dried human brain thromboplastin, prepared in accordance with the technique described by Giddings (in Thompson),[87] and of known International Sensitivity Index (ISI).

Activated Partial Tromboplastin Time (APTT)

Tests were carried out with 0,1 ml of auto-APTT reagent (General Diagnostics, Morris Plains, New Jersey) (presently manufactured by Organon Teknika) preincubated for 5 minutes and subsequently timed to a clotting endpoint after the addition of 0,1 ml of 0,025M calcium chloride in an X2 photoelectric coagulometer (General Diagnostics USA).

Samples showing a prolongation of the APTT by >5 seconds over the upper limit of the control series, were screened for the presence of an anticoagulant. This screening procedure consisted of performing an APTT by mixing equal volumes of patient and normal plasma (General Diagnostics, Verify Normal [R] Coagulation Control plasma). Failure of normal plasma to correct the prolonged APTT to within 4 seconds of the normal range, indicated the presence of a circulating inhibitor. These samples were subjected to two confirmatory tests:

(a) Platelet neutralization procedure and

(b) Thromboplastin inhibition test

Platelet neutralization procedure (PNP)

This test was performed as described by Triplett and colleagues.[79] A newly expired unit of platelet concentrate (Western Province Blood Transfusion Service, Cape Town) was aliquotted into smaller volumes, washed three times in Tris buffered saline and 3 ml aliquots containing 200 - 300 x 10^9/l platelets were frozen at -70°C until further use.

A mixture of auto-APTT reagent (General Diagnostics), patient's plasma and thawed platelet suspension was incubated at 37°C for 5 minutes then 0,025M $CaCl_2$ was added and time to clot formation determined in duplicate. A baseline APTT minus PNP APTT of >5 seconds was considered positive.

PNP results in a shortening of the clotting times of specimens with lupus anticoagulants from the baseline APTT with a median of 10 seconds (range 4 to 78 seconds). In general, the longer the initial APTT, the greater the shortening with the PNP. In contrast, there is no shortening of the PNP APTT in most patients with specific factor inhibitors.

Tissue thromboplastin inhibition test (TTI)

In 1976 Schnleider and associates introduced the concept of dilute thromboplastin test and suggested that it was a useful procedure for diagnosis of LAs.[78] However, it was

shown by Triplett not to adequately differentiate LAs from specific factor inhibitors or to the presence of heparin.[79] In view of the long standing use of the test in the study of lupus anticoagulants, it was evaluated in this study as a confirmatory procedure.

Acetone dried human brain thromboplastin was diluted 1:50 and 1:500 with normal saline (0,9%) and incubated at 37°C for 5 mins. 0,1 ml of diluted thromboplastin was added to 0,1 ml of patient plasma and the mixture incubated at 37°C for 5 minutes. 0,1 ml of 0,025M calcium chloride was then added to the mixture and the clotting times measured. The ratio of clotting times at 1:100 and 1:1000 dilutions was then determined. A ratio of 1,3 or greater was considered positive and indicative of a lupus anticoagulant.

<u>Specific factor assays of the intrinsic pathway</u>

Two factor assays of the intrinsic pathway (factors VIII and IX) were performed to rule out concurrent specific factor inhibitors using reagents and methodologies recommended by the manufacturer of the Automated Coagulation Laboratory (ACL) 800 centrifugal coagulation analyser (Instrumentation Laboratories, Milan). LAs characteristically give non-parallel curves with greater inhibition at higher dilutions.

<u>Kaolin clotting time</u>

This test was performed as originally described by Margolis [88] using 0.1 ml plasma preincubated for 3 minutes

in a water-bath at 37°C with 0.05 ml kaolin (20mg/ml in Owren's buffer, pH 7.35) and then recalcified with 0.1 ml 0.25 M calcium chloride. The manual tilt tube method was performed in duplicate on each sample of PPP and filtered PPP. Times were considered abnormal if prolonged beyond the upper limit of the normal range established for this test (>106 s). An alternative evaluation scheme is the calculation of a ratio.

A test ratio: $\frac{\text{test (seconds)}}{\text{normal (seconds)}}$

of more than 1.2 indicates an abnormal result. A normal ratio is less than 1.1 and values in between are considered borderline.

A control time of less than 60 seconds suggests contamination of the normal control plasma by platelet fragments, and invalidates the results.

<u>Dilute Russell's Viper Venom Test (DRVVT)</u>

The DRVVT was performed as previously described by Thiagarajan *et al*[81] using Russell's viper venom (Wellcome Diagnostics) reconstituted as suggested by the manufacturer and further diluted 1:200 in Tris-buffered saline to give a clotting time of between 25 and 30 seconds. The phospholipid reagent was diluted 1:8 in Tris-buffered saline. The test was performed by incubating 0.1 ml of plasma, 0.1 ml of diluted RVV, and 0.1 ml diluted phospholipid for 30 seconds

at 37°C after which 0.1 ml of calcium chloride was added and the clotting time recorded.

A clotting value of >3.8 SD above the mean is considered abnormal by Thiagarajan *et al.* This corresponds to approximately four seconds above the mean.

3.4 ANTICARDIOLIPIN ANTIBODY (ACA) TESTING

Sera for performing this assay were frozen and stored for later batch testing. The assay was performed using a modification of the ELISA method described by Lockshin *et al.*[50 89] Cardiolipin (Sigma St. Louis, MO) was used as substrate. Results were standardized to a single positive serum sample. Arbitrarily, 100 units of reactivity corresponded to the reaction produced by 20 µl of this serum. Significant levels were defined as 4 standard deviations (SD) higher than the mean of the normal controls (described previously).

The OD (optical density) values were interpreted as follows:

High positive	> 100 units IgG > 60 units IgM
Moderate positive	15 - 100 units IgG 6 - 60 units IgM
Low positive	5 - 15 units IgG 3 - 6 units IgM

The VDRL test was performed on all patients samples to eliminate cross-reactivity between positive ACA and syphilis. All positive VDRLs were FTA negative and therefore considered to be biological false positives.

3.5 STATISTICAL METHODS

An analysis of the normal range and patients data was performed using the upper 97.5% confidence interval ($x + 1.96 \times SEM$) to determine the *out of normal* range for each variable. An univariate analysis was performed on the whole sample to establish those with abnormal values. The Student t test was used to determine the significance of differences between the mean values of filtered and unfiltered tests.

CHAPTER 4

RESULTS

4.1 ESTABLISHMENT OF THE NORMAL RANGE

Clotting times of the tests performed on forty normal controls using platelet poor and filtered plasma, is shown in Table 6.

Platelet counts were determined on all 40 plasma samples after centrifugation and post-filtration using a Coulter S-Plus blood count analyzer. The mean platelet count of PPP was 23.77 x 10^9/l (SD 11.09) and 11.03 x 10^9/l (SD 7.33) for the post-filtration samples. This difference is statistically significant (p <0,000001).

The values in Table 6 were analyzed statistically and the results comparing the two variables are given in Table 7.

Table 7. STATISTICAL ANALYSIS OF TEST VARIABLES.

Variable	Mean	Standard deviation	Min	Max	SE of mean	CV
APTT	29.75	2.87	24	36	0.45	9.65
F-APTT	31.92	3.82	25	44	0.60	11.98
KCT	80.50	12.99	44	110	2.05	16.14
F-KCT	145.65	34.26	61	225	5.41	23.52
DRVVT	28.62	2.60	21	35	0.41	9.11
F-DRVVT	29.37	2.48	24	34	0.39	8.47
INR	1.03	0.06	0.8	1.16	0.01	6.12
F-INR	1.04	0.06	0.8	1.17	0.01	6.39

Table 6. FORTY NORMAL CONTROLS USING PPP AND FILTERED PLASMA WITH VALUES SHOWN IN SECONDS (EXCEPT INR).

		CENTRIFUGED PLATELET POOR PLASMA							FILTERED PLASMA			
No.	SEX	INR	APTT	APTT CONTROL	KCT	KCT CONTROL	DILUTE RVVT	DRVVT CONTROL	INR	APTT	KCT	DRVVT
1	M	1.05	33	30	96	68	27	29	1.09	34	166	30
2	F	1.01	30	30	79	68	29	29	0.89	33	161	30
3	F	1.10	30	31	83	58	27	26	1.03	30	134	28
4	M	1.10	29	31	83	58	29	26	1.17	31	154	31
5	F	0.93	29	32	69	71	29	28	0.94	35	107	30
6	M	1.04	27	32	82	71	26	28	1.05	29	152	28
7	M	0.97	31	32	86	63	35	32	0.98	32	174	34
8	F	1	32	32	109	63	34	32	0.98	34	192	34
9	F	1.01	33	32	84	76	26	26	1.02	36	196	27
10	M	1.04	29	32	82	76	28	26	1.11	32	175	28
11	F	1.02	28	32	74	83	26	26	1.05	33	109	26
12	M	1.03	27	32	82	83	27	26	1.02	29	137	27
13	F	1.11	35	32	110	87	25	27	1.15	40	225	25
14	M	1.06	24	32	72	87	25	27	1.12	27	130	26
15	M	0.98	25	31	69	76	30	28	1.03	27	119	30
16	M	1.10	33	31	102	76	31	28	1.13	37	211	31
17	F	1.11	25	31	66	82	28	30	1.10	25	61	28
18	F	1.01	29	31	71	82	27	30	1.02	32	149	27
19	M	1.04	28	31	69	82	29	30	1.05	31	143	27
20	M	1.06	31	31	72	82	29	30	1.05	34	143	33
21	F	1.08	36	31	95	81	30	28	1.10	36	137	30
22	M	1.01	31	31	83	81	28	28	0.93	33	136	30
23	F	0.96	27	31	80	81	31	28	0.98	28	127	29
24	M	1.02	31	31	80	81	31	28	1.02	32	143	30
25	F	0.83	30	34	83	81	27	27	1	30	86	27
26	M	1.05	27	34	80	81	29	27	1.04	28	128	29
27	F	1.16	29	34	86	81	27	27	1.16	31	152	27
28	M	1.13	32	34	110	81	28	27	1.12	33	186	27
29	F	1.08	30	33	85	85	29	28	1.02	30	178	29
30	M	0.96	27	33	76	85	29	28	1	27	108	30
31	F	1	32	31	72	81	29	30	1	32	155	30
32	F	1.1	29	31	82	81	29	30	1.1	31	165	31
33	M	1	29	31	90	81	30	30	1	31	177	30
34	M	1.1	28	31	73	81	30	30	1.1	30	141	32
35	M	1	26	30	73	80	32	27	1	26	110	32
36	F	1	33	31	64	85	27	27	1	36	146	29
37	F	1	26	34	44	81	21	29	1	28	76	24
38	F	1.04	33	34	68	81	34	29	1.07	35	129	33
39	M	1.14	34	34	82	81	27	29	1.17	44	168	32
40	F	1	32	34	74	81	30	29	1	35	140	34
Mean:		1.03	29.75	31.87	80.5	78.07	28.63	28.25	1.04	31.92	145.65	29.37

The *out of range* values were calculated using the 97.5% confidence interval of the normal distribution curve for each variable. The values used in this study are:

APTT .... > 35.377 s
KCT .... >105.959 s
DRVVT .... > 33.738 s
INR .... > 1.160

Filtered APTT ..... > 39.423 s
Filtered KCT ...... >212.811 s
Filtered DRVVT .... > 34.251 s
Filtered INR ...... > 1.176

Filtering of plasma produced as significant difference in the KCT only ($p < 0.000001$). This prolongation in test time is attributed directly to the removal of platelets as source of phospholipids.

4.2 ABNORMAL CONTROLS

Seven known lupus anticoagulant positive patients were retested using APTT, KCT and DRVVT. The results shown in Table 8, reflect a prolonged APTT in six with an uncorrectable 1:1 mixture of patient and normal plasma.

Table 8. CLOTTING TESTS PERFORMED ON ABNORMAL CONTROLS.

No.	INR	APTT	APTT MIX	KCT	DRVVT
1	1.1	46	40	124	25
2	1.3	73	44	149	30
3	1.1	52	42	140	33
4	1.1	54	45	94	39
5	1.1	59	53	138	43
6	1.2	58	45	137	37
7	1.0	35	31	103	44
Control	1.0	32		79	29

The INR was abnormal in patients 2 and 6. In our laboratory, however, the normal range accepted for this test is 1.0 to 1.3.

The KCT was out of range in all except nos. 4 and 7 using 106 seconds as upper limit of normal. This result was compared with the KCT ratio obtained by dividing test time by control time and expressing the result as a ratio described by Machin *et al.*[67] A value of >1.2 is regarded as abnormal.

KCT RATIO		
	1	1.54
	2	1.85
	3	1.74
	4	1.17
	5	1.71
	6	1.70
	7	1.30

The ratio obtained for patient no. 4 is interpreted as being borderline (values >1.1 but less than 1.2).

The DRVVT was abnormal in patients 4, 5, 6 and 7 (≥34 s). This was compared with the DRVVT ratio where a value of >1.1 is abnormal.

DRVVT RATIO		
	1	0.87
	2	1.05
	3	1.15
	4	1.36
	5	1.50
	6	1.29
	7	1.52

Five of the ratios were abnormal using this calculation, including the borderline case no. 3.

Filtration of the plasma (data not shown) produced no difference in interpretation of the results of these seven patients.

4.3 SLE PATIENT GROUP

Coagulation tests were performed on fifty consecutive untreated patients with systemic lupus erythematosus. There were 48 females and 2 males with a mean age of 36.9 years (range 22 to 54 yrs). Results are shown in Tables 9 and 10.

In the **PPP group**, 10 APTTs (20%) were abnormal but only 6 (12%) fulfilled the ICTH criterion: The APTT of a 1:1 mixture with normal plasma should be greater than 4 seconds over the control,[66] (i.e. 32 + 4 s). Eight KCT (16%) and five DRVVT (10%) results were abnormal.

Using **filtered** plasma (Table 10), 6 APTTs (12%) were out of range with four (8%) failing to correct on mixing with normal plasma. Seven KCT (14%) and three DRVVT (6%) results were abnormal.

The six plasma samples with prolonged APTT mixtures identified in the PPP group, were subjected to confirmatory procedures listed in Table 11 and to factor VIII and IX assays. The PNP confirmed the diagnosis of lupus anticoagulant in 4 of the 5 tested. Patient no. 29 failed to show a correction of >5 seconds while no. 43 was not tested.

Table 9. ***COAGULATION RESULTS ON FIFTY SLE PATIENTS USING PLATELET POOR PLASMA.***

NO.	SEX	AGE	INR	APTT	1:1 MIX	KCT	DRVVT
1	F	22	1	27		61	28
2	F	31	1.12	28		80	28
3	F	35	0.97	24		63	30
4	F	53	1.13	31		79	28
5	F	51	1.04	26		82	30
6	F	43	1.02	30		83	27
7	F	33	1.24 §	29		82	26
8	F	25	1.19 §	31		90	27
9	F	26	1.11	39 §	35	99	29
10	F	28	1.03	30		90	31
11	F	32	1.1	29		77	25
12	F	52	1.1	58 §	52	138 §	43 §
13	F	25	1	37 §		112 §	26
14	F	38	1	32		97	25
15	F	42	1	27		76	26
16	F	33	1.2 §	66 §	48	295 §	42 §
17	F	34	1	33		79	25
18	F	43	1	27		73	26
19	F	27	1.1	32		89	25
20	F	31	1	36 §		77	26
21	F	26	1.1	29		86	27
22	F	48	1.1	50 §	39	187 §	40 §
23	F	54	1.06	27		95	27
24	F	28	1.06	35		92	28
25	F	28	1.03	32		73	25
26	F	49	1.01	24		67	22
27	F	39	1.01	25		71	23
28	F	25	0.98	26		85	22
29	F	24	1.02	42 §	37	117 §	28
30	M	36	1	63 §	40	149 §	30
31	F	41	1.06	36 §		118 §	28
32	F	29	1	27		67	25
33	F	33	1.02	29		82	26
34	F	41	1.1	28		75	34 §
35	F	25	1.08	29		70	27
36	F	58	1.04	33		75	28
37	F	33	1.06	32		84	28
38	F	54	0.98	31		80	26
39	F	48	1.19 §	34		93	25
40	F	51	1.19 §	31		67	29
41	F	42	1.06	28		78	29
42	F	26	1.04	27		80	26
43	F	28	1	41 §	39	108 §	34 §
44	F	43	0.98	29		76	27
45	M	39	0.98	26		92	25
46	F	28	0.98	27		72	26
47	F	35	1.04	27		77	25
48	F	48	1	35		94	25
49	F	54	1.02	29		73	24
50	F	28	1.02	30		87	26

§ denotes out of range

Table 10. COAGULATION RESULTS ON FIFTY SLE PATIENTS USING FILTERED PLASMA.

NO.	SEX	AGE	INR	APTT	1:1 MIX	KCT	DRVVT
1	F	22	1	28		103	32
2	F	31	1.11	28		155	30
3	F	35	0.98	24		106	29
4	F	53	1.14	33		151	29
5	F	51	1.05	27		145	32
6	F	43	1.02	31		127	27
7	F	33	1.29 §	31		82	26
8	F	25	1.23 §	33		158	28
9	F	26	1.12	44 §		206	30
10	F	28	1.05	32		189	29
11	F	32	1.1	30		148	25
12	F	52	1.1	63 §	55	>300 §	45 §
13	F	25	1	40		215 §	28
14	F	38	1	35		151	27
15	F	42	1	28		122	28
16	F	33	1.2	70 §	59	>300 §	46 §
17	F	34	1	33		185	26
18	F	43	1	27		130	25
19	F	27	1.1	32		175	26
20	F	31	1	37		136	28
21	F	26	1.1	30		180	29
22	F	48	1.2	54 §	41	>300 §	42 §
23	F	54	1.08	28		145	27
24	F	28	1.06	36		135	32
25	F	28	1.05	38		99	29
26	F	49	1.03	26		74	27
27	F	39	1.03	29		125	25
28	F	25	0.98	26		132	24
29	F	24	1.04	45 §		244 §	30
30	M	36	1	70 §	45	192	32
31	F	41	1.09	39		216 §	28
32	F	29	1	28		118	24
33	F	33	1.02	31		165	27
34	F	41	1.08	28		130	32
35	F	25	1.1	30		105	28
36	F	58	1.06	34		145	30
37	F	33	1.08	33		203	29
38	F	54	1	32		159	27
39	F	48	1.21 §	36		232 §	28
40	F	51	1.21 §	34		150	32
41	F	42	1.08	29		147	29
42	F	26	1.04	29		135	28
43	F	28	1.02	42		163	30
44	F	43	0.98	30		130	28
45	M	39	0.98	28		132	26
46	F	28	0.98	28		123	27
47	F	35	1.06	27		142	26
48	F	48	1	36		195	26
49	F	54	1.02	30		135	24
50	F	28	1.08	32		132	27

§ denotes out of range.

TABLE 11. Confirmatory tests performed on suspected LA positives:
(A) Platelet Neutralization Procedure and
(B) Tissue Thromboplastin Inhibition test.

No.	PNP	SALINE CONTROL	DIFFER-ENCE		TTI 1:50	CONTROL	RATIO	TTI 1:500	CONTROL	RATIO
12	59	74	15	C	56	30	1.87	120	56	2.14
16	60	75	15	C	57	29	1.97	127	61	2.08
22	40	47	7	C	66	32	2.06	129	54	2.39
29	39	42	3	NC	30	26	1.15	65	50	1.3
30	43	59	16	C	49	30	1.63	100	58	1.72
43	ND	-	-	-	32	24	1.33	71	46	1.54

C : Correction
NC : No correction
ND : Not done

The Tissue Thromboplastin Inhibition test at the 1:50 dilution confirmed 5 of the 6 cases, while all the samples at 1:500 dilution were positive (>1.3). Coagulation factor assays produced non-parallel curves on all samples as addition confirmation of the nature of the inhibitors.

There were 5 out of range INR (10%) results in the PPP group but only no. 16 corresponded with a positive LA. In the filtered group, 4 (8%) were out of range showing no correlation with LA positivity.

4.4 ANTICARDIOLIPIN ANTIBODIES

The IgG and IgM anticardiolipin antibody levels determined by ELISA assay, were performed on single serum samples. The values are tabulated (Table 12) and the levels demonstrated graphically in Figures 2 and 3.

Three individuals tested positive by VDRL at a low titre of 1/2 but all were negative by FTA, demonstrating false biological positive tests for syphilis.

4.5 CLINICAL CORRELATIONS

A history of clinical complications reported in association with LA and/or ACA, namely repeated abortions, venous and arterial thromboses and other associated problems, was evaluated. This information was extracted partly from a database maintained on some of the SLE patient group by the Department of Rheumatology, Groote Schuur Hospital and by reviewing hospital records. The information is summarized in Table 13. The six LA positive patients with SLE and all moderately elevated ACA levels are indicated on the table.

Six patients were found to have a history of two or more abortions; one patient had two episodes of deep vein thrombosis of the leg and one patient a right external jugular vein thrombosis. There was one case of myocardial infarction, two cases of neuropsychiatric disorders and one case each of thrombophlebitis and pulmonary hypertension.

Table 12. IgG AND IgM ANTICARDIOLIPIN ANTIBODIES (ACA) LEVELS AND VDRL POSITIVE RESULTS IN SLE GROUP.

No.	SEX	AGE	ACA IgG	Ab LEVEL	ACA IgM	Ab LEVEL	VDRL
1	F	22	25	M	26	M	
2	F	31	17	M	25	M	
3	F	35	0		23	M	
4	F	53	56	M	22	M	
5	F	51	20	M	17	M	
6	F	43	13	L	13	M	
7	F	33	3		11	M	
8	F	25	0		6	L	
9	F	26	6	L	14	M	POS 1/2
10	F	28	20	M	30	M	
11	F	32	5		5	L	
12	F	52	6	L	13	M	
13	F	25	5		11	M	
14	F	38	10	L	5	L	
15	F	42	28	M	10	M	
16	F	33	6	L	12	M	
17	F	34	4		10	M	
18	F	43	5		6	L	
19	F	27	5		42	M	
20	F	31	33	M	14	M	
21	F	26	13	L	13	M	
22	F	48	50	M	10	M	
23	F	54	0		1		
24	F	28	0		3		
25	F	28	5		9	M	
26	F	49	0		0		
27	F	39	0		0		
28	F	25	0		0		
29	F	24	48	M	10	M	
30	M	36	0		35	M	
31	F	41	28	M	0		
32	F	29	12	L	33	M	
33	F	33	7	L	33	M	POS 1/2
34	F	41	0		0		
35	F	25	5		0		
36	F	58	3		2		
37	F	33	0		0		
38	F	54	0		9	M	
39	F	48	5		39	M	POS 1/2
40	F	51	0		1		
41	F	42	0		0		
42	F	26	4		4	L	
43	F	28	0		0		
44	F	43	0		0		
45	M	39	6	L	3		
46	F	28	0		0		
47	F	35	0		34	M	
48	F	48	0		2		
49	F	54	0		0		
50	F	28	0		0		
f	Low (L):			9		5	
f	Moderate (M):			10		26	

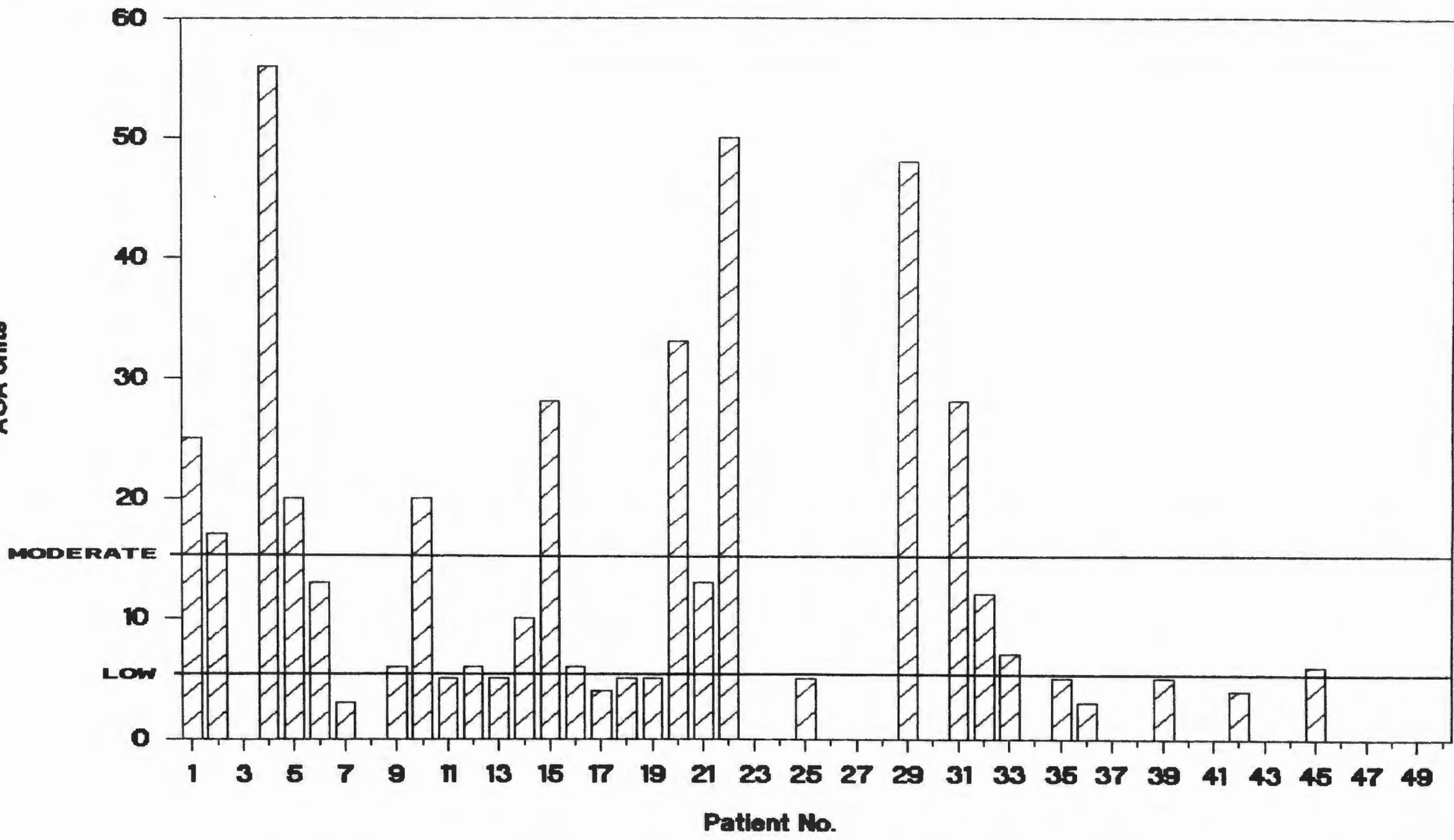

Figure 2: IgG ACA LEVELS.

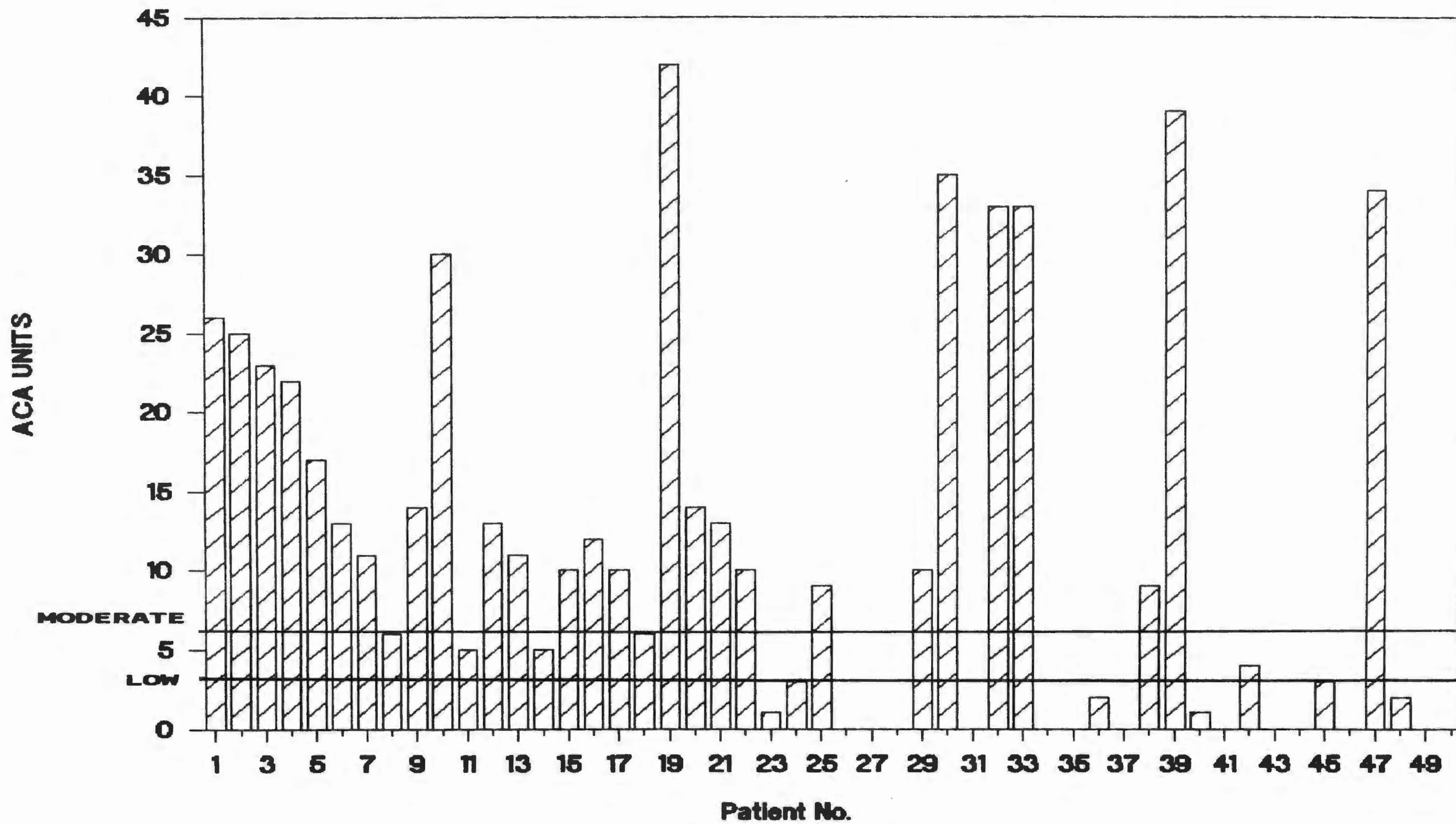
Figure 3: IgM ACA LEVELS.
ACA UNITS
45
40
35
30
25
20
15
10
MODERATE
5
LOW
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49
Patient No.

A positive association with the presence of lupus anti-coagulants was noted in two patients in the SLE study group. A history of neuropsychiatric disorder was recorded in patient no. 12 and a history of three pregnancies ending in spontaneous abortion in patient no. 43.

Table 13. THE CLINICAL COMPLICATIONS: ABORTIONS, THROMBOTIC EVENTS AND OTHER PROBLEMS SEEN IN ASSOCIATION WITH LA POSITIVITY AND MODERATE LEVELS OF ACA.

NO.	SEX	LA	IgG ACA	IgM ACA	PREG	ABORT	VENOUS THROMB	ARTERIAL THROMB	ASSOCIATED PROBLEMS
1	F		M	M	0	0			
2	F		M	M					
3	F			M	0	0			
4	F		M	M	1	1			
5	F		M	M	5	0			
6	F			M	4	0		MI	
7	F			M	3	0			
8	F				2	1			
9	F			M					
10	F		M	M	1	1			
11	F				1	0			
12	F	LA		M	1	1			Neuropsychiatric disorder
14	F			M	0	0			
15	F		M	M	3	0			
16	F	LA		M	2	0			
17	F			M	2	2 §			
18	F				3	0			Thrombophlebitis
19	F			M	1	1			
20	F		M	M	2	0			
21	F			M	0	0			
22	F	LA	M	M	0	0			
23	F				0	0	DVT x 2		
24	F				1	0			
25	F			M	0	0			
26	F						R ext jug		
27	F				6	0			
28	F				1	1			
29	F	LA	M	M	1	1			
30	M	LA		M					
31	F		M		2	0			
32	F			M	2	0			
33	F			M	0	0			
34	F				4	0			
35	F				4	1			
36	F				0	0			
37	F				3	3 §			
38	F			M	5	5 §			Pulmonary hypertension
39	F			M	6	0			
40	F				3	0			
41	F				1	1			
42	F				1	1			
43	F	LA			3	3 §			
44	F				0	0			
45	M								
46	F				5	0			
47	F			M	4	1			
48	F				3	2 §			Psychosis
49	F				0	0			
50	F				2	2 §			
Frequency						6	2	1	4

§ denotes two or more abortions

CHAPTER 5

DISCUSSION

Studies on lupus anticoagulants have been hampered by a variety of problems in the preparation, transportation and storage of samples and the lack of standardized diagnostic procedures. This has favoured the wide spread use of the solid phase anticardiolipin assay as an alternative. The ACA assay is not subject to the same inherent handling problems and this facilitates its use in large population surveys, where associations occurring in patients with antibodies to phospholipids have been confirmed. However, the LA tests and ACA assay do not detect the same abnormality and therefore, it is advisable to perform both procedures. Laboratories offering this service need to be proficient in providing the most sensitive and reliable tests available.

In the design of this project, the above issues were addressed. The 1983 ICTH criteria for the laboratory diagnosis of lupus anticoagulants as recommended by Green *et al*, [66] were strictly applied using a prolonged APTT screening test as a primary requirement. General Diagnostics auto-APTT reagent used routinely in this study, has been shown to be one of the most sensitive reagents in detecting lupus anticoagulants.[68] Routine use of insensitive agents can lead to underdiagnosis of lupus anticoagulants.

5.1 NORMAL RANGE

Tests and assays performed on forty control subjects provided values and times similar to published data. Use of standardized methodology and reagents of the same batch or lot numbers throughout the study, permitted subsequent evaluation of the two test groups on the basis of *out of range* values derived from the normal range.

Filtering of plasma produced a significant prolongation of the mean kaolin clotting time only. This is attributed directly to the removal of platelets as shown by an equally significant fall in the mean platelet count of the post-filtration samples. The increased coefficient of variation (CV) noted in the filtered KCT, highlights the need for meticulous attention to procedural detail to ensure adequate removal of platelets and a meaningful increase in test times.

5.2 ABNORMAL CONTROL GROUP

Evaluation of seven patients who had previously tested positive for LA, served as a useful exercise in determining the effectiveness of the test procedures. The presence of strong lupus anticoagulants were confirmed in six of the seven cases using approved ICTH criteria. The APTT screening procedure was most sensitive detecting 6 (85%) while the KCT was abnormal in 5 (71%) and the DRVVT in 4 (57%). The INR being marginally abnormal in 2 (28%) was the least sensitive. It is interesting that case no. 7, which

was interpreted as LA negative, had the most prolonged DRVVT, confirming the heterogeneity of results in testing for lupus anticoagulants.

All seven patients had confirmed SLE and it is important to note that cases 3, 5 and 6 were receiving corticosteroid therapy at the time of testing without causing a loss of LA activity.

A retrospective analysis of the data using KCT and DRVVT ratios as described by Machin *et al* in 1991,[67] reduces the cut-off level considerably and increases the sensitivity threshold, with 6 abnormal and one borderline KCT and 5 abnormal DRVVT results. Use of the KCT or DRVVT ratio are calculations that have merit in a laboratory where tests for the detection of LAs are seldom performed or where a normal range, applicable to local conditions, has not been established. The calculation based on a control time and patient value, is readily performed and the interpretation unambiguous.

5.3 SLE STUDY GROUP

Lupus anticoagulants were proven in six cases (12%) using platelet poor plasma and ICTH criteria. This is a low percentage for a high risk population of systemic lupus erythematosus patients by modern standards of investigation. In an analysis of 21 reported series (comprising over 1100 patients with SLE), Love and Santoro [90] found an average prevalence of 34% for lupus anticoagulant. When series that

included only unselected or consecutive patients were considered separately, the frequency of LA was slightly, although significantly, lower (28%). This prevalence figure is still appreciably higher than the widely quoted figure of 10% reported by earlier estimates based on fewer patients.

The relatively low frequency of LA in this series may be attributed to a number of reasons:

1. The patient population was selected by excluding those who were receiving corticosteroids or other immunosuppressive drugs or who were on long-term anticoagulant therapy. By this selection, patients with active disease were not included in the sample and a bias may have resulted in the detection of fewer positive LA and ACA.
2. The 1983 ICTH criteria used for the diagnosis of LA are rigid and poses restraints on evaluation and acceptance of newer sensitive test procedures.
3. From previous experience in our laboratory, it was found that the APTT screening procedure should be prolonged by at least 5 seconds above the upper limit of normal so that mixing studies could be adequately evaluated. Several weak LA may have thus gone undetected. This is apparent when considering the 10 abnormal APTT and 8 abnormal KCT results in this study with only six cases of LA confirmed.

4. Use of a 1:1 mixture of patient to normal plasma, was the ICTH recommendation. Recent research has shown a 4:1 ratio is preferable as it does not swamp weaker inhibitors.[71]
5. The source of normal control plasma used to perform mixing studies is crucial. Kaczor *et al* showed that Verify Normal [R] plasma (General Diagnostics) which was used in this study, was inferior when compared to another commercially prepared reagent (Helena ARP [R]).[71] This difference may be especially apparent when evaluating minimally prolonged screening tests.
6. Although generally accepted that LA are immediate acting inhibitors, Clyne and White [77] showed that 40% of LA require incubation to exhibit maximal anticoagulant activity and that some may not be detected if mixing studies are restricted to the preincubation phase. This aspect was not evaluated in the study.

Comparing the PPP and filtered plasma study groups, shows that filtration reduced the sensitivity of all tests to detect lupus anticoagulants. The procedure is time-consuming, adds expenditure with no additional benefit and could not be recommended for routine use.

The lupus cofactor which causes a paradoxical prolongation of the mixture time (sometimes exceeding that of the initial APTT) was not detected in any of the specimens tested in this study. This cofactor effect was probably suppressed by

the use of 1:1 mixture ratios as it has usually only been reported in laboratories using a 4:1 ratio.[1 71]

Confirmatory tests performed on plasma samples of suspected lupus anticoagulants analysed in Table 11, shows a similar trend in both PNP and TTI results. In patient no. 29, however, the PNP showed no correction while the TTI was positive only at the 1:500 dilution. This was presumably due to a low titre inhibitor, but as the tests are based on divergent principles, the value of performing both procedures in confirming suspected LA is endorsed. Misgivings have been expressed regarding the lack of specificity of the TTI test for LA,[79 80] but this was not, however, apparent in this study.

Use of the INR can not be advocated as a sensitive screening procedure. In the PPP group, of the five out of range results, only one occurred in a LA confirmed case while none of the four in the filtered group showed a positive correlation.

5.4 ANTICARDIOLIPIN ANTIBODIES

The frequency of anticardiolipin antibodies in patients with SLE have varied widely from 21 to 63% with a mean of 44%, as reported by Love and Santoro in a computer assisted search of the literature.[90] Discrepancies have arisen from differences in assay methodology as earlier work utilized markedly different methods. An international workshop addressed this issue in 1986, [91] resulting in standard-

ization of the ELISA assay technique, including the use of fetal calf serum as blocking agent to prevent non-specific binding. Agreement on the definition of a positive result remained unsettled until the Kingston Anti-phospholipid Study (KAPS) Group published a special report in 1990 on the second international standardization workshop where the use of six standards to obtain semiquantitative measure of anticardiolipin activity of both IgG and IgM isotypes was proposed.[92]

The idea that titre or isotype (specifically IgG) of anticardiolipin may be important in determining which patients have thrombosis has received considerable attention of late. There is a strong association between high-titre anticardiolipin and a history of thrombosis.[93] Several cases have been reported in which ACA titres have been shown to fluctuate markedly over time (with and without immuno-suppressive therapy) and changes may be accompanied by alteration in antibody isotype.[58] For this reason, it is questionable whether the clinical significance of antibody isotype or titre can be accurately assessed on the basis of a single test.

One main drawback of the anticardiolipin test is its sensitivity, which can give rise to frequent false positive results (albeit that most such results are low positive).[92] IgM ACA assay is particularly prone to this fault and to nonspecific binding. It is, therefore, sensible to consider only moderate or high positive ACA test results (on repeated

testing) when attributing clinical associations to their occurrence.

In the SLE group, 33 (66%) had at least one ACA isotype and 17 (34%) had both IgG and IgM antibodies. If the low positive results are disregarded, the frequency of ACA positivity is 27 (54%) which is well within the reported range for patients with systemic lupus erythematosus. There were 10 moderate IgG (20%) and 26 IgM (52%); 9 (18%) were positive for both isotypes. Five of the six LA positive cases (83%) also had moderate IgM ACA levels and a two (33%) had moderate IgG positivity.

5.5 CLINICAL ASSOCIATIONS

The frequency of clinical complications in the SLE group was relatively low with 13 evaluable events occurring in 11 patients (22%). This is less than the estimated one third of all patients with SLE in which complications can be expected.

Associations were noted with a history of two or more abortions recorded in one patient with LA and in two with moderate IgM ACA. One myocardial infarction and one patient with pulmonary hypertension had IgM ACA in the moderate range and one case of LA with moderate IgM levels had a history of a neuropsychiatric disorder.

The importance of moderately elevated IgM ACA levels in this study, is borne out by a strong correlation between LA

positivity and IgM antibodies (83%) and to the occurrence of this antibody isotype in 38% of patients with clinical complications. It is highly probable that if SLE patients had not been excluded from this study on the grounds of current immunosuppressive therapy, a greater number of symptomatic patients, with associated clinical complications could have been evaluated. Corticosteroids have a suppressive effect on lupus anticoagulants, but to a lesser extent on anticardiolipin antibodies.[90] This is due in part to the sensitivity of the ACA ELISA technique and further highlights the versatility of this test procedure. It is recommended that ACA testing be introduced routinely in the evaluation of patients with SLE, irrespective of concurrent anticoagulant or immunosuppresive therapy.

CHAPTER 6

CONCLUSIONS

In concluding this investigation of lupus anticoagulants, it is necessary to formulate recommendations on the most appropriate investigative procedure in a patient in whom this condition is suspected.

1. Double centrifugation of patient plasma is a step that needs to be adopted to enhance detection of minimally prolonged APTTs.

2. A normal range can be used to determine upper limits provided reagents, methodologies and apparatus remain constant. Alternatively, recently advocated test ratios which rely on control times to quantify results, with a published cut-off values, are preferable where a normal range is lacking.

3. An APTT screening test using a reagent with proven sensitivity to detect weak lupus anticoagulants, is the best primary test to perform. The procedure is automated and routinely performed. The KCT is equally sensitive but usually a manual method, as the recently described automated KCT [72] requires a programmable centrifugal coagulation analyzer to read the clotting times which are not available in all coagulation laboratories.

4. Mixing studies should be performed on all prolonged APTT results. A 4:1 ratio of patient to normal plasma recommended by most researchers today to avoid swamping weaker inhibitors, although not tested in this study, requires serious consideration.

5. Source of normal plasma used to perform mixing studies must be platelet poor. Manufacturers specifications should be examined to ensure that steps are taken to remove platelets from pooled plasma lots (preferably by filt-ration), as residual platelet fragments may neutralize weaker LA.

6. In our hands, the dilute RVVT is not sufficiently sensitive to be advocated for routine screening or confirmation. However, it is occasionally the only test time to be prolonged in the presence of LA.

7. The PNP and TTI are both equally effective as confirmatory test. The 1:500 dilution of TTI seems marginally more sensitive but a tendency to false positive results has challenged its specificity.[69]

8. Anticardiolipin antibody assays should be performed on all positive and suspected cases of LA as this test detects a different antiphospholipid epitope. Single estimations are not easily evaluated, and as in any serological investigation, a follow-up specimen is recommended.

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