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Preanalytical variables that affect coagulation tests need to be controlled to insure the accuracy of results. A wide range of variables affect the accuracy of global tests for the assessment of coagulation status.

Requirements for accurate monitoring of heparin therapy using sensitive tests that assess factor $X_a$ inhibition require minimization of platelet aggregation and release of contents of platelet granules. Preanalytical errors in the monitoring of fibrinolytic therapy are manifold and require minimization. The choice of anticoagulants for blood collection also becomes critical in minimizing preanalytical error in newly emerging assays that provide a sensitive assessment of coagulation status.

Preanalytical variables affecting global coagulation tests

Preanalytical variables affecting global tests for coagulation such as prothrombin time (PT) and activated partial thromboplastin time (APTT) include choice and concentration of anticoagulant, the anticoagulant to blood ratio, pH, concentration of divalent cations, hematocrit and storage temperature, to mention a few.

Choice and concentration of anticoagulant

Citrate is the preferred anticoagulant as compared to oxalate. This is due to the fact that factor $V$ is more stable in citrate than in oxalate. Furthermore, citrate rapidly complexes with calcium by forming a soluble complex, in contrast to the slow formation of the insoluble complex of calcium with oxalate.$^{1,2}$

The concentration of citrate used as anticoagulant for tests such as PT and APTT is generally either 0.129M or 0.105M. It is important to note that the effective molarity will be dependent on the choice of the dihydrate or the anhydrous citrate salt. For instance, a 3.2% solution of sodium citrate using the dihydrate salt is 0.109M. However, a 3.2% solution of sodium citrate prepared with the anhydrous salt will be 0.124M. Similarly, a 3.8% solution of sodium citrate prepared with the dihydrate salt will be 0.129M, while a 3.8% solution of sodium citrate prepared with the anhydrous salt will be 0.147M.

Anticoagulant to blood ratio

The ratio of anticoagulant (sodium citrate, either 0.129M or 0.105M) to blood that is traditionally used is 1:9. If less blood is collected than the nominal volume required to maintain a 1:9 ratio the effective concentration of citrate increases. This has a serious effect on the APTT result.

Table 1 demonstrates the effect of anticoagulant to blood ratio on the APTT result. From the table it is apparent that at a 1 to 7 ratio of anticoagulant to blood there is a significant increase in the APTT result compared to the result obtained at the nominal anticoagulant to blood ratio of 1 to 9. The effect on PT of the anticoagulant to blood ratio is noticeable only when the ratio reaches 1 to 4.5, that is when the blood collection tube is just less than half of its nominal volume.$^3$

Effect of pH

Because of the buffering effect of hemoglobin the blood pH is maintained close to normal in blood collected in buffered citrate. However, plasma pH will be higher in blood collected in buffered citrate, and higher still in blood collected in nonbuffered citrate whose initial pH is...
well on the alkaline side. This lack of buffering effect in plasma is due to the absence of hemoglobin, the imidazole group of which is responsible for the buffering effect in whole blood. The pH of the assay mixture for coagulation tests such as PT and APTT should be maintained within narrow limits (7.1 to 7.35). Utilizing normal plasma it has been demonstrated that the PT increases if pH is less than 7.1 and greater than 7.35. With abnormal plasma the pH range for PT measurements is even narrower, and lies approximately between 7.3 and 7.45. As such, pH has to be maintained rigidly within the range required for accurate measurement of PT and APTT. Normally if the blood collection tube is kept well stoppered changes in pH of blood will be minimal.

Indeed it has been shown that in an unopened evacuated blood collection tube, the PT is stable for 24 hours in blood stored at room temperature. Apparently pH changes are minimized by leaving the stopper of blood collection tube in place, thereby preventing the loss of carbon dioxide, and the resultant increase in pH, had the stopper been removed.

**Concentration of divalent cations**

The biphasic effect of divalent cations such as calcium on APTT has been well recognized. Thus while the addition of 0.025M calcium chloride to citrated plasma has no effect on the APTT result, increasing the calcium chloride concentration to 0.065M and above or decreasing it to 0.004M will both result in the artifactual elevation of the APTT result.

The effect of zinc ions at lower levels of 10 mg/L is to abolish the effect of heparin on the APTT result, by yielding a normal APTT result on a heparinized sample. However, when the zinc ion concentration is increased 10 fold to 100 mg/L the APTT results obtained on both heparinized and non-heparinized samples are increased thus mimicking the effect of heparin in non-heparinized samples.

The PT is also artifactualy increased when zinc ion concentrations are between 30 to 100 mg/L. The practical implications of this finding is that the contamination of blood sample with zinc ions could affect heparin monitoring using the APTT, and also influence results obtained on PT.

**Effect of hematocrit**

The hematocrit has a bearing on PT and APTT measurements. When a subject has a very high red blood cell count such as in polycythemia the plasma compartment is very low. Since citrate used for blood collection is concentrated in the plasma compartment the effective concentration of citrate in the plasma compartment is high.

This results in extra citrate being present in the plasma compartment which will complex with calcium added during PT and APTT measurement thus artifactualy elevating both PT and APTT, since the clotting process is slowed down due to insufficient calcium. This effect can be minimized by adjusting the citrate concentration in accordance with the hematocrit value by using empirical formulas or by using a 1:19 ratio of anticoagulant to blood.

**Effect of storage temperature**

It has been demonstrated that when plasma is stored in contact with cells and maintained at 4°C, the PT is not artifactualy affected up to 7 hours. Beyond 7 hours factor VII is activated shortening the PT.

In another study whole blood samples and plasma stored at 4°C showed a shortening of PT beyond 24 hours. In this same study it was noted that in samples stored at room temperature (25°C) or centrifuged in a non-refrigerated centrifuge, the PT was stable for up to 48 hours. Furthermore, freezing plasma at −20°C and at

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<td>1:8</td>
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Table 1. The effect of anticoagulant citrate (0.129M) to blood ratio on APTT results.
–70°C did not activate factor VII. In addition, both PT and APTT results were stable in plasma frozen at –20°C for 10 days and at –70°C for 21 days.

Factors II, VII and X were stable in plasma maintained under refrigeration for up to 6 hours. Beyond 6 hours plasma frozen at –20°C and at –70°C showed no deterioration in the levels of the above factors for up to 14 days. Factor V was stable for 6 hours when plasma was stored at 4°C. However, 20% of the activity of factor V was lost even when plasma was stored frozen at –20°C for over 7 days. Even in samples stored frozen at –20°C, after 7 days, 10% of factor V activity was lost. Factor VIII is least stable with 10% of activity lost in 4 hours when plasma is stored at 4°C. Even when plasma is stored frozen at –20°C for 3 days, 20% of factor VIII activity is lost.

Effect of centrifugation

Some residual platelet contamination is to be found even when blood is centrifuged at high speeds attainable in ordinary laboratory centrifuges (1800 g) for 15 minutes, as judged by the shortening of thrombin clotting time by 10% in plasma frozen for 48 hours and thawed to room temperature prior to testing. However, when the blood is centrifuged at 11,000 g there is very little platelet contamination. Tests such as PT, APTT, AT-III, fibrinogen, D-Dimer and dilute Russel Viper venom test were reported to be unaffected on centrifugation at such high speeds.

Minimization of platelet aggregation and release of contents of platelet granules

To assess accurately platelet activation in-vitro one must prevent platelet activation in-vitro. Platelet activation can be assessed by the measurement of one of the many constituents within the platelet α-granule which is released upon activation of platelets. Thus when platelets are activated the contents of α-granules such as platelet Factor IV (PF4), β-thromboglobulin (βTG), platelet derived growth factor (PDGF), thrombospondin (TSP) fibronectin, fibrinogen, albumin and factor VIII-related von Willebrand factor polymers (VIII:vWF) are released. Platelet activation also results in the release of contents of platelet dense granules such as adenosine diphosphate (ADP) and serotonin.

One must prevent in vitro release of these constituents to permit accurate assessment of in vivo platelet activation. Among additive mixtures that have been proposed for inclusion in blood collection tube to prevent in vitro platelet activation the following deserves mention: a mixture of acid citrate dextrose (ACD, 1 to 5 dilution), 30 μM acetylsalicylic acid (aspirin) and 1 μM prostaglandin E₁ (PGE₁). The rationale is that aspirin by acetylating and inhibiting fatty acid cyclooxygenase enzyme in platelets will inhibit release of contents within the platelet α-granules and ADP-induced platelet aggregation by preventing formation of thromboxane A₂. Because of the stability problems associated with PGE₁ and the need to use ethanol to dissolve aspirin and PGE₁ an alternative inhibitor of platelet aggregation and release has found application. This additive mixture is called CTAD (citrate, theophylline, adenosine and dipyridamole). The rationale for the utilization of CTAD is based on the maintenance of increased intra cellular levels of cyclic AMP (C-AMP) which is a potent inhibitor of platelet aggregation. Increased levels of C-AMP are maintained by activation of adenyl cyclase by the addition of free adenosine. Although red blood cells are a source of free adenosine formed form adenine nucleotides, they avidly take up the formed adenosine, as a result of which, the level of plasma adenosine is low. The uptake of adenosine by the red cell is inhibited by dipyridamole, thus permitting increased activation of adenyl cyclase by adenosine resulting in increased C-AMP levels needed to prevent platelet aggregation and release. One must prevent degradation of C-AMP by the enzyme phosphodiesterase. This is achieved by theophylline and to a certain extent by dipyridamole present in the CTAD mixture. Citrate, of course, by chelating calcium functions as an anticoagulant.

CTAD mixture can be used in the blood collection tube for monitoring heparin therapy by measurement of either heparin by chromogenic
substrate assay, or by using the APTT determination. Since in vitro aggregation and release of contents of α-granules in platelets will be inhibited by CTAD, release of PF4 present in the platelet α-granules will be minimized thus permitting a reliable assessment of circulating heparin level in plasma. CTAD can also be used to measure in vivo release, if any, of the contents within the platelet α-granules such as PF4, fibronectin, βTG, and PDGF, by ensuring inhibition of in vitro platelet aggregation and release. The exact composition of CTAD mixture is as follows: 0.11 M citric acid, 15 mM theophylline, 3.7 mM adenosine and 0.198 mM dipyridamole, with pH adjusted to 5.0. Nine volumes of blood are mixed with 1 volume of the CTAD additive anticoagulant mixture. The efficacy of CTAD additive in minimizing in vitro platelet aggregation and release of contents of α-granules was demonstrated by measurement of PF4 levels in blood collected from healthy subjects in CTAD and the conventional buffered citrate anticoagulant. While typically PF4 levels were in excess of 100 ng/mL in the conventional buffered citrate anticoagulant tube, levels measured in the CTAD tube were in the range of 12 to 14 ng/mL.

The use of CTAD as a blood collection additive permits reliable monitoring of heparin therapy by either the chromogenic substrate assay or the APTT.

Preanalytical error in assessment of fibrinolysis

Laboratory tests for the assessment of fibrinolysis are affected if the in vitro activity of the fibrinolytic enzyme such as plasmin is not inhibited. Until the advent of specific tests for D-Dimer, assessment of fibrinolysis in the laboratory were generally done by the measurement of fibrin degradation products (FDP):X, Y, D and E that are formed as a result of the action of the enzyme plasmin on fibrin and fibrinogen. To measure FDP, one must inhibit the in vitro action of plasmin by including in the blood collection tube an inhibitor of plasmin. Either aprotinin or soybean trypsin can be used as inhibitor of plasmin.

One must also effect a complete conversion of any residual fibrinogen that is present to fibrin by the addition of thrombin to the blood collection tube. Otherwise, one might get a spurious increase in the FDP. However, in patients receiving heparin, the conversion of residual fibrinogen even in presence of thrombin is slow, giving rise to spurious increases in FDP value due to the remaining unconverted fibrinogen. This can be overcome by the addition of snake venom, also known as Reptilase which can rapidly convert any residual fibrinogen to fibrin. Thus on patients receiving heparin the ideal blood collection additive mixture for the measurement of FDP is reptilase, aprotinin or soybean trypsin and thrombin.

Assessment of the efficacy of recombinant tissue plasminogen activator (rt-PA) therapy by measurement of FDP requires inhibition of in vitro activation of plasminogen to plasmin by rt-PA. Although aprotinin inhibits plasminogen activation by urokinase it is ineffective against inhibiting the rt-PA activation of plasminogen.

Specific synthetic peptides of arginine chloromethyl ketone have found application in the activation of trypsin-like enzymes. Of these D-phenylalanine proline-arginine-chloromethyl ketone (PPACK) has been proven to be effective in inhibiting in vitro rt-PA activity. PPACK irreversibly inactivates rt-PA by alkylating the active center amino acid histidine. PPACK is also a potent thrombin inhibitor. Five mM PPACK added to either 10 mM citrate or 4.2 mM EDTA was effective in inhibiting rt-PA in blood collected in these additive mixtures. Unlike aprotinin which inhibits kallikrein, coagulation factors in the preliminary phase of blood clotting and plasmin, PPACK apparently does not interfere with the potential active site of the proenzyme plasminogen as demonstrated by lack of interference in the measurement of plasminogen levels in anticoagulated blood containing PPACK.

Aprotinin in addition to interfering in the assay of plasminogen will also interfere with the α2-antiplasmin assay since both these assays are dependent on the activity of plasmin which is inhibited by aprotinin. In contrast, the concentration of PPACK used (5 mM) in blood collection may be too low to inhibit the plasmin
which is added to the \( \alpha_2 \)-antiplasmin assay.\(^{15} \)

Thus a blood sample collected with the appropriate anticoagulant and 5 mM PPACK can be used for the accurate measurement of plasminogen, \( \alpha_2 \)-antiplasmin, fibrinogen, F.D.P. and immunoreactive rt-PA.\(^{15} \)

**Considerations in the measurement of fibrinolytic activators and inhibitors**

Some physiological variables need to be noted. For instance, both t-PA and plasminogen activator inhibitor (PAI-1) levels in plasma are subject to diurnal variation in a 12-hour period.

Furthermore, even in samples taken during the same time of the day the coefficient of variation (C.V.) in measured PAI-levels range from 8 to 143%\(^{15} \). To account for diurnal variation one needs to collect blood samples spaced over several time intervals during a 24-hour period.

Consumption of alcohol induces PAI-level in plasma. The half-life of t-PA is 360 seconds; however, in presence of trauma or inflammation, when the PAI-1 level is expected to be elevated 10-fold, the half-life of t-PA is reduced to 36 seconds. There is also variability in the sensitivity of assays for the measurement of t-PA.

While previously insensitive assays reported a normal plasma t-PA level of 0.05 I.U./mL, current sensitive assays have reported levels as high as 1 I.U./mL.

Since smoking causes an acute increase in t-PA levels, it is advisable to refrain from smoking for at least an hour before collection of blood.\(^{16} \)

Measurement of free t-PA in plasma presents challenges, since it is important to prevent t-PA from complexing to PAI-1 released from platelets after blood collection. To dissociate any pre-formed t-PA-PAI-1 complex, the anticoagulant pH has to be nearly 3.0.

Furthermore, even when blood is collected with such an anticoagulant the blood pH will rise due to the powerful buffering action of hemoglobin. So even after collecting blood in such an anticoagulant, the pH of the plasma has to be adjusted to pH 3.0 to dissociate the t-PA-PAI complex.\(^{17} \)

**Concluding remarks and perspectives for the future**

The scope of the coagulation laboratory is changing. Newer tests are being introduced. It is imperative that the laboratory understand the preanalytical variables associated with new tests.

The case in point is the recent interest in the measurement of prothrombin fragment 1+2 (F1+2), which is an index of prothrombin activation by the prothrombinase complex made up of factors \( X_a, V_a \), calcium ions and platelet phospholipid.

Anticoagulants such as citrate, oxalate or EDTA are unable to inhibit the *in vitro* formation of F1+2 after collection of blood. Only heparin is able to inhibit the *in vitro* increase in F1+2 level by inhibiting factor \( X_a \). Hence blood intended for F1+2 measurement should be collected in heparin. If analysis cannot be performed promptly, heparinized plasma should be frozen at \(-20^\circ\text{C}\) within 4 hours of collection.\(^{18} \) Since the cut-off level of F1+2 to distinguish between prethrombotic state and healthy subjects is 2.7 mM,\(^* \) it is clear that the usefulness of this assay will be invalidated by preanalytical error associated with *in vitro* F1+2 generation.

In conclusion, the recognition of the impact of preanalytical error on coagulation measurements is paramount to insuring the quality of results reported by the coagulation laboratory.

\(^*\)Note. The author refers to the cut-off value observed with one commercial kit (Organon Teknika); it has been pointed out that heparin is not the generally recommended anticoagulant for F1+2 measurement.

**References**

The prothrombin time (PT) is still the most important coagulation test, especially for the monitoring of oral anticoagulant treatment. The lab to lab variability of the PT results could be reduced significantly after the introduction of reference thromboplastins, a calibration procedure of reagents and the introduction of a new reporting system for the PT in long-term oral anticoagulated patients. With the availability of recombinant tissue factor for the production of PT reagents different groups have reported the development of a new class of thromboplastins which are potentially well suited to replace the less characterized tissue thromboplastins. The following article will summarize the present state of the clinical experience of a thromboplastin which is manufactured from synthetic phospholipids and recombinant human tissue factor (Innovin, Baxter Diagnostics, ref. #2 and #3). About one and a half year after the introduction of this product numerous articles and abstracts have described the properties of this reagent versus various traditional thromboplastins in several clinical conditions. The main focus will be, however, the application of this standardized reagent for the monitoring of long term oral anticoagulation.

Materials and Methods

Innovin is a reagent based on recombinant human tissue factor expressed in E. coli and relipidated with synthetic phospholipids. All assays were calibrated using CoagCal N or locally prepared fresh normal plasma pools of >20 donors. Factor assays were performed with reagents from Baxter. Other commercial thromboplastins are indicated in the test. PT and other assays were run on optical coagulometers (Electra 1000 C, MLA, distributed by Baxter, CA 5000, Sysmex, or ACL from International Laboratory, Milan). The diluted prothrombin time for the detection of antiphospholipid antibodies (lupus anticoagulants) was performed as described.

Results

Influence of phospholipids
Relipidation studies with various synthetic phospholipids from the class of phosphatidyl serine (PS) and phosphatidyl choline (PC) were performed in which the ratio of PS/PC was kept constant but the phospholipid fatty acids were changed. Based on these results it was evident that the type of fatty acid in the phospholipid molecule was of major importance (Table 1). These lipids may be variable in purified natural phospholipids, e.g. depending on the dietary situation of the animal from which the phospholipid is extracted. Therefore, it was decided that well defined standardized synthetic lipids are preferable over mixtures from natural sources. Since the type of fatty acid side chains determines also the product’s sensitivity, e.g. in order to detect coumarin induced reductions of the activity of vitamin K-dependent coagulation factors, we expected a more narrow lot to lot consistency by the introduction of these >99% pure (HPLC) standardized synthetic lipids and thus an improved quality for coumarin therapy monitoring.

Lot to lot consistency
The precision of the ISI value of the first Innovin lots (TFS 11-29) (which is determined on various instrument types in an independent international reference institution) is summa-
The precision of the ISI value for Innovin (kit TFS 11-29) is summarized in Table 2a). The cv is in the same range which is described for the precision of an ISI determination. Therefore, indeed the various lots give very similar results. This is evident from the investigation of various controls and patient plasmas using 12 unselected lots of Innovin (Table 2b). The range of the different lots is very narrow. The conversion of the results into INR values, which can be performed validly for the patient plasmas on coumarin treatment only, shows cv values which are in the order of magnitude of the precision of the assay itself. The conversion of clotting times into the INR reduces the cv which is in agreement with published data. An analysis of 60 samples with three subsequent lots of Innovin showed more or less identical results between the lots which proves that indeed the standardized production leads to a very homogenous product (Figure 1).

### Determination of the ISI value

Innovin is standardized in an external reference institution against BCT. The International Sensitivity Index is provided for mechanical instruments, Electra and ACL coagulometers. The cv of the ISI is low (see above) which proves the theoretical assumption that the standardized production using well defined synthetic materials leads indeed to a very homogenous product with excellent batch to batch consistency.

### Table 1

The influence of using different types of phosphatidyl serine or phosphatidyl choline (variable fatty acid side chains) for the relipidation of recombinant tissue factor and the preparation of a PT reagent.

<table>
<thead>
<tr>
<th>Fatty acids in PS</th>
<th>Fatty acids in PC</th>
<th>Coagulation time</th>
<th>Coumarin plasma</th>
<th>Ratio Coumarin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimyristoyl (14:0)</td>
<td>Dilauryl (22:0)</td>
<td>19.1</td>
<td>44.6</td>
<td>2.34</td>
</tr>
<tr>
<td>Dimyristoyl</td>
<td>Dimyristoyl</td>
<td>44.1</td>
<td>100.0</td>
<td>2.27</td>
</tr>
<tr>
<td>Dimyristoyl</td>
<td>Dipalmitoyl (16:0)</td>
<td>93.2</td>
<td>200.0</td>
<td>2.15</td>
</tr>
<tr>
<td>Dimyristoyl</td>
<td>Dipalmitoleoyl (16:1)</td>
<td>14.1</td>
<td>29.7</td>
<td>2.11</td>
</tr>
<tr>
<td>Di oleoyl</td>
<td>Palmitoyl oleoyl</td>
<td>11.0</td>
<td>21.8</td>
<td>1.98</td>
</tr>
<tr>
<td>Di oleoyl</td>
<td>Palmityl oleoyl</td>
<td>9.5</td>
<td>20.0</td>
<td>2.11</td>
</tr>
<tr>
<td>Di oleoyl</td>
<td>Palmitoyl oleoyl</td>
<td>10.6</td>
<td>21.8</td>
<td>2.06</td>
</tr>
<tr>
<td>PS from bovine brain (variable fatty acids)</td>
<td>PC from egg yolk (variable fatty acids)</td>
<td>10.5</td>
<td>19.4</td>
<td>1.85</td>
</tr>
</tbody>
</table>

Citrol 1 is a commercially available normal control plasma (Baxter). The coumarin plasma was a pool from patients under long term oral anticoagulation. A constant amount of tissue factor was used in all experiments.

### Table 2

a) The precision of the ISI value for Innovin (kit TFS 11-29).

<table>
<thead>
<tr>
<th></th>
<th>ISI mean</th>
<th>SD</th>
<th>CV [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanical instrument</td>
<td>0.90</td>
<td>0.02</td>
<td>2.6</td>
</tr>
<tr>
<td>Electra</td>
<td>0.98</td>
<td>0.03</td>
<td>3.5</td>
</tr>
<tr>
<td>ACL</td>
<td>0.88</td>
<td>0.03</td>
<td>3.2</td>
</tr>
</tbody>
</table>

b) The precision of various controls or patient plasmas when tested with 12 unselected lots of Innovin on three different instruments.

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Citrol 1</th>
<th>Citrol 3</th>
<th>FNP</th>
<th>Liv.</th>
<th>Cou 1</th>
<th>Cou 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electra Mean</td>
<td>12.0</td>
<td>49.4</td>
<td>10.5</td>
<td>13.9</td>
<td>34.5</td>
<td>23.3</td>
</tr>
<tr>
<td>S. D.</td>
<td>0.22</td>
<td>3.08</td>
<td>0.16</td>
<td>0.36</td>
<td>2.08</td>
<td>0.12</td>
</tr>
<tr>
<td>C. V. (%)</td>
<td>1.80</td>
<td>6.25</td>
<td>1.55</td>
<td>2.61</td>
<td>6.02</td>
<td>3.71</td>
</tr>
<tr>
<td>KC 10 Mean</td>
<td>10.9</td>
<td>47.6</td>
<td>9.40</td>
<td>12.7</td>
<td>35.4</td>
<td>22.7</td>
</tr>
<tr>
<td>S. D.</td>
<td>0.41</td>
<td>3.07</td>
<td>0.25</td>
<td>0.35</td>
<td>2.93</td>
<td>0.13</td>
</tr>
<tr>
<td>C. V. (%)</td>
<td>3.75</td>
<td>6.46</td>
<td>2.63</td>
<td>2.77</td>
<td>8.28</td>
<td>4.07</td>
</tr>
<tr>
<td>ACL Mean</td>
<td>10.0</td>
<td>39.7</td>
<td>8.80</td>
<td>14.5</td>
<td>32.1</td>
<td>22.8</td>
</tr>
<tr>
<td>S. D.</td>
<td>0.29</td>
<td>2.02</td>
<td>0.19</td>
<td>0.52</td>
<td>1.80</td>
<td>0.13</td>
</tr>
<tr>
<td>C. V. (%)</td>
<td>2.89</td>
<td>5.09</td>
<td>2.13</td>
<td>3.59</td>
<td>5.60</td>
<td>4.32</td>
</tr>
</tbody>
</table>

FNP = Fresh normal plasma pool, Liv = pool from patients with liver disease, Cou 1 and 2 = pools from patients with oral anticoagulation.
Comparable to conventional thromboplastins, however, there is a clear tendency towards systematic instrument specific differences in ISI values. This can account to more than 10% and thus is of a clinical importance when samples with relatively strong anticoagulation are tested. Since it will be impossible to determine ISI values for all different and sometimes rarely used instruments we were looking for a simple procedure how to generate ISI values directly in the customer’s laboratory.

Innovin was used on an instrument for which an ISI value was available and on the different instrument for which the ISI should be determined. The procedure is outlined in Figure 1. It follows the procedure for ISI determination as described by Kirkwood but adding a subsequent step. This subsequent step is the use of Innovin on a coagulometer with known ISI value (as determined previously against BCT) as a reference and to calibrate it on the instrument of choice by analyzing normals and patients on oral anticoagulation with the two types of coagulometers. The slope of the regression line of the log transformed clotting times was then multiplied by the ISI value for the reference method (i.e. Innovin on a coagulometer with known ISI value).

The validity of this protocol was analyzed in a laboratory in Bremen by Gurr. He tested first plasmas from normals and patients on stable anticoagulation on a mechanical instrument.
using BCT or Innovin respectively in order to investigate if the ISI value from the box insert was applicable under the conditions of his laboratory. Using this approach an ISI value of 0.86 was found which agreed very well with the indicated ISI of 0.88 of the respective lot of Innovin. Based upon this result patient samples were tested on a CA 5000 instrument (TOA, distributed by Digitana, Hamburg) and the mechanical instrument. Using the ISI value of Innovin for mechanical instruments the ISI for the CA 5000 could be calculated. The ISI for this instrument was 0.8. This result confirmed previous studies in which a relatively low ISI had been described for the CA 5000 and placenta thromboplastin by the same group before. In a different study these results could be confirmed by others. In a very similar approach Seyfert could demonstrate the validity of this approach also for a capillary blood adaptation of Innovin which we had developed. In this application the ISI for the capillary blood procedure, which works at slightly different plasma/reagent/anticoagulant ratios the ISI was determined by analyzing capillary blood and plasma samples from the same patients with Innovin and calculating the ISI from the regression line as described above.

Using this approach or a different one in which the Hepatoquick (Boehringer) was used as a reference a good agreement was found between the Innovin capillary blood and plasma methods and of both to the Hepatoquick respectively in INR.

**Factor sensitivity**

Due to the standardized manufacturing process any contamination with coagulation factors can be excluded in recombinant thromboplastins. This is a clear difference compared to the classical tissue thromboplastins which may contain residual coagulation factors which have escaped the washing steps of the organ material, which is rich in blood and thus in
coagulation proteins.

The *in vitro* factor sensitivity of Innovin was tested in dilution experiments in which normal plasma and factor deficient plasmas where used. The *in vitro* results showed a higher sensitivity at lower levels in comparison to rabbit brain or placental thromboplastins of especially factor VII, but also of very low levels of factor II and X. Our own results were confirmed by others. In patients with congenital deficiencies, however, most of the cases showed very similar results when Innovin was compared either with placental or rabbit brain reagents, with the exception of some cases with very low levels (Table 3). In some samples, most likely of the factor VII Padova type rabbit brain reagent was more responsive as it has been described before already for this particular mutation.

In patients with liver disease Innovin behaved very similar to conventional reagents. Since PT seconds, prothrombin ratio (PR) or percentages are hardly comparable in these patients we determined the sensitivity (number of correctly detected abnormals) and found very similar sensitivities of Innovin and tissue thromboplastins (Figure 3).

This is clearly different from some of the results in patients on coumadin treatment, in which the factor levels are often much lower than in liver disease and where the vitamin K dependent factors are all at relatively low levels at the same time.

**Patients on oral anticoagulant treatment**

Innovin was tested in various clinical trials, mainly in Europe. It was compared against BCT or various commercially available reagents on various instrument types. In general we saw a good and linear correlation for most of the samples with all reagents. Especially when tested against BCT the correlation was linear and narrow (Figure 4). In some cases, however, we saw a tendency that above 3.5 INR Innovin gave slightly higher INR's in some oral anticoagulated patients. This was mainly seen in some studies in which Innovin was tested against placental thromboplastin or rabbit brain thromboplastins with ISI values >1.3.

It was evident from factor assays in those patients that the higher INR with Innovin was found mainly in plasmas with either generally low levels of the vitamin K-dependent factors,
in some cases also in cases with isolated very low (<10%) levels of factor VII or factor X respectively. Some of the results have been published.\textsuperscript{17}

In a systematic attempt to investigate these findings in more detail we took 50 samples from patients with relatively strong long term oral anticoagulation and studied then with 6 thromboplastins. In this study we could not find major significant deviations between the 6 reagents which were used (Figure 5) in the entire collective of samples but results of individual patients showed some differences. Only one thromboplastin which was very similar to Innovin and BCT/253 when results are reported in percentages showed always higher INR values than the other thromboplastins. We interpreted these results for this reagent as a erroneous ISI value that was indicated for the particular lot that was used. Using the statistical procedure according to Passing and Bablok\textsuperscript{18} only this thromboplastin showed a significant deviation of the regression line of the INR values against BCT/253. All other reagents had no statistically different INR values compared to BCT/253.

These data are not consistent to some of the results that had been previously described. The reason for this finding is difficult to understand because the same thromboplastins were used in our study and in others. Since the distribution of patients and normals was not identical in all of these trials this may contribute to some of the results that were seen before. In addition, we speculate that perhaps also lot specific differences may contribute for the tissue thromboplastins but not for Innovin which is manufactured from standardized raw materials.

Discussion

The use of a standardized thromboplastin prepared from synthetic raw materials offers a variety of theoretical advantages. Batch to batch consistency, the lack of ethical problems in getting access to human or animal tissues, the extremely low risk of infectious disease transmission in the manufacturing and use of this reagent and, of course, the purity of the new thromboplastins show a theoretical benefit over the tissue thromboplastins. It should not be ignored, however, that also these reagents have reached a high technological standard.

Therefore, a comparison is not necessarily a comparison between bad and good as long as the recombinant reagents are compared against the tissue thromboplastins with ISI values <1.5.

Clinical data in batch to batch consistency and, of course, handling and precision data of the new reagent show some advantages. In addition some clinical results may show a potential benefit of the new reagents. Some patients with oral anticoagulation are found in the therapeutic range when using tissue thromboplastins but have sometimes relatively low factor activities. Using a recombinant reagent those samples are identified with higher INR values. Similar data have been reported.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Comparison of 6 different sensitive thromboplastins in 50 patients (BCT n = 26) patient on long term anticoagulation.}
\end{figure}

\textit{(TIS = Thromboplastin IS, Baxter, T = Thromborel S, Behring, M = Manchester Comparative Thromboplastin, B = BCT/253, R = RecombiPlasTim, Ortho Diagnostic Systems, I = Innovin)}

The study was performed on a mechanical instrument versus ISI values provided by the manufacturer. The INR was calculated using a fresh normal plasma pool for the determination of the normal clotting time.
previously, however, when tissue thromboplastins of different sensitivities have been compared.\textsuperscript{19} Rosenberg group\textsuperscript{20} have observed a similar trend in patients with low dose anticoagulation in which only a sensitive reagent could detect the coumarin effect whereas a low sensitivity reagent with high ISI value missed many of those patients in which the F1+2 assay clearly demonstrated the reduced coagulation activity in those patients.

It has to be demonstrated, however, if the dose adjustment of coumarin drugs may cause less bleeding complications using a standardized recombinant reagent instead of a tissue thromboplastin.

References

7. van den Besselaar AMHP, personal communication.
Thrombosis and hemostasis is becoming a very open area which concerns all the aspects of medicine. Any body dysfunction may affect blood circulation and the cardiovascular system, and has an incidence on the coagulo-lytic equilibrium. The field of investigation of this discipline has become very widespread and it is now extending outside the conventional coagulation approach. Heredity, way of life, sedentariness, existence of pathological episodes (traumatisms, surgery, infectious diseases, metabolic disorders, autoimmunity, malignancies, ...), mental stress, ageing are among the features which can influence the hemostasis equilibrium. These past decades, many progresses have been realized in the understanding of mechanisms permitting to fulfill the double role of blood: prevention of hemorrhage and keeping body from developing thrombosis. At the same time much has been known on the different pathological triggers which can induce blood activation and on the slow cardiovascular alteration progressing with age and with disease states. Blood activation is controlled for a long time by the body’s thromboresistance and then remains clinically silent. However, if this barrier is overwhelmed thrombus formation may occur. Knowledge of these functions has been extended to vessels and blood cells which participate to the hemostasis equilibrium. This connection has been elucidated with the finding of the major role of cytokines. These latter are links between immunological conflicts, inflammation states, abnormal lipid metabolism, stimulation of monocytes and blood activation. On the other side, progresses in biotechnology have allowed to develop highly sophisticated methods for specifically measuring the different parameters involved in blood coagulation or those concerned with thromboresistance and its regulation. Assay methods have also become more and more impressive and they can be processed using advanced instrumentation. In addition, automation has allowed an efficient management of routine testing.

A major contribution has been that of International Committees and standardization organisms which have introduced universally accepted standards and working procedures. This has greatly facilitated scientific exchanges and communication between laboratories thanks to a better harmonization.

Lastly, clinical studies and long term epidemiological programs are required in order to define the clinical significance of the various analysis developed, or to propose diagnostic profiles and to promote prevention of disease. An important breakthrough is also ongoing for the therapeutic approach. Pharmaceutical industry has developed new drugs which present a tightly targeted action at molecular or receptor levels and which can show a specific activity with few side effects. These evolutions, undertaken years ago, are nowadays amplified by the impact of new biotechnologies, which have many practical repercussions on the diagnostic approach, or on therapy and its monitoring. Furthermore all these technological advances stimulate and speed up fundamental and clinical research. Scope of investigation in thrombosis and hemostasis changes as a consequence. This situation requires new strategies for research and develop-

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opment. They must integrate all these different aspects inasmuch that research programs are becoming very expensive in an environment of hard competition. The ratio cost/benefits is then an important parameter as well as the time required for running a project.

The present analysis will be restricted to the diagnostic area where was acquired the here reported experience. In this field new diagnostic approaches are now available and concern: a) diagnosis of a decreased thromboresistance and of biochemical defects associated to thrombophilia; b) evaluation of markers which might be of predictive value for the existence of an underlying disease; c) identification of pathological states which are frequently associated to cardiovascular alterations and to increased thrombotic risk; d) follow-up of high risk clinical contexts; e) monitoring of prophylactic or curative therapies; f) validation of new drugs presenting anticoagulant, antithrombotic and thrombolytic activity.

This review aims to discuss some research and development strategies which can be set up in hemostasis and thrombosis and it keeps the attention on the different considerations which must be evaluated when running the projects.

Research and development goals in hemostasis and thrombosis

The main goals for research and development in hemostasis and thrombosis are: a) to diagnose or to predict situations at risk for hemorrhage or thromboembolism; b) to characterize dysfunctions of the coagulo-lytic system inducing the disease states; c) to monitor substitutive, curative or preventive therapies; they are presented on Table 1.

This approach requires therefore a very comprehensive understanding of biochemical mechanisms implicated in the control and in the regulation of hemostasis pathways, of the factors which trigger blood activation, of the markers which objectivate the existence of an underlying disease in the clinically silent phase and of the different therapeutic behaviors which can be decided for preventive or curative indications. In addition, achieving these objectives requires clinical validations and epidemiological studies which are always necessary for establishing the significance and the value of the diagnostic approach developed. This is why the first step before starting a research project is to clearly identify its overall objective and to describe the main characteristics which must be obtained. This will orient all the work performed until the proposed goal is reached.

Nowadays, diagnosis in hemostasis and thrombosis usually concerns identification of pathological states, follow-up of thrombotic diseases until normalization, and monitoring of therapies. For any research program, at the beginning of the study it is always necessary to evaluate the environment of the field concerned. This means that the working conditions and the using sites concerned with the new diagnostic approaches proposed must be anticipated very early in the research initiation. The repercussions that this development may have in terms of health care and medical orientation must also be evaluated at this time. Then all the means necessary for conducting the project must be analyzed with concern to technologies required, human or economical resources necessary, partnerships expected and difficulties to face. Lastly, before starting the work, an accurate state of the art must be established for focussing all the efforts to the right direction.

In hemostasis and thrombosis, if considerable progresses have been realized these past years many questions remain unsolved and research and development is very active in different directions. Most of the hemorrhagic diathesis have been described today and they can now be
diagnosed very safely. In addition, well standardized methods are available for evaluating the anticoagulant activity of drugs used for treating or preventing thrombotic events. Many advances have also been performed for standardization of assay methods and well accepted reference materials are available for calibrating these ones; furthermore they are validated by comparison to international standards. Much has also been understood on body’s thrombore-sistance and cardiovascular alterations which favor the risk of thrombosis, or on the epidemiological factors which can progressively induce blood activation. However, many aspects remain unexplained and much must still be explored and understood with concern to thromboem-bolic diseases, their diagnosis, their prediction, their recurrence and their management.

Today only 25 to 30% of familial thrombotic diseases are characterized and are explained by decreased body’s defenses involving an anticoagulant or a fibrinolytic factor. In addition, if some congenital diseases which induce blood activation have also been identified, many phenomenons triggering this pathological state, or dysfunctions resulting from disturbed blood and vessel cell interactions or from other complementar-y factors associated to thrombosis, remain unexplained. Moreover it is now well established that blood activation is usually initiated a long time before the appearance of the first clinical symptoms preceding thromboem-bolic diseases, but it remains silent thanks to the existence of the efficient body’s thromboresis-tance. Nevertheless, few diagnostic possibilities are available at this early stage of disease evolution. So, this is still a constant need for developing and validating new tools which can diagnose individuals with advanced cardiovascular alterations and then predict those at risk for thrombotic pathologies. Preventive therapy and an adequate medical care could then be envisaged for keeping from this disease or at least for delaying its occurrence.

Another main feature in this field is the very active development of new drugs by industry. Those latter present a precisely targeted function and allow to develop new therapeutic strategies. All these evolutions have been allowed by the better knowledge of hemostasis mechanisms and their dysfunctions, and by the breakthrough of biotechnologies concerning peptide and protein engineering, the new genetic approaches, the molecular biology advances and the progresses in immunological and biochemical technologies. But first of all, they are the result of a close association and of an efficient partnership between fundamental and academic research, clinical investigation, and industry. Routine clinical practice takes advantage of these modern and automated diagnostic trends. In addition, establishing activity and benefit of the new drugs developed, proposing therapeutic strategies accurately designed for pathological states and validating these approaches require also sophisticated diagnostic tools for exploring all the repercussions on body, during the preclinical phase studies. Development of specific techniques for clinical validation of these new drugs becomes nowadays an important goal which requires a specific strategy. Lastly academic research needs also sophisticated and innovative diagnostic techniques for investigating all the regulations of hemostasis and its interactions with the other body functions (and vice versa), either globally or in blood, at the cellular level or at the molecular one. Studies on cell to cell interactions, connections between the major body’s functions and factors modulating them, molecular modifications which progressively can induce chronic and evolutive pathological states require advanced research approaches. Therefore, these research objectives in hemostasis and thrombo-sis associate very sophisticated and specific investigation tools with a global understanding of the various function relationships as well as with the knowledge of their clinical and epidemiological consequences.

Establishing the state of the art and preparing the strategy

Once the objective defined and before deciding a strategy for a research and development project in hemostasis and thrombosis, the scientific field concerned must be comprehensively appreciated and the state of the art must be well
established. This approach allows to correctly situate the project and to start it properly.

Different means are available for this step. Obviously, literature brings useful information which must be synthesized respectively to the program envisaged. However, scientific relationships and participation to congresses complete the previous information, put the ideas in a concrete format and help to establish partnerships or to understand all the problems that must be faced. Then the next consideration must concern evaluation of the competencies and of human resources required for performing the work, and it is followed by the initiation of the necessary partnerships with academic or hospital research groups, or with companies which can offer a complementary expertise.

At this step, the project becomes more structured and the major development stages must be established. A synthetic report will be required when each one of these stages is completed. A review of the work done and a global presentation of the results obtained are expected to be presented at this time. Actually these stages are important review points for following a project and they are usually associated to decisions concerning its continuation, the costs involved or the means necessary. Lastly, before the project starts, all the active raw materials which will be required must be specified as their performances will closely govern the characteristics of the diagnostic techniques designed. All the methods for analyzing specifications of these raw materials must also be set up at the beginning of the work. All these materials are the basis for developing the adequate assays and for establishing investigation methods, which can be adapted to instruments or, when required, automated.

In the following step, these methods must be optimized, standardized and validated in clinical studies performed on normal population and on selected pathological groups. Sometimes predictive value can only be established through running long time and large scale epidemiological studies. But not only is it necessary to develop new diagnostic approaches and to propose many sophisticated analyzes and technologies, it is also needed to include these aspects in a philosophical and prospective analysis. These various points are summarized on Table 2. This means that all projects must be run within a frame defining a long term strategy which allows to orient and select the research and development programs in a comprehensive synthesis. This analysis allows to keep projects coherent within a global objective. But, this is important to remind at any time that in hemostasis and thrombosis running a project requires a close relationship and an efficient collaboration between academic research, clinical investigators, practices, epidemiologists and technology developers.

Table 2. Different aspects of R.D. in hemostasis and thrombosis.

<table>
<thead>
<tr>
<th>Aspect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fundamental: understanding the mechanisms and identification of the factors involved</td>
</tr>
<tr>
<td>Biochemical: protein chemistry and purification, development of analytical tools</td>
</tr>
<tr>
<td>Technical: design of adequate assays and of investigation methods; automation; instrumentation</td>
</tr>
<tr>
<td>Clinical: studies on normal population and on selected pathologies</td>
</tr>
<tr>
<td>Epidemiological: predictive value and prognosis</td>
</tr>
<tr>
<td>Philosophical and prospective: comprehensive synthesis</td>
</tr>
</tbody>
</table>
Consequences on the present diagnostic needs in hemostasis and thrombosis

The present understanding of hemostasis and thrombosis offers new diagnostic possibilities and creates new needs for the research axes. Blood irrigates all parts of the body bringing them what is necessary for maintaining cells and organs alive. It is also the pathway eliminating metabolism waste and one of the connections between organs. Everything which occurs in the body has repercussions on blood and vice versa. This is why hemostasis which maintains blood fluid and prevents from hemorrhage presents so many links with all the body functions and then reflects the various abnormalities occurring. Most of the diseases or metabolic disorders affecting body have incidences on the coagulo-lytic equilibrium, and these ones can frequently induce localized or disseminated blood activation, which remains asymptomatic for a long time thanks to the body's thromboresistance. When this one is overwhelmed, thrombin becomes biologically active, fibrin is formed and accumulates at the site of its generation producing vessel obstruction and the sudden occurrence of a thrombotic event. This event can occur in presence of a minor trigger if body's defenses against thrombosis are decreased, which is observed in presence of a deficiency of an antithrombotic (ATIII, protein C, protein S, ...) or of a fibrinolytic factor, or when there is an excess of a factor reducing thromboresistance (for example histidine-rich glycoprotein). This situation is associated with some of the familial thrombophilias and this thrombotic pathology usually concerns young individuals. Of a more general concern is the second clinical situation resulting from an excess of blood activation which overwhelms a normal body's thromboresistance. The stimuli which trigger the initiation of blood coagulation pathways can be produced by many different causes such as stress, ageing, sedentarity, way of life (food, smoking habits, oral contraceptive users, ...), but they can more frequently be generated and strongly enhanced by pathological contexts such as atherosclerosis and lipid metabolism, diabetes and metabolic disorders, hypertension, homocystinuria, infections, inflammation, autoimmunity and drug induced allergy, malignancy, traumas, surgery, obstetrics and complications of pregnancy, etc... Figure 1 shows the action of these triggers which induce blood coagulation pathways activation. These ones are controlled and inhibited by the body's antithrombotic and fibrinolytic defenses. No pathology occurs until excessive fibrin generation obstructs a vessel. Thrombus formation is then the final stage of disease evolution. Even though chronic and compensated activation can remain silent for a long time, biochemical indicators are formed. Assays for these molecular markers are now available for exploring the early disease states.

These analyses concern the major functions of hemostasis pathways. A panel can be envisaged for profiling blood activation at different levels: endothelium and vessels, blood cells, thrombin formation pathways, thrombin generation and fibrinolysis, fibrinoformation, fibrinolysis. These molecular markers which indicate the initiation of disease are reported on Figure 1 at the level where they are produced in presence of blood activation which can lead to occurrence of thrombosis. However, the strong amplification efficiency of clotting pathways limits the predictive value of most of these indicators and this poor predictivity is still reduced by their short half lives. This is why D-dimer remains the marker which has been found the most useful, despite it becomes only positive in well established clinical contexts. Moreover, for demonstrating the diagnostic and predictive values of these markers it is necessary to perform epidemiological studies, which require much time and important means and are mainly lacking today. All these considerations identify new diagnostic needs in hemostasis and thrombosis. They concern measurement of new parameters, characterization of pathological conditions associated to thrombotic risk and development of valid laboratory technologies.

Practical aspects

This strategy has been applied for developing specialized diagnostic tools for anti-thrombotic
proteins, markers of blood activation and factors triggering onset of disease. In this last group endothelial damage, atherosclerotic lesions and drug induced- or auto-antibodies are well identified causes. The laboratory structure permitting to conduct these projects includes all the various technical steps which are required for performing a research and development program, and which are reminded on Table 4. First of all the basic laboratory techniques must be correctly established and standardized and the different technical groups with the complementary competencies must work together in a synergistic coordination. Our structure involves a biochemistry laboratory for protein purification and for obtaining the active enzymes implicated in hemostasis and fibrinolysis. This is completed by a laboratory producing monoclonal and polyclonal antibodies. These immunological reagents can be used directly or alternatively antibodies can be extracted and, following sophisticated protein chemistry, they can be transformed to the active principles which are used for designing the assays. According to the different needs expressed these antibodies can be digested to F(ab')2 or to F(ab) fragments and coated onto reactive supports such as micro ELISA plates or latex beads (magnetic, fluorescent or polystyrene), or coupled directly to a label (enzymatic, fluorescent, chemiluminescent or particular) or even directly linked to matrixes used for immuno-adsorbant columns. Aside this technology, active principles can also be prepared by cell culture and they are then obtained in the growth medium supernatant (this is the case of PAI-1 released by fibrosarco-

Figure 1. Scheme showing the way leading from hemostasis activation to the occurrence of a thrombotic event: the various molecular markers which are available for evaluating the different stages of disease evolution are indicated.
Table 4. The various steps of a R&D project.

- Research goal and diagnostic need (objectives, clinical applications, specifications)
- State of the art: what is known? What is available?
- Feasibility evaluation of the means necessary, technologies used, raw material, human resources, ...
- Analysis of difficulties to face, estimation of costs, time required, partnerships expected, ...
- Development: optimization, standardization, scale-up, ...
- Clinical validation of new drugs under development

In close collaboration with the research unit is the development group that has in charge all the optimization of procedures and the establishment of reproducible conditions, then their standardization and scale up. At this stage, definition of performance characteristics and validation in the true clinical applications must be realized. A tight relationship with clinical groups ensures efficiency and value of this step. Lastly, when the project is fully developed and validated, transfer to the manufacturing unit and establishment of standardized manufacturing methods can take place. In parallel registration files must be prepared for the various applications claimed and for the different countries. Their approvals is required before commercial launch and use of the new diagnostic tools.

However, these different steps are not isolated and a global view must drive the various stages all along the application of the decided strategy. It means that the clinical use and the final specifications must be considered before preparing the raw active material and all along during the project progresses a close relationship must link the research, the development and the clinical validation aspects. Of importance is also the instrumentation and automation problem, which is managed in a development unit where the adaptations to the analyzers and the development of automation procedures are performed.

In order to fit more closely the end user's needs, and to reach the highest synergy between reagents, and their applications to specific instrumentation, these aspects must be developed together with the biological research. Figure 2 summarizes the different laboratory structures which have been set up for conducting the research and development programs in hemostasis and thrombosis, and their mutual relationship. The documentation service brings to everybody all the information they need for fulfilling their role, respectively to scientific data, technical solutions, patent situation and expected partnerships.

Example of a research and development strategy

The strategy we are following for research and development of diagnostics in the field of
Strategies in hemostasis and thrombosis

Thrombosis and hemostasis can be illustrated by one example which is briefly summarized hereunder and which concerns the diagnosis of heparin-induced thrombocytopenia (HIT), type II, which is antibody dependent. This complication of heparin therapy occurs rarely (0.1 to 1% of patients), but it is severe and it must be diagnosed very rapidly as it is life threatening.

Within the scope of our collaboration with the clinical research groups of Bicêtre and Clamart hospitals (Prof. D. Meyer, Paris, France), we planned to develop a new diagnostic test for HIT, more sensitive and more specific than the platelet aggregometry assays and more practical than the $^{14}$C-serotonin release test. The project specifications were then clearly identified as well as the application context in clinical situations. Our strategy involved these different steps: a) state of the art and understanding of the clinical complications and the biological associations with HIT; b) preparation of a panel of 10 plasmas from patients clinically characterized for type II HIT and of 10 normal controls; c) identification of the platelet component which binds HIT antibodies in the presence of heparin; d) research of an assay method for specifically measuring HIT antibodies; e) preparation of the active materials required and development of a well-established assay method, then optimization and standardization of it; f) validation, clinical studies and technical considerations concerning biological functions and mechanisms; g) industrial transfer and scale up; h) registration, patent filing and definition of clinical applications. This last step (h) can concern the different levels of the project conduction, as for example the patent must be filed as soon as the approach is found and if it is actually new and patentable.

This strategy allowed to identify PF4 as being the platelet component which binds HIT antibodies in the presence of heparin and this finding allowed to develop a specific ELISA for measuring these antibodies binding PF4-heparin complexes. A patent was filed for protecting the industrial rights for this assay and the development period started. For cost considerations, PF4 extracted from platelets was not usable and a source of recombinant PF4 was preferred and validated for this application. The other main
The reagent required was anti-IgGAM antibodies coupled to peroxidase. This immunoconjugate was prepared in order to offer a homogeneous reactivity to IgG, IgA and IgM whatever the isotype. Separate conjugates for isotyping were also prepared. When all the reagents were available and when the assay method was developed, the clinical studies were performed and they showed the potential predictive value for this new assay. This was particularly outpointed by one patient studied retrospectively, who was found positive in this assay 3-5 days before the dramatic drop of platelet count (<10⁹/L).

Clinical studies also demonstrated that three isotypes may be present, IgG, IgA and IgM and that in patients under heparin therapy for more than 7 days but without clinical or biological signs of HIT, antibodies to PF4 heparin were already present in 10-15% of cases. These latter must be considered at risk for developing a clinical HIT if heparin is continued. Nevertheless, among the patients studied in this group (n=80) only IgM and IgA were identified and the IgG isotype was never found. This work, when associated to all the former literature on HIT, allowed to propose a new mechanism for explaining the development of type II HIT under heparin therapy. The target antigen for HIT antibodies is a macromolecular complex of heparin and PF4 formed at a ratio of 25 IU of heparin per mg of PF4. These complexes are fixed by the platelet PF4 receptors which are expressed on platelets following a slight activation by thrombin, and they bind HIT antibodies. These latter when they are of IgG isotype, bind to platelet Fc-RII receptors, enhancing platelet activation, their aggregation and occurrence of thrombosis. Our present work suggests that the platelet PF4 receptors play a key role as patients presenting only with IgM and (or) IgA antibody isotypes developed thrombocytopenia and thrombotic complications. Industrial methodology and scale up lead to establish reproducible and standardized working conditions for manufacturing this assay which can now be newly introduced for diagnosis of HIT.

This diagnosis is usually performed in situations of emergency when HIT is suspected. Micro ELISA techniques poorly fit this application and one a time assays would be better adapted. This is why the next step is to develop a technology allowing a single and rapid testing. The final considerations are the registrations preceding the market approval; they must be filed in each country before launching the diagnostic method and they constitute the reference basis for the application context. In summary, bringing this project to achievement involved the different laboratory structures and a very close relationship with the clinical groups, what was an absolute necessity for succeeding in this research.

**General considerations and conclusions**

The very complex medical and biological environment in hemostasis and thrombosis, and their associated repercussions, the very important challenge that thrombotic diseases represent for public health and the very dynamic activity of drug industry in this promising field, have created new conditions which must be considered when a research and development strategy is set up.

In this report different aspects have been analyzed and they are illustrated by the description of the laboratory structure involved in conducting the programmed projects and by the presentation of a practical example. Obviously, the general considerations governing biotechnology research must continuously be taken into account for designing a strategy and for running the project. Among them are the bioethical considerations and the guidelines for animal experimentation or for using products of human origin. Procedures must be well established and laboratory staff correctly informed and continuously aware on the application of the various rules and on the respect of the safety recommendations.

The recent issue of HIV infectiosity has brought many constraints in the use of products of human origin, which, in our field, concern mainly blood or platelet concentrates. Accurate identification of material origin and full testing for HIV antibodies, HBs antigen and HVC antibodies is required as well as a close follow up of the different steps of the material treatment.
Moreover, restrictions in using animals limit in some countries conventional technologies for producing monoclonal antibodies. These techniques must be substituted for in vitro production methods which are now well established, efficient and cost effective. Another major issue is the financial support required for accomplishing the research and development strategy. Project costs are becoming more and more expensive as it has been illustrated previously. Companies have to restrict and to focus their programs or they have to share the costs by putting together their efforts in initiating projects presenting a common interest in complementary fields. This partnership is now encouraged in Europe by Eureka authorities which support joint research projects involving companies from different countries. This helps small and medium size companies to initiate more ambitious and more innovative research and development studies than their own means would have allowed.

In hemostasis and thrombosis this approach exceeds very widely national programs and many projects now implicate laboratories worldwide. International collaboration has become the usual rule. This is also illustrated by the important need for harmonization of procedures and for reference materials: in this field a very active and hard work is being performed by international standardization committees in order to achieve this goal and to propose consensus panels. An important characteristic is also the more and more heavy registration that must be applied for each diagnostic technique proposed. According to countries, different rules govern these registrations. There are 3 main specific situations in North America, in Europe and in Japan. There is now a tendency to harmonize regulations towards a common basis, and this will be greatly enhanced by the ISO 9001 certification which recommends working conditions and procedures universally accepted.

In conclusion, research and development strategy in hemostasis and thrombosis requires a long term overview for defining broad axes supported by a close relationship with the scientific community. Bringing competencies together, planning each step and establishing international partnerships are new important features which condition success. Within this large frame specific projects are managed in compliance with the overall objective.

Lastly, clinical validation is necessary before industrial scale up and release. Academic and industrial researches, which have very separate histories and different cultures are meeting together, for more synergy and efficiency, in launching strategies for projects which have positive repercussions for the various partners.

References

Venous thromboembolism is a major health problem in the western world. In spite of many studies the pathogenesis of this condition is still not completely defined. A brief survey of the physiology of venous circulation suggests that venous blood flow is more complex than that of the arterial circulation. At any given time, the greatest part of the circulating blood volume is contained in the low pressure venous circulation. Even in light of new evidence that endothelium has an active secretory function, i.e., prostacyclin, endothelium-derived relaxation factor and endothelin, a potent vasoconstrictor, etc., we still view the venous system as a passive, non-specific capacitance chamber. There are three major types of veins, namely the cutaneous, the splanchnic, and the skeletal muscular veins. The veins within the skeletal muscles have little or no sympathetic innervation, whereas the cutaneous and splanchnic veins are rich in this regard. This deficiency in the innervation of skeletal muscle veins explains why the emptying of leg veins in human relies heavily on the muscle pump. This pump effect results from leg muscle activity and maintains the upward flow of blood along the deep venous system. A series of deep venous system valves prevent distal blood reflux. During the relaxation phase of muscle activity, the valve cusps are in direct apposition. The closure of these valves subdivides the venous column into short segments and reduces the pressure exerted on the adjacent cutaneous veins by breaking up the overall hydrostatic venous pressure that normally would be higher in a straight, uninterrupted blood column. Also, a series of perforator valves prevents blood reflux from the deep to the superficial venous system. Flow through the venous system is phasic and is also largely influenced by diaphragmatic movements during the respiratory cycle.

Flow is decreased during inspiration owing to the increase in intra-abdominal pressure. Conversely, it is increased during expiration owing to the decrease in intra-abdominal pressure. During a Valsalva maneuver, flow stops completely, but the higher arterial pressure prevents reverse flow. Patterns of flow are also altered under normal physiologic conditions. Blood flow through the deep and superficial veins of the leg is augmented during exercise, and the direction of flow in the legs is from superficial to deep veins (the opposite is true in the feet). Venous pressure is also affected by several physiologic conditions (for example positional changes, from lying to sitting). Compliance (i.e., elasticity) of the vessel wall plays also an important role. Venous compliance is reduced in many of the conditions that predispose to DVT, such as low output states (e.g., congestive heart failure, dysautonomic conditions e.g., diabetes mellitus), stroke (via interference with the muscle pump), and aging. The main features playing a role in the pathogenesis of venous thromboembolism can be summarized as follows: 1) alteration in the clotting system; 2) reduction in blood flow and 3) alterations of the vessel walls.

Changes in blood coagulation

The changes in the plasmatic and platelet components of blood coagulation appear as the most important events in the pathogenesis of thrombosis. As the matter of fact, in recent decades, the description of several conditions, both congenital and acquired, strictly associated with thrombosis have added additional impact to this contention. It seems that changes
in blood coagulation are always present in venous thrombosis, be they primary or secondary to decreased blood flow and/or to vessel damage.

The discussion of the changes in blood coagulation is synonymous to deal with prothrombotic or hypercoagulable state.\textsuperscript{6,19,21,37,39,55}

The thrombophilic states could be divided in:

a. congenital hypercoagulable state;
b. acquired hypercoagulable state;
c. mixed congenital and acquired hypercoagulable state.

### Congenital hypercoagulable states

Congenital hypercoagulable states are represented mainly by the deficiencies of clotting inhibitors such as ATIII, protein C and protein S. They could also be divided in conditions characterized by the lack and/or malfunction of a clotting inhibitor and those due to an increased level of protein (example PAI increase). The term familial thrombophilia indicates a clinical condition characterized by an increased tendency to recurrent venous thrombosis developing at young age in one or more members of a family which cannot satisfactorily be explained by known risk factors. The main congenital conditions predisposing to thrombosis are gathered in Table 1.

The most important of these congenital conditions are those represented by defects of ATIII, protein C and protein S. Altogether they may be found in about 50% of the patients with a history of juvenile thrombosis associated with a positive family history. It is interesting to note that only about 50% of these patients are asymptomatic (Table 2). The reason for such behavior is still unknown but triggering events may play an important role.

The incidence and significance of the resistance to activated protein C is still being evaluated even though it seems to be quite widespread.\textsuperscript{10} The other conditions are less frequent and for some of them, heparin co-factor II deficiency, for example, a sure correlation with thrombosis is not yet firmly established.

#### Hereditary antithrombin III defects

ATIII is a plasma glycoprotein (58,000D) synthesized in the liver and endothelial cells. It inhibits mainly thrombin and factor X\textsubscript{a} and IX\textsubscript{a} but also factor XI\textsubscript{a} and XII\textsubscript{a}. Its action is dramatically accelerated by heparin and mucopolysaccarides like heparin present on the endothelial cell surface.\textsuperscript{50} The complete amino acid sequence is known. The gene for ATIII is localized on chromosome 1 and its organization has been defined.

Numerous families with hereditary deficiency have been reported.\textsuperscript{14,22,36,71} ATIII deficiency is an autosomal dominant trait and heterozygotes are at risk to develop venous thrombotic disease. Homozygotes of type IIC defect have been described. The other ATIII defects, with a few exceptions (ATIII Budapest, for example)\textsuperscript{52,63} seem to be incompatible with life at the homozygous level. In the general population a prevalence of ATIII deficiency of 1:2000 to 1:5000 has been reported.

The classification of ATIII defects proposed by Sas in 1980\textsuperscript{53} seems to comply for practical

### Table 1. Main congenital conditions predisposing to thrombosis.

<table>
<thead>
<tr>
<th>&quot;Coagulative&quot; familial thrombosis</th>
<th>&quot;Non-coagulative&quot; familial thrombosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antithrombin III (ATIII) defects</td>
<td>Homocystinuria</td>
</tr>
<tr>
<td>Protein C defects</td>
<td>Lipoprotein (a) increase</td>
</tr>
<tr>
<td>Protein S defects</td>
<td>Histidine-rich glycoprotein (HRGP) changes</td>
</tr>
<tr>
<td>Defects of second protein C co-factor</td>
<td></td>
</tr>
<tr>
<td>Heparin Co-factor II defects</td>
<td></td>
</tr>
<tr>
<td>Dysfibrinogenemia</td>
<td></td>
</tr>
<tr>
<td>Disorders of the fibrinolytic system</td>
<td></td>
</tr>
<tr>
<td>Combined defects</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Prevalence of thrombotic events in patients with congenital defects of clotting inhibitors.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Year</th>
<th>Factor</th>
<th>No. of Subjects</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thaler &amp; Lechner(66)</td>
<td>1991</td>
<td>ATIII</td>
<td>194</td>
<td>107 (55%)</td>
</tr>
<tr>
<td>Engesser et al (15)</td>
<td>1987</td>
<td>Protein S</td>
<td>711</td>
<td>39 (55%)</td>
</tr>
<tr>
<td>Breemans et al (4)</td>
<td>1988</td>
<td>Protein C</td>
<td>188</td>
<td>113 (60%)</td>
</tr>
<tr>
<td>Girolami et al. (27,28)</td>
<td>1992</td>
<td>ATIII, Protein S, Protein C</td>
<td>211</td>
<td>107 (50%)</td>
</tr>
</tbody>
</table>
use. Genetic study in the coming years might definitely clarify this point.

A large number of families with abnormal ATIII (type II defect) have been reported during the past 10 years and were divided into subgroups on the basis of electrophoretic mobility in presence or absence of heparin, impairment of heparin cofactor activity and/or of progressive antithrombin activity. Where the abnormality consists only of a reduced affinity of ATIII for binding to heparin, the frequency of thromboembolism is rather low.

For several ATIII variants the amino acid substitution has been identified. On the basis of the sites of amino acid substitutions in ATIII molecules, an impairment of heparin binding and/or co-factor activity may occur as for Arg 47 to His, or Pro 41 to Leu substitutions (ATIII Paris, Basel, Padua, etc.). Substitutions in other sites may give a defective protease or thrombin inhibition activities (ATIII Northwick Park, ATIII Chicago, ATIII Denver, etc.). As far as thrombotic manifestations are concerned, deep vein thrombosis, pulmonary embolism, splancnic thrombosis and superficial thrombosis are as frequent as in other coagulation inhibitor defects. Arterial thrombosis have also been described.

Hereditary protein C defects

Protein C is a vitamin K-dependent glycoprotein (63000 D) synthesized in the liver. It is the zymogen of a serine protease. Once activated by the thrombin-thrombomodulin complex at the endothelial cell surface, it inhibits factor Va and stimulates fibrinolysis. The complete amino acid sequence is known and the gene is on chromosome 2. The deficiency is transmitted as an autosomal dominant trait. Heterozygotes are at risk of developing thrombotic manifestations (ATIII Northwick Park, ATIII Chicago, ATIII Denver, etc.). As far as thrombotic manifestations are concerned, deep vein thrombosis, pulmonary embolism, splancnic thrombosis and superficial thrombosis are as frequent as in other coagulation inhibitor defects. Arterial thrombosis have also been described.

Hereditary protein S defects

Protein S (PS) is a vitamin K-dependent plasma glycoprotein (84000 D) synthesized in the liver and in the endothelial cells. PS is not the zymogen of a serine protease. It is the co-factor of activated protein C and forms complex with C4b-binding protein (C4b-bp). In plasma, PS can be free or bound to (C4b-bp), but only free PS is active. The complete amino acid sequence is known and the genes (\( \alpha \) gene and \( \beta \) pseudogene) are on chromosome 3. The classification of hereditary PS defects is not clearly established. Comp\' has proposed a classification with an attempt to fit the complex laboratory findings (total and free PS antigen and PS activity levels). A simplification has been suggested by
our group on the basis of the functional role which only free PS possesses. In this simplified classification, Type I includes the cases with a concomitant decrease of both antigen and activity whereas patients with Type II defect exhibit normal free antigen and decreased activity levels.\textsuperscript{27,28}

Hereditary protein S deficiency has been reported to inherit as autosomal dominant trait and the clinical manifestation are very similar to those of hereditary Protein C deficiency. Patients with different free and normal total protein S antigen patterns have been described.\textsuperscript{7,24,25,56} Acquired form have also been described.\textsuperscript{12} The role of C4b-bp in these variants is not well understood. Arterial thrombosis have been maintained to be relatively frequent in this condition.\textsuperscript{8,62}

Deficiency of second protein C co-factor

Recently a new clinical condition associated with thrombosis has been described.\textsuperscript{10} The main feature of this defect consists of poor anticoagulant response to activated protein C (APC) of patients’ plasma. It has been suggested that this laboratory finding may be due to the lack of a second protein C co-factor on an inherited basis. The significance and the importance of this condition remain to be proven. At least hypothetically, the APC resistance may be due to other conditions such as the presence of autoantibody to APC, a fast-acting protease inhibitor to APC, protein S deficiency, abnormality of factors VIII or V in the APC cleavage sites.

Heparin co-factor II defects

Heparin co-factor II (HC II) is a plasma glycoprotein with a molecular weight of 65,000 D. It inhibits thrombin with a very narrow specificity. It has no anti-factor Xa activity. The reaction is accelerated both by heparin and dermatan sulphate.

Although some families with HC II deficiency and thrombotic manifestations have been reported,\textsuperscript{60,61} there is no clear evidence of an association between inherited defect and thrombosis.\textsuperscript{2} HC II plasma levels have been shown to increase with age.

Dysfibrinogemias exhibit a low plasma level of functional fibrinogen in the presence of a normal antigen value. About 10\% of the families described showed thrombophilia.\textsuperscript{54} The abnormalities seem to be associated to impaired fibrinopeptide release, reduced affinity for thrombin, polymerization defects and/or increased resistance to lysis by the fibrinolytic system.

Disorders of the fibrinolytic system

They can be classified in:
a. disorders of plasminogen;
b. disorders of plasminogen activators;
c. disorders of plasminogen activator inhibitor.\textsuperscript{24}

Disorders of plasminogen

Plasminogen is a plasma glycoprotein (89,000 D) synthesized in the liver. It is the precursor of plasmin which degrades insoluble fibrin. A few families have been described with hypo- or dysplasminogenemia and recurrent thrombosis. The true deficiency state seems to be less frequent than dysplasminogenemias and this is a peculiar feature. The clinical picture is similar to that seen in ATIII deficiency. Bidimensional immunoelectrophoresis may be useful for detecting both low levels of protein and/or abnormal migrations.

Defective release and/or function of plasminogen activator

Tissue-type plasminogen activator (t-PA) is a glycoprotein with a molecular weight of 64,000 D synthesized in endothelial cells. It activates plasminogen into plasmin. The release of t-PA by endothelial cells can be stimulated by venous occlusion, DDAVP, exercise. Families with impaired tPA release and thrombosis have been described.

Increased levels of plasminogen activators inhibitor

PAI is considered an acute phase protein. Several patients with thrombosis have been found to have high PAI levels. A few families with hereditary elevated PAI levels and thrombophilia have been described.
Altogether we have studied in our laboratory 12 patients with defects of the fibrinolytic system and familial thrombosis.

**Combined defects**
Combined inherited defects of coagulation inhibitors or the association of one of them with a congenital defect of the fibrinolytic system have been reported. Although severe thrombotic manifestations may often occur also in uncommon sites, at least 50% of the patients reported remained asymptomatic. In other words, the thrombotic diathesis did not appear to be more severe in combined rather than in single congenital defect. The defects appear to segregate independently. The combination of a defect of an inhibitor together with a defect of another clotting factor has also been shown. In some of these cases it seems that the bleeding defect may protect patients from thrombosis.

**Non-coagulative familial thrombosis**

*Homocystinuria*. Homocystinuria is a metabolic disease that may be due to several genetic disorders. More frequently there is deficiency in cystathionine \(\beta\) synthase. Increased plasma homocysteine levels may induce connective tissue damage and trigger the coagulation system. It is suggested that the mechanism involved might be an interference with thrombomodulin activity or an enhancement of factor V activity. Homozygotes are at risk for developing thrombosis. Heterozygotes are predisposed to premature vascular disease. Arterial and venous thrombosis are very important clinical manifestations of the disease.

*Lipoprotein(a) increase*. High levels of Lp(a) have been associated with arterial thrombosis. A possible mechanism may be an interference with plasminogen receptors and/or plasmin by competing for binding sites on molecules and cells. High plasma levels are associated with coronary artery disease.

*Changes in histidine-rich glycoprotein (HRGP)*. HRGP is a plasma glycoprotein with a molecular weight between 60,000 and 80,000 D. It has no enzymatic activity and exhibits anti-fibrinolytic activity since it forms complexes with plasminogen present in plasma. In addition, HRGP inhibits plasminogen binding to fibrin. *In vivo*, HRGP may act as procoagulant when heparin is given. In fact, it binds heparin thereby reducing its availability for interaction with thrombin-antithrombin complex. Although the occurrence of elevated plasma level of HRGP and thrombophilia has been reported, there is no sufficient data to establish a clear relationship. The matter is further complicated by the fact that recently, a family with decreased level of HRGP and thrombosis has been described.

**Acquired conditions predisposing to thrombosis**

Acquired conditions predisposing to thrombosis are more frequent but more difficult to define. Often two or more than two conditions listed below are present in the same individual at the same time. The individual responsibility of all these factors remains to be quantitated in most instances. The main acquired hypercoaguluable states are gathered in Tables 3 and 4.

All these conditions are of marked clinical relevance but the actual impact each of them may have on the pathogenesis of thrombosis is unclear. It is important to emphasize that these conditions are often reversible and that a given patient may show during the course of a given disease several bouts of hypercoagulability and of a normal coagulation pattern. An association between cancer and venous thrombosis has been known since a long time. Recently it has been demonstrated that this association has a statistical significance. In fact cancer manifested itself more frequently in patients with idiopathic thrombosis than in those with secondary thrombosis.

<table>
<thead>
<tr>
<th>Table 3. Physiologically or paraphysiological conditions associated with increased incidence of thrombosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Pregnancy and puerperium</td>
</tr>
<tr>
<td>2. Advanced age</td>
</tr>
<tr>
<td>3. Smoking</td>
</tr>
<tr>
<td>4. Low fluid intake (dehydration)</td>
</tr>
</tbody>
</table>
Mixed congenital and acquired hypercoagulable states

The occurrence of both congenital and acquired conditions in the same person represent a common event in clinical practice (surgery or pregnancy in a patient with a defect of a clotting inhibitor or of the fibrinolytic system). The best example of such a combination and one that has received recently considerable attention is well represented, for example, by the intake of oral contraceptives by a patient with ATIII defect. As shown in Table 5, several patients with ATIII deficiency who showed their first thrombotic event shortly after the onset of oral contraceptive therapy have been investigated.26

The incidence of thrombotic complication in patients with congenital defects of inhibitors, under given triggering factors, is in fact greater than that of normal counterparts. This may represent a new approach to the study of thrombotic manifestations in patients with inhibitor defects.27,28

Hypercoagulable conditions represent common clinical events. They may represent about 25% of patients usually admitted in a general hospital. Diagnosis is reached on the basis of family history, evaluation of clinical status and laboratory tests. Female patients with defects in coagulation inhibitors should never take oral contraceptives. This triggering factor seems to be in these patients even more important than pregnancy (Table 6).

Furthermore it has to be remembered that thrombosis in patients with defects in the inhibitor system seem to develop after 1 or 2 cycles of OCT (early appearance). On the contrary thrombosis in normal women on OCT require several cycles to occur (late appearance) (Table 8). This discrepancy may have diagnostic implications in the sense that if a woman develops thrombosis during the first or the second cycle of OCT one may strongly suspect the existence of an underlying inhibitor defect.

Congenital and acquired clotting changes, isolated or in combination, probably constitute the major pathogenetic mechanism of venous thromboembolism.

Changes in blood flow

It is more appropriate to talk about decreased blood flow rather than stasis. Decreased blood flow may in fact be the result of decreased cardiac function (stasis) or increased blood viscosity (hyperviscosity syndrome). Blood flow is regulated by the following Bernouille formula, namely:

$$F = \frac{\Delta P \times \pi \times r^4}{8 \times \eta \times L}$$

Where $\Delta P$ is the difference in pressure, $L$ is the length of the vessel. The two major ele-
ments which control flow are $\Delta P$ (cardiac function) and blood viscosity. In fact the formula may be simplified as

$$F = \frac{\Delta P}{\mu}$$

Increased blood viscosity is independent of stasis due to heart failure since it may be present even without it (for example in polycythemic patients with normal heart function). Conversely stasis may be present also in patient with low blood viscosity (for example heart failure in patient with anemia). The main conditions causing stasis and/or decreased blood flow are listed in Table 8.

The fact that most venous thrombi originate in valve pockets, where flow is more stagnant, and that the frequency of thrombosis increases with advancing age and immobilization are strongly suggestive of a causal relationship between stasis and thrombogenesis. McLachlin et al.,$^{40,41}$ studied the retention of contrast material, administered through a dorsal vein of the foot to a group of patients with no history of venous disease. These investigators observed retention of the dye in the valve pockets of the thigh and calf and in the venous saccules of the calf muscles. Patients who were motionless while supine exhibited a prolonged clearance time with mean stagnation times in the thigh and lower leg of 21 and 27 minutes, respectively. Stagnation times were reduced by leg muscle contractions or a 15° elevation of the leg. Sevitt et al.$^{57,58}$ and others proposed that anatomy and function of venous valves predispose to disturbances of flow that, when augmented by the presence of stasis, lead to venous thrombosis. However, in the experimental animal the ligation and clamping of jugular veins are not followed by the appearance of thrombosis. The contrary occurs when serum is injected in the veins.$^{70}$

In humans, several of the conditions that predispose to DVT involve the combination of venous stasis and the activation of blood coagulation. In post-operative DVT, for example, blood coagulation is activated during surgery via the release of thromboplastin, and stasis occurs as a result of decreased mobility both during and after operation.$^{38}$

Patients with strokes and hemiparetic sequelae have a higher frequency of DVT in the paralyzed limb as compared to the non affected one.$^{9}$ Stasis increases the contact time between blood and venous segments and turbulences along the valve cusps lead to the deposition of erythrocytes, granulocytes, platelets, factor $X_a$ and thrombin, within the valve pockets. Stasis may also prevent adequate mixing between coagulation enzymes and their inhibitors. ADP may be released from stagnant red blood cells, leukocytes or both and trigger further platelet aggregation. Extension of the thrombus is usually anterograde but it may become retrograde when complete occlusion of the vein occurs. In conclusion it seems that decreased blood flow is an important factor in the pathogenesis of venous thrombosis.

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<table>
<thead>
<tr>
<th>Table 6: Thrombosis in patients with AT III deficiency during untreated pregnancy or during OCT. The difference was statistically significant ($p&lt;0.01$).</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N° of pts</strong></td>
</tr>
<tr>
<td>Patients during pregnancy*</td>
</tr>
<tr>
<td>Patients on OCT*</td>
</tr>
</tbody>
</table>

*One patient has been considered in both groups.

<table>
<thead>
<tr>
<th>Table 7: Duration of therapy and average age at time of first thrombotic event in patients on OCT.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N° of sympt. patients</strong></td>
</tr>
<tr>
<td>Patients with ATIII defect</td>
</tr>
<tr>
<td>Patients with PC and PS defects</td>
</tr>
<tr>
<td>Patients without defects</td>
</tr>
</tbody>
</table>
Changes in the venous vessel walls

Anatomic variants of vein may be a predisposing factor for thrombosis even without endothelial lesions.

It is well known that endothelial injury plays an important role in arterial thrombosis. On the contrary, there is still controversy whether endothelial damage and platelet interaction play a role in the formation of venous thrombi. Platelets normally do not adhere to the intact endothelium. When the endothelium is denuded, platelets adhere to the exposed subendothelial structures. This process is followed rapidly by platelet activation and aggregation. Endothelial damage also causes the activation of blood coagulation. Antiblastic drugs, contrast media and in-dwelling catheters have been reported to cause venous endothelial damage in clinical practice.

In the rabbit, venous thrombi form in the absence of damage to the vessel wall. The pathologic studies of valve pocket thrombi by Sevitt, did not reveal any evidence of intimal damage. Also, Borgstrom et al. failed to induce thrombi in the legs of animals after ligation of the femoral veins and vessel wall injury by cauteryization. In humans, endothelial damage may play a role in the pathogenesis of DVT after major hip surgery. Endothelial damage occurs as a result of intraoperative manipulations and/or thermal injury from the acrylic compounds with cemented prosthesis. Some authors suggested hypoxic injury to the endothelium may play a role in the formation of DVT. The endothelium of venous valve cusps receives little or no oxygen from its avascular tissue and therefore depends on the oxygen content of the blood. During hypokinetic conditions, oxygen supply to valve cusp endothelium is reduced.

Oxygen consumption by the static leukocytes might also be increased.

Experimental work by Hamer and Malone in dogs showed a strict correlation between length of anesthesia and the incidence of leg thrombosis. The highest incidence of leg thrombosis were seen in a group of dogs which were allowed periodic wear off of anesthesia before receiving the additional barbiturate dose. The authors related the presence of venous thrombi in all three groups to hypoxic endothelial damage. They attributed the higher frequency of thrombi in the unevenly anesthesized dogs to the periodic introduction of fresh blood within valve pockets. The authors postulated that freshly oxygenated leukocytes and platelets adhere more readily to the endothelium and, therefore, constitute the nidus for thrombus formation. It is conceivable that hypoxia may represent also in humans an important thrombogenic trigger.

The main recognized factors causing vein walls damage are listed in Table 9.

Pulmonary embolism: the complication of deep vein thrombosis

The evolution of DVT is along three different lines:
1. complete healing and resolution,
2. post-phlebitic syndrome,
3. fatal or non-fatal pulmonary embolism.

Pulmonary embolism is responsible for 5% of all postoperative deaths and is said to occur in as many as 20% of all patients admitted to the hospital. An understanding of the pathogenesis of pulmonary embolism is of paramount importance in order to attempt an adequate prophylaxis. The predisposing causes to pulmonary embolism are, almost always, those already dealt with in the pathogenesis of DVT. Few additional, non-coagulative causes have also to be taken into consideration (see Table 10).

Sources of emboli

The source of about 90% of all thromboemboli is the lower extremity. Thrombi in veins in the calf usually migrate to an above-knee location before embolization. However, this
sequence of events may be apparent since emboli from the calf must of necessity be small and therefore may be undetected. Certainly, the origin of most fatal pulmonary emboli is the large (proximal) veins of the leg.43,44 The use of 125I-labeled fibrinogen scanning in the diagnosis of calf vein thrombosis has led to the appreciation that only a minority (15%) extend above the knee, and that embolization is preceded by such extension.43,44 Superficial thrombophlebitis, however, does not lead to embolization, except in the event of communication with the deep venous system, which occur mainly in the presence of venous incompetence. Deep venous thrombosis may also arise directly in veins above the knee; for example, after hip surgery or direct trauma, or during the last trimester of pregnancy. The source of the remaining diagnosed pulmonary emboli is variable. An upper extremity source is uncommon but, it may be noted in specific circumstances. The use of subclavian venous catheterization leads to venous thrombosis in about 25% of patients.35 Embolization occurs in about 10% of these cases, even after anticoagulation has been instituted. An axillosubclavian source of emboli also may be noted in patients with malignancy associated with hypercoagulable states. Extrinsic venous obstruction related to thoracic outlet obstruction, neoplasms, or congenital venous malformation may also be responsible for upper extremity thromboembolic disease.13 Septic pulmonary embolism is one of the more common pulmonary complications of intravenous drug abuse secondary to subacute bacterial endocarditis or supplicative thrombophlebitis.47 Septic emboli may also occur in association with pelvic inflammatory disease or secondary infection of ventriculoatrial shunts, cardiac pacemakers, and arteriovenous fistulas. The right atrium or right ventricle is a relatively common source of clot in the setting of right ventricular failure of any cause, e.g., infarction, cor pulmonale, or rheumatic heart disease. Congenital cardiac disease, such as tetralogy of Fallot with pulmonic stenosis and secondary polycythemia, is an uncommon but important cause of thromboembolism.1

The pelvic veins can be a source of thromboembolism, but such emboli generally are small. These emboli are noted in the setting of prostatic disease, surgical intervention, parturition, or abortion.

**Conclusions**

Deep vein thrombosis is often or always the result of a combination of factors. Quantitation

---

**Table 9. Main factors causing endothelial damage.**

1. Surgery (hip surgery in particular)
2. External trauma
3. Intravenous drugs (antiblastics, analgesics, etc.)
4. Hypoxia from any cause (anesthesia, preexisting cardio-pulmonary incompetence, smoking, etc.)
5. Contrast media
6. Venous in-dwelling catheters

**Table 10. Sources of pulmonary emboli.**

**Thoracic vein thrombosis**
- Superior vena cava thrombosis

**Abdominal vein thrombosis**
- Inferior vena cava thrombosis
- Iliac vein thrombosis

**Veins of the leg**
- Proximal extension of calf thrombosis
- Femoral vein thrombosis

**Veins of upper extremities**
- Subclavian or jugular veins (catheterization and/or in-dwelling catheters)
- In association with malignancy (compression, hypercoagulability)
- Extrinsic venous compression (thoracic outlet syndromes, congenital malformation)
- Suppurative thrombophlebitis owing to intravenous drug abuse
- Infected arteriovenous fistula

**Heart**
- Thrombosis of right heart chambers
- Right-sided endocarditis
- Right ventricular failure
- Infected pace-maker wires
- Mixture of right heart

**Pelvic veins**
- Prostate disease and prostate surgery
- Pregnancy
- Abortion (septic)
of the single components is difficult and may vary from patient to patient. In most cases, with exception of the hyperviscosity syndrome, more than one factor is present. Often the entire spectrum of factors is evident. In the hyperviscosity syndrome only the decreased blood flow seems important even though, secondarily, clotting changes may occur.

It is important to emphasize that these factors, in a given patient, may vary from time to time and therefore may be difficult to pinpoint.

Another important consideration is the one pertaining to the triggering factors (surgery, trauma, OCT, etc.). There are several patients (about 50% of total) with congenital clotting changes predisposing to venous thrombosis who never develop thrombosis. Nobody knows for sure the reason for this discrepancy. It is likely however that asymptomatic patients had never had major triggering events or associated changes in vessel wall or blood flow during their lifetime. In the view of the complexity of the problem and of the frequent interdependency among several factors in the pathogenesis of thromboembolism, the obsessive insistence on strict rules such as, for example, the suggested adherence to fixed PT ratios, seems unjustified. This approach appears typical of single disease or, worse, single laboratory test physician and not that of experienced clinicians.

References

The magnitude of the problem of venous thrombosis is recently increasing since the results of extensive data of post-mortem and prospective studies; these studies have shown a strong association between pulmonary embolism (PE) and deep vein thrombosis (DVT) of the lower limbs, and have also shown that patients with DVT are at risk for PE.1-8 Death from PE represents the major complication, while chronic pain, swelling and the development of post-thrombotic syndrome are the most common complication of DVT. Chronic major vessel pulmonary hypertension is a serious but uncommon complication of venous thromboembolism (VTE).

Incidence and prevalence of venous thromboembolism

Information on the incidence and prevalence of venous thrombosis of the lower limbs are derived from several studies performed on medical and surgical patients objectively tested for DVT. About 90% of cases are silent and DVT has been usually detected by 1) radioactive fibrinogen leg scanning, which is moderately sensitive for calf thrombosis but less sensitive for proximal DVT, 2) impedance plethysmography and doppler-augmented ultrasound which are insensitive for distal and non occlusive thrombosis, but sensitive to occlusive DVT, 3) compression ultrasound, which is high sensitive for proximal vein thrombosis and less sensitive for distal, small or non occlusive DVT as occur in asymptomatic patients, 4) venography, which represents the most accurate and sensitive method in either symptomatic or asymptomatic patients and it represents the reference standard test for detecting DVT of the lower limbs.

The prevalence of DVT in the general community has been estimated from large descriptive studies on symptomatic patients: the annual incidence of proximal DVT has been reported to be 48 cases for 100,000.9 Estimates of the incidence and prevalence of PE are less reliable than those described for DVT, because the ante-mortem diagnosis of PE is difficult and the post-mortem diagnosis highly selective. Moreover, the diagnosis of PE has been usually performed by ventilation/perfusion lung scanning; in this diagnostic method the sensitivity and specificity in overall patients with high-, and low- or intermediate-probability scanning were 93% and 10% respectively.10,11 Therefore, studies based on V/Q lung scan produce falsely high estimates of PE.

One available index of the frequency of fatal PE in the general population is based on official statistics derived from death certificates and the clinical diagnosis provides the basis for these certificates. Nevertheless, the clinical diagnosis has low sensitivity and specificity, and even when autopsy results become subsequently available, death certificates are seldom updated. In Horowitz and Tatter’s study, 11,000 autopsies were analyzed and 316 of these showed macroscopic pulmonary emboli; 11% of these cases had the diagnosis before death, while 32% of the patients were diagnosed as having died of myocardial infarction, 15% of cerebrovascular disease and 14% of pneumonia.12 Hospital autopsy results show a wide discrepancy in the prevalence of PE at death due to the differences in the nature of the population studied as well as in how pulmonary emboli are detected and

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Moreover, the significance of such small emboli is uncertain, as with all autopsy-based data. Therefore, estimates of fatal and non-fatal PE are extremely unreliable. Finally, there are no satisfactory criteria to distinguish between fatal and non-fatal emboli detected at autopsy. Angiographic studies suggest that young and healthy people may survive major occlusive embolism obstructing 50% of the pulmonary vasculature. The occurrence of chronic major vessel pulmonary hypertension without clear history of pulmonary embolism in the preceding years is an indirect confirmation of this hypothesis.

An equally difficult problem is estimating the frequency of venous thrombosis and non-fatal PE in the general population. Several studies have provided information on the frequency with which venous thromboembolism and non-fatal PE occur in selected groups of hospitalized patients. These patients are obviously different from the general population, and they are also very different from each other.

In conclusion, there are no valid estimates of the incidence of fatal pulmonary embolism or total venous thromboembolism in the general population. The most satisfactory estimates of the incidence of venous thromboembolism are in selected groups of hospitalized patients. The availability of objective means of diagnosing venous thromboembolism in hospitalized patients had led to the identification of several important risk factors for venous thromboembolism in this population.

**Surgery and other trauma**

Through the use of objective diagnostic criteria based on autopsy examination, 125I-fibrinogen leg scanning, impedance plethysmography, venography, lung scanning, and angiography, reliable data have been gathered on the frequency of post-operative venous thromboembolism in general abdominal, urologic, gynecologic, orthopedic, and other surgery and following trauma of the lower limbs. Most reported studies have concluded that orthopedic procedures and trauma of the lower limbs are particularly high-risk situations with an overall incidence of venous thromboembolism of about 50%.

In a prospective study performed in our Institution, post-operative DVT occurred in 41% of patients who underwent hip replacement procedures. In the same study, we assessed also the very low sensitivity of IPG and the relatively low sensitivity of compression ultrasound in the detection of asymptomatic proximal DVT.

The incidence of fatal PE after hip surgery based on clinical diagnosis or post-mortem assessment has been reported to be between 0.3% and 10.0%. However, the three highest reported values of 6.6%, 7.0% and 10.0% were all based on a series of at least 100 patients and had autopsy rates between 83% and 100%, suggesting that some of the other reported figures are underestimates. There are no equivocal studies of the effect of the anesthesia in patients receiving antithrombotic agents for prophylaxis, and some studies reported a significant difference in proximal DVT (73% reduced to 20%) with regional anesthesia.

Based on the 125I-fibrinogen leg scanning, the reported incidence of VTE in studies of patients undergoing abdomino-thoracic surgery ranges from 14% to 33%. Urologic surgery places patients at significant risk of venous thromboembolism, particularly open operations on the prostate gland. Some studies totaling more than 200 patients undergoing open prostatectomy reported a 35% average incidence of DVT detected by leg scanning. In gynecologic surgery, the incidence of post-operative DVT was reported to be 7% following vaginal hysterectomy, 12% following abdominal hysterectomy, and 27% following Wertheim’s hysterectomy.

**Pregnancy and puerperium**

Pregnancy and the puerperium have been regarded as high-risk conditions for the devel-
opment of venous thromboembolic disease. However, most of the reported major studies were done before the development of objective diagnostic tests for venous thrombosis. Extensive data of clinically diagnosed thrombosis in pregnancy are available. The reported incidence per 1,000 pregnancies in the antepartum period ranged from 0.08 to 0.15. These values are similar to those reported in non pregnant women of childbearing age. In contrast, the reported monthly incidence of post-partum thrombosis per 1,000 pregnancies ranged from 2.7 to 20. The post-partum period has consistently been found to be higher-risk period than the ante-partum period with a relative risk in excess of 20-fold.

Cardiac and neurologic disease

Descriptive studies using fibrinogen leg scan to diagnose DVT in patients who underwent myocardial infarction report an incidence of 20% to 40% over a period of 10 to 14 days (37). Among patients with acute myocardial infarction, the incidence of ante-mortem PE unknown, but emboli have been found at autopsy in about 8% of cases. Congestive heart failure has been also associated with high incidence of autopsy-proven pulmonary emboli. Although congestive heart failure complicating myocardial infarction appears to be an additional risk factor, it is unknown whether the value reported for congestive heart failure alone would differ from those described for other sick patients confined to bed.

Disease associated with immobility and paralysis might be expected to be associated with a high incidence of DVT. Recently, Turpie et al., found an incidence of DVT of 12% in patients with minor, partial or complete stroke. Thrombosis occurred only in patients with complete paralysis. Other studies reported higher values of DVT incidence.

Malignancy and cancer chemotherapy

The presence of cancer has been associated with an increased rate of post-operative venous thrombosis, detected by leg scanning, but the presence of confounding factors such as age, extent of surgery, preoperative and postoperative management makes it difficult to assess the real role of malignancy in the etiology of venous thrombosis. An association between cancer and thrombosis has also been described in patients with spontaneous VTE. Cancer is also associated with a rapidly progressive thrombotic process of both superficial and deep veins (thrombophlebitis migrans), and there are anecdotal reports of failed anticoagulant therapy in patients with cancer and VTE. Several studies reported higher incidence of cancer development in patients who had idiopathic DVT than in patients with DVT of known cause, other than cancer. The reported association between venous thrombosis and the subsequent development of cancer has important therapeutic and prognostic implications. The association between VTE and malignancy has been attributed to a procoagulant material released from the tumor; this association also raises the question of cancer screening in patients who present with idiopathic venous thrombosis or with VTE in unusual sites. Patients with cancer are particularly prone to thrombotic complications during chemotherapy. Thromboembolic episode have been reported in 5% of patients with breast cancer undergoing chemotherapy, and more frequently in patients with advanced disease.

Age, obesity and varicose veins

Advanced age has consistently been found to be associated with an increased incidence of venous thromboembolic disease. One hospital-ized-based prospective autopsy series showed the incidence of PE to be relatively low in patients younger than 40 years, but thereafter the incidence increased steadily with age.

Obesity was suggested as a risk factor for venous thrombosis some 60 years ago, but the evidence from more recent studies is equivocal. Some studies have examined the relationship between varicose veins and venous thromboembolism in postoperative patients, but no information based on reliable objective tests is available concerning this association in
general population. The currently available studies do not provide convincing evidence that varicose veins are a risk factor for the development of postoperative venous thrombosis.

Previous venous thromboembolism

Results of studies in outpatients support the view that previous venous thrombosis is an important independent risk factor for VTE. In studies in outpatients presenting with symptomatic venous thrombosis, previous venous thrombosis was a strong predictive factor. In a study of secondary prevention in patients with DVT, the recurrence rate over the first 12 months after the end of treatment in patients who had a single episode of DVT was about 6%, significantly lower than the 20% incidence in another cohort of patients who had more than one episode of thrombosis.

Prolonged immobilization, blood group and ethnicity

Venous stasis is of major importance in the formation of venous thrombosis. Dynamic angiographic studies in humans show that in absence of calf muscle contraction, prolonged venous stasis occurs in the soleal veins. It would be expected that disease that paralyze or immobilize the lower limbs would be associated with a high incidence of venous thrombosis, and this hypothesis is supported by both clinical and autopsy studies. Moreover, although it may argued that the underlying condition is the principal determinant of the development of VTE, the consistency of the pattern of thrombosis observed in diverse groups with different causes of limb inactivity supports the concept that prolonged immobilization is an independent risk factor in the development of VTE.

There are no conclusive data on the association between venous thrombosis and blood groups. In a previous analysis, a relative lack of patients with blood group O among patients receiving anticoagulants for VTE was observed. This stimulated a retrospective study that showed, despite the diverse methods of selection of both cases and controls, a reduced risk of VTE among patients with blood group O. In contrast, no relation was found between blood group and the development of venous thrombosis objectively documented.

Epidemiologic studies have shown that a combination of genetic and environmental factors can explain the observed geographic variation in disease. The incidence of postoperative VTE has been reported to vary in different part of the world, and it has been suggested that ethnicity may therefore be a risk factor. However, interpretation of these reported differences is often confounded by the lack of direct comparability in such matter as patient selection and management, type of operation and diagnostic methods.

Oral contraceptive and postmenopausal estrogen replacement

The combined incidence of venous thrombosis and PE for users of oral contraceptive pills is low, but the large number of healthy young women using the pills makes any relation between oral contraceptive and thrombosis a potentially important problem.

Interest in thrombogenicity of the pills was initiated by a series of case reports and descriptive studies. Nevertheless, in these retrospective studies there is considerable potential for bias, such as diagnostic suspicion bias, recall bias, and reporting bias. Some studies had prospective design; all studies showed contraceptive pill users to be at greater risk for venous thromboembolism. However, knowledge that the patients was taking the contraceptive pill and the lack of objective diagnosis are consistent sources of bias. Thus, although the overall weight of evidence appears to support the hypothesis that oral contraceptive predispose to VTE, these findings should be confirmed in an appropriately designed study.

In comparison with the oral contraceptive, there have been few studies of the relationship between the use of post-menopausal estrogens and venous thromboembolism. The doses of estrogen used for postmenopausal replacement are much lower than those used for contraception. Some studies reported a trend in favor of
the increased risk for developing VTE in patients undergoing estrogen replacement, but this association was not statistically significant. Therefore, none of the available studies found a strong association between the use of postmenopausal estrogens and venous thromboembolism; however, none of these studies was large enough to rule out a clinically significant association. In addition, these studies did not use objective diagnostic tests and were potentially biased.

References

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Deep-vein thrombosis of the lower extremities may result in the development of late sequelae. The clinical manifestations of the post-thrombotic syndrome are reported to vary from minor signs (i.e., stasis pigmentation, venous ectasia) to severe manifestations such as chronic pain, intractable edema and leg ulceration.\(^{1-9}\) The post-thrombotic syndrome develops as a result of the combination of venous hypertension, due to persistent outflow obstruction and valvular incompetence, and abnormal microvasculature or lymphatic function.\(^{10,11}\)

The precise incidence of the post-thrombotic syndrome following confirmed venous thrombosis is unknown but has varied in the published studies between 20 and 100%.\(^1,9,12-15\) Thus far, most studies have been limited to small,\(^12-15\) or retrospective series of patients.\(^1-5,7,9\)

Furthermore, in all studies the potential for bias was high due to either the selection of patients with extensive thrombotic disease or to failure to distinguish post-thrombotic sequelae from recurrent vein thrombosis. Moreover, often no objective diagnostic method was used for the confirmation of the presence or absence of venous thrombosis.\(^1-3\) Finally, different and nonstandardized criteria for the presence of the post-thrombotic syndrome were applied in the respective studies.

It is important to know the precise incidence and severity of the post-thrombotic syndrome since various therapeutic strategies are suggested for its prevention, including surgical thrombectomy and fibrinolytic therapy.\(^16,21\) These therapeutic strategies are associated with high rates of complications, and, therefore, the benefit-risk ratio of these strategies can only be positive if the post-thrombotic syndrome occurs frequently. Furthermore, it remains uncertain if these interventions will lead to reduction of the incidence of the post-thrombotic syndrome.\(^19,21\)

We have conducted a prospective follow-up study in a large cohort of more than 200 consecutive patients with their first episode of venography documented symptomatic deep-vein thrombosis to determine: the incidence of the post-thrombotic syndrome during long-term follow-up; the clinical severity of this condition; the period elapsed between the acute thrombotic episode and the development of the syndrome; whether a relationship exists between the duration of symptoms at the time of initial referral for venous thrombosis and the development of post-thrombotic syndrome; the relationship between the extent of the initial venous thrombosis and the occurrence of subsequent sequelae. In addition, we assessed whether patients with recurrent episodes of ipsilateral deep-vein thrombosis were at a higher risk for developing post-thrombotic syndrome. All patients were treated with heparin followed by oral anticoagulant therapy for a period of three months and wore elastic compression stockings.

**Methods**

**Patients and eligibility**

All outpatients who were referred by their general practitioners to the Thrombosis Center of the University of Padua from May 1985 through May 1990 were considered for this
study. Patients with a first episode of venography proven deep-vein thrombosis were potentially eligible for the study. Patients were excluded if they were referred because of recurrent venous thrombosis, if they could not be followed as outpatients because they lived too far from the hospital to return for visits, or if they had a poor life expectancy (defined as less than six months). Patients who fulfilled the inclusion criteria and passed the screen of exclusion criteria were eligible for the study. Eligible patients who gave informed consent were entered in the study. This study was approved by the institutional review board of the University of Padua.

Study design

All patients were treated with heparin followed by a three-month period of oral anticoagulant therapy. After discharge from the hospital at approximately day ten, patients were instructed to wear elastic compression stockings (40 mm Hg at the ankle). Patients were asked to return to the Thrombosis Centre for follow-up assessments at 6 months from the acute episode, and then every six months until 5 years. At each follow-up visit, the presence and severity of post-thrombotic signs and symptoms were scored using a standardized scale. This score was developed in a separate series of patients with overt post-thrombotic syndrome and patients without any sign and symptom of the syndrome after an episode of deep-vein thrombosis (Table 1). For this score, five subjective symptoms (heaviness, pain—spontaneous or during deambulation; cramps, pruritus, paresthesia) and six objective signs (pretibial edema, induration of the skin, hyperpigmentation, new venous ectasia, redness, and pain during calf compression) were considered. Each item was graded from 0 to 3. In addition, the presence or absence of ulceration of the leg was assessed.

A patient was regarded to have a severe post-thrombotic syndrome if ulceration of the leg was observed or if a score of ≥ 18 was obtained on a single occasion. Moderate post-thrombotic syndrome was defined if on two consecutive follow-up visits a score from 9 to 17 was reached (including at least one objective sign). Patients with a score of 3 to 8 on two consecutive follow-up visits were defined to have mild post-thrombotic syndrome, whereas all other patients were considered not to have the post-thrombotic syndrome. The score was determined in all patients by a single trained physician who was unaware of the results of the previous evaluations. The minimum period of follow-up was 18 months, and patients who were unable to attend the scheduled visit were seen at home.

Table 1. Standardized scale for the assessment of the post-thrombotic syndrome.

<table>
<thead>
<tr>
<th>Subjective symptoms*</th>
<th>Objective signs*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heaviness</td>
<td>Pretibial edema</td>
</tr>
<tr>
<td>Pain</td>
<td>Induration of the skin</td>
</tr>
<tr>
<td>Cramps</td>
<td>Hyperpigmentation</td>
</tr>
<tr>
<td>Pruritus</td>
<td>New venous ectasia</td>
</tr>
<tr>
<td>Paresthesia</td>
<td>Redness</td>
</tr>
<tr>
<td>Pain during calf compression</td>
<td>Ulceration of the skin</td>
</tr>
</tbody>
</table>

Definition of post-thrombotic syndrome

- Severe: ulcer or a score ≥ 18 measured on one occasion.
- Moderate: a score between 9 and 18 (including at least one objective sign measured on two consecutive follow-up visits).
- Mild: a score between 3 and 8 on two consecutive follow-up visits.

*Each sign or symptom was graded with a score between 0 and 3. The presence or absence of a leg ulcer was noted.

Recurrence venous thrombosis

Patients were instructed to return immediately to the Thrombosis Centre if they developed signs or symptoms suggestive of recurrent venous thrombosis or pulmonary embolism. Recurrent venous thrombosis was diagnosed by contrast venography. Patients who presented with pulmonary embolism were examined for the presence of concurrent leg symptoms. If the venogram was not diagnostic, recurrent thrombosis was diagnosed on the basis of an abnormal 125I fibrinogen leg scanning. Patients with documented recurrent venous thrombosis were treated with heparin, followed by oral anticoagulation for a period of at least one year. For the purpose of the study, contralateral vein thrombosis was not taken into consideration, nor were episodes of pulmonary embolism apparently not related to recurrent manifestations of ipsilateral vein thrombosis.
Contrast venography

Contrast venography was performed as described previously.\textsuperscript{23,24} The criteria for the presence of venous thrombosis were a constant intraluminal filling defect confirmed in at least two projections, or persistent nonopacification of a vein (or a segment thereof) despite repeated injection with contrast material. A diagnosis of recurrent venous thrombosis was made if a new intraluminal filling defect was observed on venography. All venograms were adjudicated by a panel of independent experts.

Analysis

The incidence of the post-thrombotic syndrome was assessed for severe, moderate and mild manifestations. A comparison was made between the incidence of post-thrombotic syndrome in patients with isolated calf-vein thrombosis and patients with proximal-vein thrombosis. Furthermore, a comparison was performed between the time elapsed between the duration of symptoms prior to the diagnosis of the initial episode of venous thrombosis and the presence of the post-thrombotic syndrome.

The cumulative incidence of severe and moderate post-thrombotic syndrome combined was calculated for all patients as well as for those with and without recurrent thrombosis. The method of Kaplan-Meier was used and the significance was assessed with the method of Mantel-Haenszel.

The incidence of the post-thrombotic syndrome in patients with recurrent thrombosis was calculated as function of the time elapsed since the day of (the first) recurrence. The relation between the incidence of post-thrombotic syndrome and the extent of the thrombus was calculated for patients who did not develop recurrent venous thrombosis.

The chi-square test, Fisher’s exact test and Student t-tests were used when appropriate. Two-sided p-values of less than 0.05 were considered statistically significant.

The Common Odds Ratio of the risk of developing post-thrombotic syndrome and their 95% confidence intervals (CI) were calculated according to Mantel Haenszel.

Results

Patients selection

During the recruitment period of this study, deep-vein thrombosis was demonstrated by contrast venography in 298 patients. Of these patients, 74 (25%) were excluded for the following reasons: poor life expectancy (39 patients), geographical inaccessibility (14 patients), and previously documented episodes of ipsilateral thrombosis (21 patients). Thus, a total of 224 patients were ruled eligible for the study, of whom 3 refused to give informed consent. Therefore, a total of 221 patients were entered in the study. Five patients died before the first follow-up visit. All remaining 216 patients completed at least 18 months of follow-up.

Patients characteristics

Of the patients included in the analysis, 118 (55%) were males and 98 females. The mean age was 59.3 years (range, 19 to 86). Isolated calf-vein thrombosis was documented in 20 (9%) patients, and proximal-vein thrombosis in the remaining 196 patients. Four (2%; 95% CI, 0.7% to 5.5%) patients had isolated proximal-vein thrombosis. Thrombi involved the popliteal vein in 18 (9.2%; 95% CI, 5.7% to 14%) patients, the popliteal and the femoral vein(s) in 101 (52%; 95% CI, 44% to 59%) patients, the femoral and the iliac vein(s) in 17 (8.7%; 95% CI, 5.3% to 13.7%) patients, whereas thrombi involved the entire proximal deep venous system in the remaining 60 (31%; 95% CI, 24% to 37%) patients.

Incidence of the post-thrombotic syndrome

Of the 216 patients, 9 (4%) died during the period of follow-up and three patients were lost to follow-up (after 24, 36 and 48 months, respectively). The mean follow-up in all patients was 44.8±16.2 months. The post-thrombotic syndrome occurred in 64 (29.6%; 95% CI, 23.7% to 36.3%) of the 216 patients. The syndrome was severe in 15 (6.9%; 95% CI, 4% to 11.4%), moderate in 9 (4.2%; 95% CI, 2% to 8%), and mild in 40 (18.5%; 95% CI, 14% to 25%) patients. No relation was found between the duration of symptoms at

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the time of referral for the initial episode of venous thrombosis and the occurrence of the post-thrombotic syndrome (p=0.76).

Post-thrombotic syndrome in patients with proximal or calf-vein thrombosis

Post-thrombotic syndrome developed in 58 (29.6%) of the patients with proximal-vein thrombosis compared with six (30%) of the patients with isolated calf-vein thrombosis (p=0.83; Common Odds Ratio, 0.98; 95% CI, 0.3 to 3.0).

The extent of deep-vein thrombosis in patients who did not develop recurrent thrombosis is shown in Table 2. Mild, moderate or severe post-thrombotic syndrome developed in five (26%) of the 19 patients with isolated calf-vein thrombosis, in three (20%) of the 15 patients with popliteal vein thrombosis, in 24 (26%) of the 92 patients with popliteal and femoral vein(s) thrombosis, in 16 (31%) of the 51 patients with thrombosis of the entire proximal system, and in two (15%) of the 13 patients with thrombosis of the femoral and iliac vein. There was no relation between the extent of thrombosis and the occurrence of the post-thrombotic syndrome (p=0.98).

Discussion

The findings of this large prospective follow-up study in consecutive patients with a first episode of venography proven deep-vein thrombosis challenge various widely accepted dogmas about the development of the post-thrombotic syndrome. Firstly, more than 70% of the patients in our study did not develop any manifestation during a mean follow-up period of almost four years. Secondly, only about ten percent of the patients developed moderate or severe manifestations of the post-thrombotic syndrome. Interestingly, in the majority of patients these manifestations became apparent within the first and second year following the acute thrombotic episode. Although it is widely believed that the majority of patients with a first episode of extensive proximal-vein thrombosis are at risk for developing the post-thrombotic syndrome, the results of our study indicate that its development was not related to the extent of proximal thrombosis. Thus, patients with minor proximal-vein thrombi (i.e., thrombi involving the popliteal vein only) were as

Table 2. The relation between the extent of the venous thrombosis and the development and severity of the post-thrombotic syndrome in patients with a single episode of deep-vein thrombosis of the leg.*

<table>
<thead>
<tr>
<th>Isolated calf (N=19)</th>
<th>Popliteal (N=15)</th>
<th>Popliteal femoral (N=92)</th>
<th>All proximal veins (N=51)</th>
<th>Femoral iliac (N=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No PTSD</td>
<td>14 (74%)</td>
<td>12 (80%)</td>
<td>68 (74%)</td>
<td>35 (69%)</td>
</tr>
<tr>
<td>Mild PTSD</td>
<td>8 (21%)</td>
<td>2 (13%)</td>
<td>18 (20%)</td>
<td>12 (23%)</td>
</tr>
<tr>
<td>Moderate PTSD</td>
<td>0</td>
<td>0</td>
<td>1 (1%)</td>
<td>2 (4%)</td>
</tr>
<tr>
<td>Severe PTSD</td>
<td>1 (5%)</td>
<td>1 (7%)</td>
<td>5 (5%)</td>
<td>2 (4%)</td>
</tr>
</tbody>
</table>

*Results are not significantly different (p=0.92).
likely to develop late sequelae as patients with thrombosis of the entire venous system of the thigh and pelvis. Surprisingly, similar proportions of patients with isolated calf-vein thrombosis developed the post-thrombotic syndrome as patients with proximal venous thrombosis. Furthermore, the duration of symptoms before the diagnosis of the first episode of venous thrombosis was not related to the occurrence of the post-thrombotic syndrome.

The results of our study clearly demonstrate that manifestations of the post-thrombotic syndrome are relatively rare after a first episode of venous thrombosis, however, this risk increases significantly with the development of recurrent venous thrombosis in the same leg.

For the assessment of the post-thrombotic syndrome, we applied a scale which was developed prior to this study in a separate series of patients with and without post-thrombotic sequelae. The scale specified both objective signs and subjective symptoms, and required the confirmation on two consecutive follow-up visits to assess mild and moderate post-thrombotic syndrome. Thus, we believe that this scale is valid and reflects those clinical signs and symptoms of the post-thrombotic syndrome, which, in particular for the severe post-thrombotic manifestations, interfere with quality of life. Selection bias was avoided by including consecutive symptomatic patients with a first episode of venography proven venous thrombosis. Observation bias was minimized by having the follow-up visits of all patients performed by a single investigator who was blinded to the results of previous assessments. Furthermore, we achieved almost complete follow-up in all patients, thereby minimizing the possibility that patients with post-thrombotic sequelae were missed for the analysis. All patients were instructed to wear elastic compression stockings in order to prevent bias occurring when some patients were going to wear these and other patients not. Therefore, our results apply to patients with a first episode of deep-vein thrombosis who use elastic compression stockings. The use of the elastic compression stockings might have lowered the incidence of the post-thrombotic syndrome.

The clinical implications of this study are the following. First, in view of the low absolute incidence of the post-thrombotic syndrome demonstrated in this study, it becomes questionable whether the routine use of aggressive therapy to prevent its development, (i.e., surgical thrombectomy and thrombolytic therapy) can be justified. However, patients with recurrent venous thrombosis constitute a group at high risk for developing the post-thrombotic syndrome and, therefore, this would be a potential group of patients to evaluate these therapeutic strategies. Secondly, prevention of recurrent vein thrombosis is important to reduce the risk of developing the post-thrombotic syndrome.
Therefore, patients should be treated with heparin followed by oral anticoagulants for a minimum period of three months. 21-30

The major limitation of this study is the relatively short period of follow-up (mean follow-up, 45 months). It can be argued that the post-thrombotic syndrome might develop several years after the first episode of deep-vein thrombosis. However, of the 24 patients with moderate or severe post-thrombotic manifestations, the diagnosis was made within the first two years in 19 (79%), suggesting that the follow-up time in our study might be adequate to give a valid estimate of the incidence of the post-thrombotic syndrome. These findings are in agreement with those of another prospective follow-up study. 31 It is possible that some of our asymptomatic patients will develop the syndrome after a longer period. This possibility will be addressed by continuing to follow these patients.

We conclude that about 10% of patients with a first episode of venous thrombosis who are treated with heparin and oral anticoagulants and who wear elastic compression stockings, develop moderate or severe post-thrombotic manifestations. This incidence is considerably lower than rates suggested from previous clinical trials. 1-9 Delay in referral for the initial venous thrombosis and the extent of the thrombi at that time are not related to the occurrence and severity of the post-thrombotic syndrome, however, recurrent vein thrombosis is a major determinant for its development.

References

The mechanisms that regulate blood coagulation remain the focus of intense investigation. One of the reasons for this interest is the emerging concept that these regulatory pathways may contribute to acquired conditions that predispose to thrombosis. The protein C pathway is an excellent example of mechanisms that allow acquired deficiencies to develop, especially those associated with inflammation. Both clinical and basic studies indicate that the protein C anticoagulant pathway plays a major role in regulating the hemostatic process. Preclinical studies and preliminary clinical studies suggest that optimal function of the protein C anticoagulant pathway is critical to preventing deep vein thrombosis, reperfusion injury in the myocardium, septic shock, microvascular coagulation in response to warfarin, and potentially in limiting the extent of coronary artery occlusion in response to injury.

To appreciate the role of the pathway in thrombotic disease, it is useful to first review the nature of the molecules involved in the system.

**Components of the protein C pathway**

A model of the protein C anticoagulant pathway is shown in Figure 1. The activation complex is formed when thrombin complexes with TM on the endothelial cell surface (for a review of the pathway see ref. 16-19). Activated protein C binds with protein S to cell surfaces and then functions as an anticoagulant by inactivating factor Va and VIIIa by limited proteolysis, with activated protein C showing a marked preference for the activated coagulation factors. Protein S circulates free and bound to C4b-binding protein (C4bBP) that may have associated serum amyloid P(18). Only the free form of protein S is capable of functioning in the anticoagulant complex, although both forms can interact with model membrane surfaces.

Activated protein C is cleared slowly from the circulation ($T_{1/2} \approx 15$ min) by complex formation with $\alpha_1$-antitrypsin ($\alpha_1$-AT) and the protein C inhibitor (PCI). The contribution of the two inhibitors to activated protein C inactivation in vivo is approximately equal. $\alpha_2$ macroglobulin can also inhibit activated protein C.

**A model of the impact of inflammation on the protein C pathway**

Inflammation can perturb the function of the pathway (Figure 2). C4bBP behaves as an acute phase protein, and hence its levels rise in inflammatory situations. By mass action, under conditions where protein S levels stay nearly normal, elevation in C4bBP levels results in increased complex formation between protein S and C4bBP with a concomitant decrease in free, and hence functional, protein S. TM also appears to be down regulated by inflammation. Vascular injury associated with severe inflammatory insult leads to elevations in circulating TM probably due to proteolytic release, possibly mediated by neutrophils, or vesiculation. Cytokines and endotoxin can

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induce TM down regulation by blocking transcription followed by internalization and degradation.

One clinical example where inflammation leads to reduced endothelial TM, plasma protein C and free protein S levels is in human renal allograft rejection.

The specificity and intricate regulation of the TM response to inflammatory mediators in culture certainly suggest that down regulation should occur in vivo in most severe inflammatory situations. Except for organ rejection cited above and villitis, in vivo data on TM down regulation remains limited. Some authors have failed to observe decreases in response to endotoxin infusion even when the challenge is lethal.

A possible explanation is that down regulation is also regulated. For instance, TNF down regulation of TM can be prevented by IL-4 or other agents that elevate cyclic AMP.

**Structural features of protein C, protein S and thrombomodulin**

A schematic picture of the proteins involved in the pathway is shown in Figure 3. Protein C and protein S are vitamin K dependent proteins which require Ca\(^{2+}\) and the vitamin K dependent formation of \(\gamma\)-carboxyglutamic acid (Gla) residues to allow membrane binding

Protein C is primarily a two chain protein. Activation occurs with release of a dodecapeptide from the amino-terminus of the heavy chain. TM is an integral membrane protein with the following domain structure: the amino terminal domain has no known function; the epidermal growth factor-like domains which contain the thrombin binding site; an O-linked sugar region where the chondroitin sulfate moiety is attached; a single transmembrane region and a short cytoplasmic domain with no known function. The chondroitin sulfate functions to accelerate inhibition of thrombin by
antithrombin III and to increase the affinity for thrombin. The relative importance of TM dependent acceleration of thrombin inhibition and protein C activation remains unknown. Furthermore, it is unclear what percentage of total thrombin inhibition is mediated by TM, although its high concentration in the microcirculation and high affinity for thrombin suggests that it should contribute significantly to thrombin clearance.

**Homzygous and heterozygous protein C deficiency**

Homzygous protein C deficiency is often characterized by microvascular thrombotic complications including purpura fulminans. Often, the initial presentation seems to be restricted primarily to the skin. These skin lesions are often associated with disseminated intravascular coagulation (DIC), and can be corrected with protein C concentrate.

Several reports have appeared recently on homozygous protein C deficiency with the first thrombotic episode occurring relatively late, in the second or third decade of life. These patients have low, but detectable, protein C, which may protect them through infancy. The pathology and clinical presentation of homozygous protein C deficiency and warfarin induced skin necrosis has been reviewed recently.

Heterozygous protein C deficiency is a risk factor for thrombosis in some families (see for instance reference). It is equally apparent, however, that approximately 1/300 individuals in the normal population has protein C levels of 50% or below, and the vast majority of these individuals appear to be asymptomatic.

**Protein C and warfarin induced skin necrosis**

A rare complication of warfarin therapy is skin necrosis. Involvement of protein C in this process was suggested by several observations: 1) protein C is a vitamin K dependent protein; 2) activity decreases rapidly after the onset of
anticoagulant therapy; 3) necrosis develops soon after therapy is initiated; and 4) morphological examination of the lesions often, but not always, indicates inflammatory cell infiltrates and the patients themselves often have an inflammatory disorder. Of 16 patients that developed skin necrosis, approximately 1/3 were individuals with heterozygous protein C deficiency. The prevalence of approximately 1/300 in the general population makes it unlikely that the deficiency is not a risk factor for skin necrosis. Since skin necrosis does not always occur in heterozygous individuals during oral anticoagulant therapy, it is clear that protein C deficiency alone is not sufficient to elicit skin necrosis. Since skin necrosis does not always occur in heterozygous individuals during oral anticoagulant therapy, it is clear that protein C deficiency alone is not sufficient to elicit skin necrosis. Inflammation is a good candidate for the other contributing factor.

Inflammation can impair the protein C system by elevating C4bBP thereby reducing anticoagulantly active protein S. Reduced levels of free protein S are not always associated with inflammation, however. Inflammation also initiates coagulation by inducing intravascular tissue factor expression. A potential, but unproven, mechanism for the common involvement of the skin in warfarin induced microvascular thrombosis is suggested by the observation that the skin behaves as a potent inflammatory organ capable of releasing cytokines like TNF and IL-1 that in turn are capable of promoting coagulation. The link between protein C and microvascular thrombosis is presumably related to the fact that most protein C activation takes place in the microcirculation where the TM concentration is high.

![Figure 3. Schematic representation of protein C, protein S, and thrombomodulin (TM). The vitamin K dependent Gla residues of protein C and protein S are indicated by small Y-shaped symbols. Formation of these vitamin K-dependent residues is essential to full activity of protein C and protein S. Gla, γ-carboxyglutamic acid; Th.-sens., thrombin sensitive. (Reprinted from J. Biol. Chem. 264:4743-4746, 1989, with permission of the American Society for Biochemistry and Molecular Biology, Inc.)](image)

**Impaired function of the protein C pathway can lead to DIC, microvascular thrombosis or deep vein thrombosis**

We have developed primate models to examine possible links between protein C and inflammation. One of our interests has been to determine the underlying mechanisms responsible for DIC/microvascular thrombosis and how these are distinguished from the stimuli that result in large vessel disease. Our studies began with the observation that activated pro-
tein C could protect baboons from the lethal effects of E. coli infusion. The animals are protected not only from DIC, but from the inflammatory injury to the vasculature and the organ damage that ultimately leads to death. These anti-inflammatory activities may not be directly related to the anticoagulant activity of activated protein C, since active site blocked factor Xa prevented DIC without preventing organ damage and death. The levels of APC used to prevent toxic effects of E. coli were quite high, raising the question of whether protein C played an active role in normal host response to infection. This question was approached by blocking protein C activation with a monoclonal antibody that overlaps the activation site on protein C and then challenging with E. coli at 10% the concentration required to elicit a lethal response. Under these conditions in controls, the E. coli caused only an acute phase response, but when protein C activation is blocked, a septic shock-like response ensued with DIC, organ damage and ultimate death. Activated protein C prevented the deleterious effects of the antibody. Elevating C4bBP levels resulted in decreased protein S function and, like the antibody inhibition of protein C activation, resulted in DIC and death in animals challenged with sublethal levels of E. coli. Protein S prevented this deleterious response. Thus, protein C activation during sepsis and adequate free protein S are both critical to effective host defense responses in gram negative sepsis. The latter point is important since protein C levels drop dramatically in the most severely effected individuals.

We were surprised that blocking protein C activation did not in itself cause short term massive thrombosis unless a secondary stimulus was given. Although when protein C activation is blocked, DIC occurs in response to E. coli, if TNF is infused instead, localized thrombosis results. These studies clearly implicate inflammation as a potential requirement for thrombosis in protein C or protein S deficiency, and raise the possibility that some of the affected families might have hyperinflammatory responses as a contributing factor to the thrombotic complications.

Why TNF elicited thrombosis while E. coli elicited a DIC response remains unclear. One difference in host response to E. coli vs TNF is the ability of E. coli to activate massive amounts of complement. In vitro, C5b9, the membrane attack complex of complement, leads to platelet microparticle formation. These complement generated particles have the interesting property of possessing potent coagulantly active membrane surfaces, but lack activation of the adhesion receptor GPIIb-IIIa. Thus, these particle mimic liposomes in many ways. Indeed, infusion of liposomes into the animals treated with TNF as described above elicits a DIC response (Taylor and Esmon, unpublished results). Whether the microparticles are the key difference between thrombosis and DIC remains to be determined.

Protein C is likely to be an effective antithrombotic drug since animal experiments indicate that activated protein C can prevent the extension of a thrombus in vivo at concentrations that do not increase surgical blood loss. The basis for the selective antithrombotic effect with little or no effect on hemostasis remains unclear, but this observation has been made either quantitatively or qualitatively in most of the animal studies to date and all of those presented in this review.

**Protein C and thrombosis in lupus**

Protein C activation and activated protein C function have both been shown to be decreased in some patients with lupus anticoagulants and antiphospholipid antibodies. The mechanisms can involve either direct interaction with key proteins or more generally, investigators have felt that these anticoagulants function by blocking membrane interaction. Assuming that lupus anticoagulants are not an epiphenomenon, the key question that remains unanswered is how do anticoagulants lead to thrombosis. If blocking membrane binding by protein C anticoagulant pathway components is the answer, then the question arises as to how these antibodies can have a selective effect on the protein C pathway. While this remains uncertain, a potential insight comes from recent studies which
demonstrated that the activated protein C anticoagulant activity is potently stimulated by the presence of phosphatidylyethanolamine in the membrane, whereas this phospholipid has little effect on the prothrombin activation complex. This observation is especially interesting with respect to the antiphospholipid antibody situation since lupus anticoagulants and antiphospholipid antibodies often react with phosphatidylyethanolamine. These studies suggest that selective inhibition of the protein C pathway can occur through masking the membrane phosphatidylyethanolamine that is critical to activated protein C function. Further studies are in progress to test this hypothesis.

Protein C and reperfusion injury
Reperfusion injury and septic shock appear to share many common mechanisms, i.e., free radicals, neutrophil mediated injury, complement and perhaps microvascular thrombosis. Since protein C activation was required for protection against the shock response, we examined the importance of protein C activation in reperfusion injury. Following transient ischemia resulting from occlusion of the left anterior descending coronary artery, in both a porcine and canine model, activated protein C was detectable in the region at risk in less than 1 minute following occlusion. Protein C was activated in the region at risk, but not systemically. Relative to controls, inhibition of protein C activation resulted in impaired left ventricular function and frequently resulted in ventricular fibrillation.

These studies raise the question of whether there is a physiological mechanism to impair protein C activation in ischemia. One possible mechanism is suggested by the observation that patients with myocardial infarction have been reported to have detectable circulating TNF. The TNF would presumably down regulate TM expression and hence limit protein C activation. Hypoxia has also been shown to down regulate TM expression. Decreased TM expression would presumably be similar to inhibiting protein C activation with the antibody, and hence increased ischemic injury would be anticipated.

Protein C and arterial thrombosis
Current information suggests that thrombin is the major mediator of arterial thrombosis. Activated protein C can prevent or limit platelet dependent arterial thrombosis in a baboon model. Interestingly, even though platelet deposition in this model is caused by thrombin, infusion of thrombin systemically inhibits platelet deposition. This inhibitory activity is due to protein C activation, since inhibition of protein C activation eliminates the anticoagulant response.

The possibility of the protein C system playing a major role in preventing arterial thrombosis clinically is weakly supported by case reports of patients with arterial thrombosis and protein C deficiency, though venous and microvascular thrombosis are far more common manifestations of protein C deficiency. Based on the animal models, it is possible that protein C deficiency could increase the severity of the myocardial infarction and/or contribute to thrombus formation at an earlier stage in atherosclerosis. If this is the case, obtaining clinical evidence of involvement will require very careful studies.

Conclusions
Available evidence suggests that the protein C pathway is intimately involved with inflammation. The products of the pathway can block the deleterious influence of inflammation on vascular injury and organ dysfunction, but inflammation can also lead to down regulation of the pathway by a myriad of mechanisms. Our understanding of the detailed pathways connecting these diverse responses remains limited. Future studies that delineate these pathways promise to aid in the development of new diagnostics and therapeutics.

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Recently developed assay systems for assaying the activation of blood coagulation and fibrinolysis allow rapid and specific determination of these parameters in plasma. These include thrombin-antithrombin complexes (T-AT), the activation peptide of prothrombin fragment 1+2 (F1+2) and a specific degradation product of cross-linked fibrin D-Dimer (D-Di). Experience with D-Di is by far the most extensive since TAT and F1+2 have been introduced only recently.

This paper will review: 1) the intrinsic performances of these tests, in terms of sensitivity and specificity for the diagnosis of deep venous thrombosis (DVT) or pulmonary embolism in clinically suspected patients, with special attention to false positive and false negative results; 2) data on the evolution of these markers during and after antithrombotic treatment; 3) the current problems which limit the widespread use of these markers; 4) the place of laboratory tests in a diagnostic strategy under routine clinical conditions.

Sensitivity and specificity of laboratory tests for the diagnosis of venous thromboembolism in symptomatic patients

Limited data available for F1+2 and T-AT do not encourage the use of these markers for the diagnosis. Their sensitivities, defined as the proportion of patients with marker levels above a critical cut-off among the patients with proven thrombosis, are poor (Table 1). This lack of sensitivity has been confirmed in a recent clinical trial, where the sensitivity of F1+2 and T-AT were 66 and 89% respectively. Indeed, for these two markers, there is a large overlap of the values found in healthy controls and in patients with DVT, making the definition of an appropriate cut-off hazardous. As hemostatic activation occurs in various physiopathological conditions, the specificities of these tests are also poor.

Experience with D-Di is by far more extensive. Several studies have investigated the performance of the test, using ELISA assays, in consecutive patients clinically suspected of DVT. Diagnosis of DVT was established by venography or non-invasive techniques. By pooling the data of these studies, it is possible to estimate a global sensitivity of D-Di for the diagnosis of DVT close to 97%. A similar figure is found for the diagnosis of pulmonary embolism. As the negative predictive value of a normal D-Di level is about 95%, one could propose the use of D-Di measurement to rule out the diagnosis of venous thromboembolism.

The small proportion of false-negative D-Di results remains unexplained. Details of patients with D-Di levels below the critical cut-off in spite of an established thrombosis are rarely reported. One possibility is that some patients display a defective fibrinolysis. We found indeed that D-Di at the time of diagnosis is directly proportional to the plasma levels of plasmin-antiplasmin complexes (P. Sié, unpublished). A second possibility is that D-Di levels are proportional to the fibrin mass and that thrombi of small size are missed by this test. In support of this hypothesis, a positive correlation between D-Di and the phlebographic index has been recently reported and, D-Di levels are lower in patients with distal thrombosis than in those with proximal thrombosis.

The global specificity of D-Di is poor, 35% in DVT and 45% in pulmonary embolism. False-positive D-Di results, i.e. D-Di above a critical
Evolution of D-Di in treated patients with established thromboembolism

Several studies\textsuperscript{3,5,10} indicate that, during the initial phase of treatment by unfractionated heparin, D-Di levels decrease sharply but still remain abnormal at hospital discharge. In practice, that means that D-Di results can be interpreted in patients already receiving heparin for a suspicion of thrombotic event and waiting for confirmation by an objective method. There are occasional reports of secondary increase of D-Di in patients who displayed thrombus extension under heparin treatment\textsuperscript{11} but the value of D-Di follow-up for detection of early recurrence needs to be evaluated. Three months after a DVT, when the patients have been correctly treated by oral anticoagulants, D-Di levels return to the normal range established for an age-matched population.\textsuperscript{11} In a retrospective study (P. Sié, submitted), we found that, after stopping their anticoagulant treatment, several months or years after the last DVT episode, D-Di levels remained normal in patients without symptoms of post-phlebitic syndrome, associated disease or inherited coagulation disorder. This suggest that D-Di is also of potential value for the diagnosis of long-term recurrence.

Current problems which limit the widespread use of laboratory tests for the diagnosis of venous thrombosis

Lack of convenience is a major limitation of D-Di ELISA methods. Indeed these methods are time-consuming, difficult to perform, require special equipment and are costly when run for individual patients. Semi-quantitative latex agglutination methods are fast, economical and simple, although reading is subjective. Unfortunately the sensitivity in patients suspected of DVT or pulmonary embolism is sub-optimal.\textsuperscript{1} Discordances are usually in the sense of a normal latex test result with an abnormal ELISA test result.\textsuperscript{12} As abnormal values with D-Di latex are not specific for thrombosis and normal values are endowed with an unacceptable risk of false negative results, the only interest of latex agglutination assays might be a rapid screening of patients with elevated D-Di levels, who are not candidates for D-Di ELISA measurement. Those with normal or borderline latex test results should have ELISA measurement.

Four ELISA assays are currently available. They have different test principles and antibody
reagents and do not measure the same analytes in plasma. The ranges of control values are quite different and the correlation between the results of different assays is rather poor. Comparative evaluation of the four assays has been performed in a recent study where cut-off values were determined a posteriori to achieve a sensitivity of 100%. It is thus difficult to predict whether the assays will be equivalent in operational conditions. Reproducibilities of all assays, specially at low D-Di concentrations close to the cut-off levels are poor (the order of magnitude of between-assay CV is 20%), making it highly questionable to ground a clinical decision on a borderline value obtained on a single occasion.

**Place of laboratory tests in a diagnostic strategy under routine clinical conditions**

So far, the potential utility of D-Di, measured using an ELISA method, is restricted to the exclusion of venous thromboembolism. With this objective in mind, appropriate cut-offs which provide the optimal sensitivity should be determined, but confidence on a normal blood test result is not sufficient to replace objective testing.

In patients with clinically suspected DVT, non-invasive tests such as impedance plethysmography or real-time compression ultrasonography are sensitive for occlusive proximal thrombi. However these tests miss the diagnosis of distal DVT or non-occlusive proximal thrombi in 5 to 10% of cases. To identify these patients with normal non-invasive test at presentation, who risk developing occlusive proximal thrombi if they remain untreated, a follow-up with non-invasive testing is proposed. Combining a laboratory test with non-invasive testing could increase the global sensitivity at presentation and subsequent non-invasive testing in these patients could be no longer necessary. A recent trial tested this hypothesis. By using the combined approach, a definite diagnosis decision about the presence or absence of DVT on the day of referral was reached in 42% of all patients, as opposed to 19% using non-invasive testing alone. The 23% increase represented those patients (69 of the 309 included) with negative results of both non-invasive and laboratory tests. The diagnosis of thrombosis was excluded a posteriori in all these patients using serial non-invasive testing during the week after the referral.

In patients with clinically suspected pulmonary embolism, non-invasive testing, i.e. ventilation/perfusion lung scanning, (V/Q scan) is used for initial screening. The sensitivity of this test is high and a normal result virtually rules out the diagnosis of pulmonary embolism. Unfortunately V/Q scans are inconclusive in a high proportion of patients, 50% of which have no pulmonary embolism. In these patients, combination of lower limb non-invasive testing for DVT, the source of pulmonary embolism, with D-Di measurement could identify the patients with the lowest probability of pulmonary embolism. So, pulmonary angiogram, which is invasive and risky, could be avoided in a significant number of patients. This approach should now be tested in management trials.

To sum up, laboratory tests, and specially D-Di measurement, still require improvements in reproducibility, standardization and convenience but they have a potential utility in the diagnosis of venous thromboembolism. They could be used to rule out the diagnosis in a small proportion of patients without comorbid conditions, thus reducing the cost, inconvenience, wasted time and, as far as invasive procedures are concerned, discomfort and risk, for symptomatic patients without thrombosis. The safety and real utility of this approach in routine conditions remain to be evaluated before being recommended.

**References**

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Patients with idiopathic venous thromboembolism (IVT), i.e. with deep venous thrombosis or pulmonary embolism in the absence of recognized predisposing risk factors, stand a significantly higher chance of a subsequent cancer with respect to normal subjects or patients with secondary thrombosis. Screening these patients in an attempt to find and locate an occult, preclinical tumor can theoretically lead to early intervention and thereby improve health outcomes and save resources. However, because the cancer might be everywhere and because each cancer may be screened by a number of different tests, deciding how to screen is a difficult matter.

Furthermore, there is uncertainty about the proportion of cases that, once diagnosed, are treatable with effective life prolongation. For these reasons, cancer screening utility is still controversial and it is a general policy to properly investigate only symptomatic areas or abnormalities detected by physical examination or routine laboratory tests. In this work we turned to decision analysis to measure the expected utility and costs of a screening procedure. To reduce the complexity of the clinical problem, we first operated a selection of the cancers worth to be screened according to a general utility criterion. Then, we implemented a decision model able to pursue sequences of decisions in order to decide the best sequence to screen cancers and perform diagnostic tests.

**Materials and Methods**

**IVT and cancer**

Medline key words used to assess the incidence of cancer in IVT were: deep-venous thrombosis, pulmonary embolism, cancer. We considered only studies recording incidence of cancer in venous thrombosis and/or pulmonary embolism. Fourteen studies were evaluated: 3 of them were perspective controlled studies reporting a total of 211 patients with IVT (mean age 64 years). In all these studies a pre follow-up routine examination had been performed, and the follow-ups ranged from 2 to 6 years. The incidence of cancer in the first two years of follow-up was 2% but the incidence of cancer after the second year was similar in patients with IVT and controls. Therefore IVT-related were stated to be those cancers appearing since the second year from IVT. A global incidence of 16/129 (7.6%) was recorded. This was assumed to be the prevalence of preclinical cancers at the time of IVT, i.e. 2.7 times that of the general population.

**Framing the clinical scenario**

We considered an hypothetical cohort of patients aged from 60 to 64 admitted to the hospital for venous thrombosis or pulmonary embolism, in whom a routine investigation was completed but no malignancy was identified. Routine examination consisted in a thorough history taking, thorough physical examination,
ESR, whole blood count, peripheral blood examination, protein electrophoresis, ASAT, ALAT, alkaline phosphatase, calcium and urinalysis. Moreover, chest roentgenography, digital rectal examination in both men and women, and breast examination in women, were completed as clinically mandatory acts in patients at risk of having a cancer.

Selecting cancers worth to be screened

In patients with IVT cancer may be everywhere. Since it is not feasible to screen for all possible cancers, we first questioned which cancer to screen. Two selection criteria were sequentially used. The first one was a prevalence-based rule-out criterion. An optimal screening scenario was set (performance of a single perfect and risk-free test and complete curability of screen-detected cancers), and a one-way threshold analysis was conducted to calculate the cancer prevalence that would give an estimated minimum acceptable utility of 30 days life prolongation. The second criterium was an efficacy-based rule-out criterion. Cancers were excluded according to literature based evidence (from randomized controlled trials) that the patient chance of dying from that cancer will not be decreased by screening induced early detection.

The decision model

We framed a decision tree to outline the optional sequences of cancers to screen, including the no screen option, by a series of investigation modules. Each module plans the alternative sequences of tests for each cancer (Figure 1). The tree starts with a decision node where the options are the possible answers to the question: which cancer first? After pursuing the tests through the cancer specific investigation module, if no cancer has been found, a sequence of new decision nodes models the question: which cancer next?

The investigation module retains the same basal structure for all the cancers. The decision concerns what test to do first between a series of ordinated tests (Table 1). In each module, only with the primary test positive the strategy proceeds to the subsequent test up to the final one, i.e. biopsy or laparoscopy. Probabilities of test results comes from Bayes formula and depend on cancer prevalence and test characteristics. The sequence of tests, test characteristics (sensitivity, specificity) and cancer preva-
ence were derived from literature, by considering only cancers in their preclinical phase12-25 (Table 2). We assumed that only one cancer could be present in each individual.

We defined two dimensions of outcome utility: monetary cost of screening and patient survival (cost per years of life saved), without considering either morbidity or psychologic complications. We performed the analysis both including the cost of further hospitalization due to the screening procedure, and the cost of having the screening outside the hospital. In this latter case physicians’ fees were calculated. The costs that would appear out of the screening time-frame were not included. All the costs are those estimated by the Italian Government (1991 prices).

The algorithm

The solver LISP algorithm has a recursive structure with the aim to find the best cost-effective strategy among the ones listed in the decision plan.

Results

Cancers worth to be screened

With 30 days life prolongation as the minimum acceptable screening utility value, threshold analysis resulted in a threshold prevalence for a cancer of 548/100,000 for males and of 434/100,000 for females. By considering the 2.7 times risk of having a tumor in IVT, the threshold prevalence for a cancer to be screened is 200/100,000 in males and 161/100,000 in females of the age of 64 years old. According to these data, cancers with an expected prevalence higher than the screening threshold were located in the colonrectum, prostate, bladder, buccal cavity, pharynx and larynx, lung and melanoma for males, in the breast, cervix, colonrectum, ovarion and lung in females.8,9

According to the criterium of literature-based evidence of screening effectiveness, only two cancers in males (rectocolon and prostate) and four cancers in females (rectocolon, cervix, breast, ovarion) proved worth to be screened.26-47 Moreover, routine examination provides the only possible screening test for melanoma and oral cavity, exhausting in such a way any screening strategy.

Baseline analysis

A male of 60-64 years old, who has a age-adjusted life expectancy of 13.0 years, has a life expectancy of 11.99 years (4,376 days) when affected by IVT and not screened. An ideal screen would produce an expected survival of 12.77 years (4,661 days), with a net gain of 285 days. By considering our model where rectocolon and prostate cancers alone were screened, the screening option produces an expected survival that is always greater than the no-screening option. As a matter of fact the best screening produces an expected survival of 12.13 years (4,427 days). Thus, screening would, on average, prolong life expectancy of each patient screened by 52.2 days.

Table 1. Sequence of tests for the cancer screening.

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Tests</th>
<th>Primary</th>
<th>Secondary</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rectocolon</td>
<td>FOBT</td>
<td></td>
<td>Colonoscopy</td>
<td>Biopsy</td>
</tr>
<tr>
<td>Prostate</td>
<td>PSA</td>
<td></td>
<td>Sonography</td>
<td>Biopsy</td>
</tr>
<tr>
<td>Breast</td>
<td></td>
<td></td>
<td>Sonography</td>
<td>Biopsy</td>
</tr>
<tr>
<td>Ovarion</td>
<td>CA125</td>
<td></td>
<td>Sonography</td>
<td>Biopsy</td>
</tr>
<tr>
<td>Cervix</td>
<td>PAP test</td>
<td></td>
<td>Colonoscopy</td>
<td>Biopsy</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test Characteristics for an Early Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test</td>
</tr>
<tr>
<td>-----------------------</td>
</tr>
<tr>
<td>FOBT (for colon cancer)</td>
</tr>
<tr>
<td>PSA</td>
</tr>
<tr>
<td>PAP test</td>
</tr>
<tr>
<td>CA125</td>
</tr>
<tr>
<td>Colonoscopy</td>
</tr>
<tr>
<td>Sonography (for prostate cancer)</td>
</tr>
<tr>
<td>Mammography</td>
</tr>
<tr>
<td>Sonography (for ovarian cancer)</td>
</tr>
<tr>
<td>Colonoscopy</td>
</tr>
</tbody>
</table>
The most cost-effective strategy is to screen prostate cancer first, by a PSA test first. The cost-effectiveness ratio of the best screening strategy has a value of 864,210 Liras ($513) for year-life saved when tests are done outside the hospital, and 12,704,000 Liras ($7,940) when tests are done during hospitalization.

Discussion

Decision analysis is an accepted method to shape cost-effectiveness problems and evaluate the relevance of different factors involved in a clinical scenario. In this work we used decision analysis with the principal aim of framing the utility of cancer screening in patients with IVT. We considered the clinical scenario where a patient is admitted to hospital for a IVT, and an extensive routine workup is performed without supporting any cause of venous thrombosis, in particular cancer. For practical reasons we assumed that it was worth screening only a limited number of cancers with a short battery of tests and that only one cancer could be present. All the cancers disclosed at screening were supposed to be in an early phase, and all the cancers present but not screened would emerge later in their worst stage. We first used a one-way threshold analysis to calculate which cancer prevalence under conditions extremely favorable to screening would give a minimum acceptable screening utility. A survival increase of 30 days seemed to be a reasonable utility for screening. Literature-accepted screening utilities for cancers are: 3 months for Papanicolau tests and 2 months for breast cancer; 1.4 days gained with ovarian cancer screening were defined as a toss-up. An additional criterion for screening was literature-based evidence of a specific cancer screening effectiveness. With these utilities as anchors, two cancers in males and four cancers in females proved to be worth to be screened.

In this study we considered the utility of finding a cancer only from the perspectives of economic advantages and of patient life duration. We have disregarded both psychologic disadvantages for the patient who realizes the possibility of having a cancer and physical discomfort due to testing procedures.

All strategies we have modelized produce an increased expected life for the patients screened. The mean life expectancy for a 60-64-year-old male with IVT is 11.99 years while his expected survival, i.e. that of age- and sex-controlled healthy population, is 13 years. The screening procedure would, on average, prolong each screened patient life expectancy by 52.2 days.

We implemented a decision model dealing with two questions: which test first and what cancer first. The result of our analysis is that the best procedure is to screen prostate cancer first with PSA, but the difference among test-strategies scarcely influences final outcomes.

The cost-effectiveness analysis resulted in a cost-effectiveness ratio that falls always below $50,000 per year life gained, which represents the accepted cost-effectiveness ratio for the most common medical treatments. The conclusion of this work is that screening for a cancer in IVT patients should be recommended.

References

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26. Selby JV, Friedman GD, Collen MF. Sigmoidoscopy and mor-
Preceding discussed epidemiological studies have documented the high frequency of acute deep venous thrombosis (DVT) and pulmonary embolism (PE) in hospitalized patients, particularly after surgery. The risk of venous thromboembolism is different for the different patients in relation to the type of surgery, the type of disease, and the individual risk factors. The expected probability of thrombosis and bleeding are the key parameters (apart from cost considerations) for selecting the optimal approach among the several pharmacological and physical means of prophylaxis which proved to be effective in reliable clinical studies. The pharmacological tools include: low-dose heparin (5000 u/12h; 5000 u/8h, 7500 u/12 h); adjusted dose heparin; low dose heparin/DHE; low-molecular-weight heparins and heparinoids; oral anticoagulants; dextran 40 and 70; aspirin.

Low-dose heparin (LDH) has been shown effective since the early seventies and has become the standard for comparison with other methods. It is commonly used at fixed doses, but an adjusted-dose scheme has been proposed in 1983 by Leyvraz et al. for hip surgery, a setting where traditional LDH is relatively ineffective. Although superior to LDH, prophylaxis with adjusted-dose heparin is cumbersome, since it requires daily PTT determination and dosage adjustment, and its application has been limited.

LDH/DHE: the association with dihydroergotamine 0.5 mg s.c or i.m. 12 or 8 hourly has been employed particularly in central Europe, but the potentially useful venoconstrictive effect of DHE is counterbalanced by the occasional cases of serious ergotism (for this reason the drug has not been licensed in the United States).

Moreover, the greater efficacy of this combination over LDH alone, observed in some studies, has not been confirmed in the pooled analysis (see below). This method of prophylaxis therefore may well belong to the past.

Low-molecular-weight heparins (LMWH) sure belong to the present. Obtained by chemical or enzymatic degradation of heparin, the fractions which have been developed commercially (nadroparin: Fraxiparine®, Seleparina®; enoxaparin: Clexan®, Lovenox®, Trombenox®; tinzaparine: Logiparin®, Innohep®; parnaparine: Fluxum®, Minidalton®; dalteparin: Fragmin®; RD heparin: Normiflo®; sandoparin: Mono Embolex®(NM); reviparin: Clivarin®) have an average molecular weight of 4,000-6,500 daltons and share distinctive pharmacological properties, such as an anti-Xa/anti-IIa activity ratio greater than 1.5, a bioavailability close to 100% and half-lives of anti-Xa activity after subcutaneous injections between 3 to 5 hours. The reduction in chain length of heparin decreases its affinity for plasma and vascular matrix proteins, including histidine-rich glycoprotein (HRGP), platelet factor 4 (PF4), vitronectin, fibronectin, lipoproteins, and von Willebrand factor (vWF). These plasma proteins may compete with antithrombin III in binding to heparin, thereby reducing its anticoagulant effect in vivo. The LMWHs have also a reduced affinity for endothelial cells, macrophages and platelets. Thus, in comparison with unfractionated heparin, LMWHs show greater availability (particularly at the low doses employed in prophylaxis), a longer plasma half life, a less complicated mechanism of clearance, a more predictable anticoagulant response to fixed doses, and reduced platelet-associated side-effects.
Although it is true that products with the same activity *in vitro* do not necessarily have the same clinical effect, clinical trials indicate that all preparations so far used are safe and effective for prophylaxis of DVT within a relatively narrow dose range, when expressed as units of anti-Xa activity against the International Standard for low molecular weight heparin.

Lomoparan (*Orgaran*®, previously known as ORG 10172), a low-molecular-weight heparinoid containing a mixture of heparan sulfate, dermatan sulfate and condroitin sulfates, has also achieved good results in the prevention of DVT in surgical and medical patients at high risk of venous thrombosis.

Dermatan sulfate given at high dose intramuscularly has been shown marginally effective in hip fracture.7

Oral anticoagulants are among the oldest methods of prophylaxis, but due to the risk of bleeding and the inconvenience of close monitoring they are used only in conditions carrying a high risk of thrombosis, such as orthopedic surgery. Elective hip and knee replacement are considered an indication for prophylaxis with vitamin K antagonists in the US, but a recent large trial has shown that their efficacy is not greater than LMWH in this type of surgery.a Warfarin administration is usually begun the evening before (or after) surgery, with the aim of preventing progression of small thrombi possibly formed in the immediate postoperative period. Some orthopedic surgeons prolong treatment for about two months, i.e. until full mobilization is achieved. The desired therapeutic range is 2.0 to 3.0 *International Normalized Ratio* (INR).

Dextran have been employed in two preparations of 40,000 and 70,000 daltons (mean molecular weight) mainly in Scandinavian countries; they induce hemodilution and a von Willebrand-like platelet defect and have been considered a possible alternative to LMWHs and oral anticoagulants in patients undergoing orthopedic surgery. It should be recognized however that dextran have lower effectiveness, higher cost and may be associated with serious side-effects such as volume overload and anaphylactic reactions.

Antiplatelet agents and aspirin in particular have been used in prevention of post-operative venous thromboembolism with limited success. The recent metaanalysis of the Antiplatelet Trialists’ Collaboration,9 provides evidence that they are better than no treatment, however the overall rate of isotopic or venographic DVT observed is clearly unsatisfactory: 19.4% in general surgery, 37.5% in elective hip replacement, 33.9% in hip fracture.

**Physical methods**

**Elastic stockings**

Graded compression elastic stockings have been shown effective in reducing the incidence of radioisotopic DVT in general surgery patients16,17 and their use can be advocated as an alternative to pharmacological means in patients at low thrombotic risk or in patients at intermediate thrombotic risk who have an increased risk of bleeding. Elastic stockings may be employed in combination with other prophylactic agents, such as LDH or LMWH, and this approach proved to be more effective than heparin alone in some studies.12,13 In the European Fraxiparin Study, about 2000 general surgery patients were randomized to LDH plus elastic stockings or to LMWH plus elastic stockings; the frequency of positive leg scans was very low in both groups: 4.5% in the former and 2.8% only in the latter.14

**Intermittent pneumatic compression**

Venous stasis can also been prevented with a variety of inflatable boots and leggings which produce intermittent pneumatic compression (IPC) of the lower limbs. IPC has been used in general surgery patients, where it has been shown equivalent to LDH in reducing the incidence of DVT,15-18 in orthopedic surgery, where it proved effective in elective hip replacement,19-23 in total knee replacement,24,25 and in neurosurgery,26-31 where it can be considered as the method of choice in elective intracranial operation.

However, devices using inflatable cuffs which squeeze the calf are impractical for orthopedic
surgery. Recently, inflatable shoes (A-V Impulse System, also called foot pump) have been introduced and shown effective in total knee replacement, and in elective and post-traumatic hip operations.

Electrical calf muscle stimulation
This technique which prevents venous stasis by contracting the calf muscles has been shown effective for DVT prevention in some randomized trials, but it is painful and may be used only under anesthesia or in paraplegic patients.

Venous interruption
Usually inserted for prevention of pulmonary embolism in patients with proximal vein thrombosis who cannot be anticoagulated, caval filters have also been used in primary prevention after hip fracture or immediately before elective hip replacement in patients with previous venous thromboembolism. Although effective, this procedure cannot yet be recommended, partly because it lacks a formal evaluation in a controlled trial in comparison with other simpler and less expensive means of prophylaxis, partly because the filter insertion is not without complications, such as migration of the filter, caval occlusion, and distal vein thrombosis, despite progressive improvements of filter design. It should be considered in multiple trauma patients at very high risk of venous thromboembolism, if other simpler modalities of prophylaxis are not applicable.

Let's now consider the pros and cons of the different pharmacological and physical means in the various categories of patients at risk of venous thromboembolism, with the aim of selecting the optimal approach in the different categories.

A great number of level I studies devoted to this topic have been published and owing to the completeness of data and general agreement among studies, we can obtain useful information by a simple cumulative analysis of these data, when appropriate. Even if open to criticism from the statistical point of view, because the power of the random assignment is lost in the pooled analysis, and the homogeneity of the groups of patients mixed together is debatable, this procedure is simple and allows a tentative ranking of the different methods employed. It may be considered a useful, albeit unsophisticated, compass to orient oneself in a vast and conflicting literature, and it has been used in the review article: Prevention of Venous Thromboembolism, by Clagett et al., discussed at the Third ACCP Consensus Conference on Antithrombotic Therapy.

Elective general surgery
This broad category includes patients over the age of 40 years undergoing major abdominal surgery (duration > 30 min) and also patients undergoing thoracic, urologic and gynecologic surgery, since their risk of thrombosis is similar and it is impossible to segregate these patients in most trials. Table 1 reports the cumulative rate of DVT for the commonly used prophylactic regimens, obtained by pooled analysis of randomized studies published in the English literature which used the Fibrinogen Uptake Test (FUT) to detect venous thrombosis. There is only one more entry in respect of the corresponding list reported in the paper of Clagett et al.

The overall incidence of DVT is 25% in untreated control subjects (19% in trials in which positive leg scans were confirmed by venography); 29% in patients with malignancy. The incidence of proximal DVT in general surgery without prophylaxis is about 7%, while symp-
tomatic pulmonary embolism occurs in 1-2% of patients and fatal pulmonary embolism in 0.2-0.8%.\textsuperscript{1,40,42} Low-dose heparin has been evaluated versus placebo in 29 trials totalling more than 8,000 patients, and overall a 60% reduction of DVT has been found, the rate of positive leg scans decreasing from 25% to 8%. A statistical significant reduction of proximal DVT has also been reported in 3 out of the 5 larger trials, and already in 1975 Kakkar and coworkers had provided evidence of a 70% reduction of fatal pulmonary embolism in a study including more than 4,000 patients.\textsuperscript{43} These impressive results are obtained at the expense of a small increase of the rate of wound hematoma, while no increase of the incidence of major bleeding has been found in two metanalyses.\textsuperscript{1,44} Heparin is usually given in a dose of 5,000 IU b.i.d. or t.i.d. and although an adequate direct comparison is not available, the metanalysis of Clagett and Reisch suggests that the 8-hourly regimen is preferable, since it yielded an average DVT rate of 7.5% in 15 studies, as opposed to 11.8% in 34 studies of 12-hourly heparin, without inducing a greater bleeding risk.\textsuperscript{44}

LDH/DHE: although more effective than LDH alone in 4 studies in which the two regimens were directly compared, the cumulative data do not confirm the superiority of the drug combination; moreover, dihydroergotamine may induce vasospasm.

LMW heparins are the most effective means of thromboprophylaxis. Cumulative data in general surgery show a highly significant risk reduction of 50% versus LDH, at variance with the metanalysis of Nurmohamed et al.\textsuperscript{45} who did not find a statistically significant difference between the two modalities, when considering only the studies with strong methodology. Even accepting the conservative approach, LMWHs appear to be at least as effective and safe as LDH and have the advantage of once-daily injection. Among the different preparations on the market, nadroparine has been extensively studied in general surgery patients and has been found more effective than LDH in two studies,\textsuperscript{15,46} reported in Table 2.

A recent study by Bergquist and coworkers (not included in the table) provided evidence of a dose relationship of LMWH both with the frequency of thrombosis and bleeding complications.\textsuperscript{47} About 2,000 patients undergoing elective general abdominal surgery (66% because of malignancy) were randomized to prophylaxis with 2,500 or 5,000 anti-Xa units of dalteparin every evening, beginning 12 hours before surgery. Isotopic DVT was detected in 123 of the 975 patients in the 2,500 group (12.6%) and in 65 of the 975 in the 5,000 group (6.7%) (p<0.001). Bleeding complications occurred in 2.7% in the 2,500 group and in 4.7% in the 5,000 group (p=0.016).

Two large comparative trials evaluated clinical endpoints. Pezzuoli et al. randomized 4,498 patients to nadroparine 3100 anti-Xa units beginning 2 hours preoperatively or to placebo.\textsuperscript{42} Thromboembolic mortality was 0.09% in the LMWH group and 0.36% in the placebo group, while there were more bleeding complications (minor) and transfusion requirements in the LMWH group. Kakkar and coworkers randomized 3809 patients to Fragmin 2500 anti-Xa IU once daily beginning 1-4 hours before surgery versus LDH 5000 IU twice daily for 5 days. No difference in the rate of clinical venous thromboembolism but a lower rate of severe bleeding in the LMWH group was reported.\textsuperscript{48} Although effective even in the minidose regimen of 1 mg/day developed by Poller et al.,\textsuperscript{49} warfarin does not appear to be more effective than LDH or LMWH, and its use in prevention of DVT after general surgery cannot be recommended.

Dextrans and aspirin are less effective than LDH and LMWH and there is no ground for

<table>
<thead>
<tr>
<th>Study</th>
<th>Treatment</th>
<th># pat</th>
<th>DVT (%)</th>
<th>Bleeding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kakkar &amp;</td>
<td>Nadroparine</td>
<td>196</td>
<td>5 (2.6%)</td>
<td>10 (5.1%)</td>
</tr>
<tr>
<td>Murray, 1985</td>
<td>LDH (x2)</td>
<td>199</td>
<td>15 (7.5%)</td>
<td>7 (3.5%)</td>
</tr>
<tr>
<td>Encke &amp;</td>
<td>Nadroparine</td>
<td>960</td>
<td>27 (2.8%)</td>
<td>47 (4.9%)</td>
</tr>
<tr>
<td>Breddin, 1988</td>
<td>LDH (x5)</td>
<td>936</td>
<td>42 (4.5%)</td>
<td>42 (4.5%)</td>
</tr>
</tbody>
</table>

Table 2. Studies in general surgery patients showing a significant difference in efficacy between LMWH and LDH.
the use of either drug in this patient category.

Among the devices that enhance venous flow graded pressure elastic stockings fared equally well than intermittent pneumatic compression in the overall assessment of trials in general surgery, and due to their safety, low cost and simplicity may thus be preferred for low-risk patients and for all patients at risk of bleeding because of hematological problems or because of the type of surgery (e.g. urological surgery).

Orthopedic surgery

Orthopedic surgery is associated with a high risk of venous thromboembolism. We will examine separately elective hip replacement, hip fracture and total knee replacement. The tables include data from published randomized trials (either full-length reports or abstracts) using routine venography to screen for postoperative DVT. LMW heparins are grouped together. Many new entries are present in respect to the corresponding tables by Clagett et al. and only for these studies the references are given here. For older studies the reader is referred to the bibliography of the cited paper.

Elective hip replacement

In untreated patients the incidence of all DVT is about 50%, the frequency of proximal venous thrombosis 20%, and 5% of patients develop clinical pulmonary embolism (fatal in about 1%). As can be seen in Table 3, low-dose heparin, with or without dihydro-ergotamine, gives an inadequate protection, reducing the rate of DVT to 27%.

About the same is the overall failure rate of dextran, and even worse are the results obtained with aspirin. Elastic stockings have been evaluated in only two studies, with disappointing results, while the average incidence of DVT found in the pooled analysis of 5 studies employing intermittent pneumatic compression was an appreciable 22%. Best results are seen in the central part of the table, with the following regimens: adjusted low-dose heparin, LMW heparins, lomoparan and warfarin obtaining a relative risk reduction of about 70% and a failure rate ranging from 16% to 19%. Among these, LMW heparins or heparinoids appear to be the drugs of choice nowadays, since their application is simpler than adjusted dose heparin or warfarin, and they are no less effective. LMWHs have been extensively evaluated in this particular setting (15 clinical trials, for a total of about 2,500 treated patients), and among the various LMWHs enoxaparine has undergone considerable scrutiny, with consistent success. Other LMWHs have also been shown effective. Infusion of antithrombin III concentrates plus LDH has been employed by Francis and associates with excellent results, but this prophylactic modality is very expensive and cannot be recommended as a routine procedure.

Hip fractures

In post-traumatic hip surgery venous thrombosis may be triggered by bone fracture and may precede the operation. Advanced age and immobilization add to the risk and actually 20% of total in-hospital mortality after hip fracture is caused by pulmonary embolism. Pooled analysis of randomized trials in hip fractures using venography is reported in Table 4.

The incidence of DVT in untreated patients is 46%. Aspirin appears to be virtually ineffective in the trials using mandatory venography for
screening; 30% or more is the failure rate either with LDH (with or without DHE), or with dermatansulfate or with dextran (including studies where dextran was associated with DHE or aspirin [66]).

Better results have been obtained with warfarin (average DVT rate = 25%), and particularly with LMWHs and lomoparan, which gave a relative risk reduction of 60% (from 46% to 18% [67-74]). Prophylaxis is usually started soon after diagnosis and, if LMWHs are used, an interval of at least 8 hours should be left before surgery.

Knee surgery

Negligible after arthroscopy and intermediate after meniscectomy and synovectomy, the thrombotic risk peaks after surgery for total knee replacement (TKR). As can be seen in Table 5, not only DVT rate is very high in untreated patients, but it remains high with pharmacological prophylaxis, while interesting are the results obtained with intermittent pneumatic compression in 4 small trials.23,24,31,75

Results with the LMWHs are not homogeneous and are separately reported in Table 6.

Logiparin and RD heparin were recently evaluated versus warfarin in two large trials.61 Oral anticoagulants were associated with a failure rate of 55% (152/277) in the study of Hull et al., while the incidence of DVT in the Logiparin group was 45% (116/258). Proximal DVT were 12% and 8% respectively. In the RD heparin study, the failure rate was 41% for warfarin, 25% and 28% for the two regimens of LMWH respectively. Enoxaparin 30 mg b.i.d was compared to placebo in the study of Leclerc et al.,76 the failure rate being 19% in the LMWH group and 65% in the placebo group (8/41 patients vs 35/54, relative risk reduction=70%; P< 0.0001).

Proximal DVT was observed in 19% of the placebo patients and in none of the enoxaparin patients. In the study of Faunø et al.77 enoxaparine 40 mg once daily was compared with UFH 5000 IU t.i.d in 215 patients undergoing TKR: the failure rate was 23.3% in the LMWH group and 30.5% in the LDH group, proximal DVT being 3% and 5% respectively. In theory, the best results in total hip replacement should be obtained with a combination of IPC (preferably by means of the foot pump, which is more practical) and enoxaparin 30 mg/12 hours started the morning after operation.

Neurosurgery

Patients undergoing an elective intracranial operation face a risk of venous thromboembolism which is similar to that encountered by general surgery patients. Table 7 reports the main results of the pooled analysis of the randomized trials comparing several preventive strategies with placebo and using FUT to screen for DVT. The average incidence of deep venous thrombosis in untreated patients was 24%, most of which being calf DVT. Physical means of prophylaxis have been preferred for fear of serious intracranial bleeding.

Low-dose-heparin was successfully used in

| Table 4. DVT rates in hip fractures (routine venography). |
|-------------|----------------|--------|----------------|
| Regimen     | # trials | # pat | DVT Incidence | 95% CI |
| Untreated   | 9        | 370   | 171           | 46% 41-51 |
| LDH         | 6        | 217   | 73            | 34% 26-41 |
| LMWH        | 5        | 267   | 47            | 18% 13-22 |
| Lomoparan   | 3        | 250   | 42            | 17% 12-21 |
| Dermatan sulfate | 1 | 74 | 28 | 38% 27-49 |
| Oral anticoagulants | 6 | 301 | 74 | 25% 20-29 |
| Dextran     | 9        | 596   | 168           | 30% 26-34 |
| Aspirin     | 2        | 153   | 66            | 42% 35-51 |

| Table 5. DVT rates in total knee replacement | |
|-------------|----------------|--------|----------------|
| Regimen     | # trials | # pat | DVT Incidence | 95% CI |
| Untreated   | 4        | 128   | 86            | 67% 54-78 |
| Aspirin     | 2        | 50    | 27            | 54% 36-69 |
| LDH         | 1        | 92    | 28            | 30% 21-40 |
| LMWH        | 4        | 750   | 224           | 30% 27-33 |
| Warfarin    | 2        | 471   | 204           | 42% 39-48 |
| PC          | 4        | 105   | 25            | 23% 15-31 |
one study. All the others employed IPC, alone or associated with elastic stockings.\cite{27-32} IPC and elastic stockings were compared head to head in one study, the results being equivalent.\cite{27} The choice is between these two physical methods of prophylaxis.

**Acute spinal cord injury**

After acute spinal cord injury causing paraplegia, the incidence of clinical DVT and PE is 16% and 5% respectively. The risk is especially high in the first two weeks after trauma. Fatal pulmonary embolism is rare after 3 months.\cite{78,79}

The rate of DVT diagnosed by mandatory venography is reported in Table 8.

There is some evidence therefore, although based on a limited number of small studies,\cite{80-83} that both LMWH and LDH plus electrical stimulation are very effective.

**Medical conditions**

Low-dose heparin, LMWH, elastic stockings and aspirin have all been reported to prevent DVT in general medical inpatients,\cite{84-86} while prophylactic LDH has also been used successfully in intensive care units.\cite{87} Myocardial infarction and stroke however are the best studied medical conditions.

**Myocardial infarction**

The incidence of radioisotopic DVT in untreated patients is 24% (Table 9). Two trials evaluated high dose intravenous heparin (40,000 IU/day) showing good protection;\cite{88,89} four studies have evaluated LDH in different regimens (5000 IU/12h; 5000 IU/8h; 7500 IU/12h), 3 out of 4 showing a significant reduction of calf DVT.\cite{90-93}

All these trials were performed in the pre-thrombolytic era: antithrombotic treatment has changed nowadays, since it has been documented 1) that aspirin reduces mortality and therefore should be part of the treatment after acute MI, and 2) that after thrombolysis heparin should be given at higher than prophylactic dose, especially if t-PA is used, in order to prevent reocclusion of the infarct-related coronary artery. In anterior MI, low-dose heparin has no effect in preventing mural thrombosis, and 12,500 U/12h s.c. are required.\cite{94}

**Table 6. LMW heparins in total knee replacement.**

<table>
<thead>
<tr>
<th>Regimen</th>
<th># pat</th>
<th>all DVT (%)</th>
<th>95% CI</th>
<th>proximal DVT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Logiparin 75 anti-Xa U/Kg/24h</td>
<td>258</td>
<td>45%</td>
<td>39-51</td>
<td>6.9%</td>
</tr>
<tr>
<td>Warfarin post-op</td>
<td>277</td>
<td>55%</td>
<td>49-61</td>
<td>10.5%</td>
</tr>
<tr>
<td>RD heparin 50 anti-Xa U/Kg/12h</td>
<td>150</td>
<td>25%</td>
<td>14-34</td>
<td>6%</td>
</tr>
<tr>
<td>RD heparin 90 anti-Xa U/Kg/24h</td>
<td>149</td>
<td>28%</td>
<td>16-38</td>
<td>5%</td>
</tr>
<tr>
<td>Warfarin pre-op</td>
<td>147</td>
<td>41%</td>
<td>33-49</td>
<td>10%</td>
</tr>
<tr>
<td>Enoxaparin 30mg/12h</td>
<td>41</td>
<td>19%</td>
<td>7-31</td>
<td>0</td>
</tr>
<tr>
<td>Placebo</td>
<td>54</td>
<td>65%</td>
<td>52-78</td>
<td>20%</td>
</tr>
<tr>
<td>Enoxaparin 40mg/24h</td>
<td>108</td>
<td>23%</td>
<td>15-31</td>
<td>3%</td>
</tr>
<tr>
<td>LDH 5000 IU/8h</td>
<td>105</td>
<td>30%</td>
<td>21-38</td>
<td>5%</td>
</tr>
</tbody>
</table>

**Table 7. DVT rates in elective intracranial neurosurgery.**

<table>
<thead>
<tr>
<th>Regimen</th>
<th># trials</th>
<th># pat</th>
<th># DVT</th>
<th>Incidence</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>7</td>
<td>406</td>
<td>96</td>
<td>24%</td>
<td>20-28</td>
</tr>
<tr>
<td>IPC</td>
<td>6</td>
<td>362</td>
<td>24</td>
<td>7%</td>
<td>4-9</td>
</tr>
<tr>
<td>stockings</td>
<td>1</td>
<td>80</td>
<td>7</td>
<td>9%</td>
<td>2-15</td>
</tr>
<tr>
<td>LDH</td>
<td>1</td>
<td>50</td>
<td>3</td>
<td>6%</td>
<td>0-13</td>
</tr>
</tbody>
</table>

**Table 8. DVT rates in acute spinal cord injury.**

<table>
<thead>
<tr>
<th>Regimen</th>
<th># trials</th>
<th># pat</th>
<th># DVT</th>
<th>Incidence</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>4</td>
<td>68</td>
<td>48</td>
<td>70%</td>
<td>60-80</td>
</tr>
<tr>
<td>LDH</td>
<td>3</td>
<td>95</td>
<td>33</td>
<td>35%</td>
<td>21-42</td>
</tr>
<tr>
<td>LDH + el.st.*</td>
<td>1</td>
<td>15</td>
<td>1</td>
<td>7%</td>
<td>0-19</td>
</tr>
<tr>
<td>LMWH</td>
<td>2</td>
<td>68</td>
<td>8</td>
<td>12%</td>
<td>4-19</td>
</tr>
</tbody>
</table>

*el. st. = electrical stimulation

**Table 9. DVT rates in myocardial infarction (FUT).**

<table>
<thead>
<tr>
<th>Regimen</th>
<th># pat</th>
<th># DVT</th>
<th>Incidence</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>214</td>
<td>51</td>
<td>20%</td>
<td>18-30</td>
</tr>
<tr>
<td>Low-dose heparin</td>
<td>16</td>
<td>51</td>
<td>17%</td>
<td>3-10</td>
</tr>
<tr>
<td>High-dose heparin</td>
<td>70</td>
<td>3</td>
<td>4%</td>
<td>1-9</td>
</tr>
</tbody>
</table>
Ischemic stroke

Hemiplegia and hemiparesis are major risk factors for venous thromboembolism, and the rate of DVT after acute stroke has been reported as high as 60-75%. Although the use of LDH, alone or in association with DHE, has been shown to be partially effective in patients with acute ischemic stroke, the fear of hemorrhagic transformation of the infarct area deterred from its widespread application. Experience with dextran 40 was disappointing.

Limited so far the experience with LMWHs. Prins and associates randomized 60 patients to prophylaxis with Fragmin or with placebo. There were 6 DVTs and one PE in those on LMW heparin vs 15 DVTs and 2 PEs in those on placebo (p= 0.05). On the other hand, bleeding occurred in 5 of those on Fragmin vs 2 on placebo, and 9 of the LMW-treated patients died compared to 4 of those on placebo. In their double-blind randomized trial, Sandset et al did not find any significant difference (34% vs 36% respectively) in the incidence of DVT in 103 patients with acute ischemic stroke treated twice daily either with Fragmin or with placebo. Hemorrhagic transformation of the brain infarction was observed in 8% of patients in the LMW heparin group and 6% in the placebo group.

Good results have been obtained with Lomoparan, which at the dose of 750 anti-Xa units twice daily has been shown significantly more effective than placebo (DVT rate 4% in the active treatment group vs 28% in the placebo group) and than LDH (DVT rate 9% in the heparinoid group vs 31% in the LDH group) in two randomized double-blind studies by Turpie et al. One major hemorrhage in the Lomoparan group and one minor bleeding in the placebo group were observed in the first study, while the incidence of bleeding was 2% in each group in the second study. Hemorrhagic transformation of the infarct was found in 9.3% of the patients treated with Lomoparan, as compared to 5.6% treated with LDH 5000 u twice daily.

Dumas et al. recently reported on a third randomized double-blind trial with Lomoparan 1250 anti-Xa units twice daily versus LDH 5000 units twice daily in prevention of venous thromboembolism in 179 patients with acute ischemic stroke. DVT rate occurred in 15% of 89 patients receiving the heparinoid and in 19% of 90 patients receiving low-dose heparin. Hemorrhages in the infarct area occurred in two patients in each group. Currently, a large study (TOAST) with this LMW heparinoid in ischemic stroke is underway.

Final considerations and future developments

New information rapidly accumulates in this field and any recommendation is bound to short life. However, here is firm evidence to date that low-molecular-weight heparins and heparinoids are effective and safe in prevention of venous thromboembolism. This view is synoptically expressed in Table 10, where LMWHs have been suggested as first choice drugs for thromboprophylaxis in general surgery, in elective and traumatic hip surgery, and in spinal cord injury. The LMW heparinoid Lomoparan appears to be a good competitor in acute ischemic stroke and hip fracture.

The contention is often made that LMWHs are expensive. It has been shown however that if LMWHs are used in total hip arthroplasty instead of LDH, the costs for the health system would actually be lower, since a significant number of proximal vein thrombosis would be averted, and the attendant savings in treatment expenditures would have been greater than the increased drug cost for each treatment day.

Graded pressure elastic stockings should be considered for patients undergoing low-risk general surgery, for urological surgery and for other patients at intermediate thrombotic risk who have an-over-than-average risk of bleeding. Intermittent pneumatic compression, associated with LMWH or LDH, is advisable in knee implantation, while in neurosurgery IPC and/or elastic stockings have been shown safe and effective. An association of LMWH and either elastic stockings or IPC is a reasonable approach to patients at very high thromboembolic risk.

An important point that needs to be clarified by future studies is the optimal time of adminis-
Three of these - and a non significant best documented = Enoxa - and indeed the evening regimen before surgery or to a postoperative start, and have favored the move to a longer interval day administration, studies compared the once a day and the twice a dosage regimens no longer recommended early studies with LMWHs - given in high concentration of the LMWHs. Low-dose heparin has been traditionally given 2 hours before surgery, to contrast activation of coagulation occurring during surgery and preventing thrombus formation. The increased bleeding observed in early studies with LMWHs – given in high dosage regimens no longer recommended – have favored the move to a longer interval before surgery or to a postoperative start, and the latter approach proved effective in a number of nordamerican trials. Three of these studies compared the once a day and the twice a day administration, and a non significant trend towards greater efficacy in the b.i.d. regimen was apparent.

Considering the two preoperative regimens (2 hours and 12 hours before), it has been shown both in general surgery and in hip surgery that antithrombotic activity of LMWH (enoxapar) is fully maintained when prophylaxis is given on the evening before operation as compared with the usual preoperative morning dose, and indeed the evening regimen seems to be appealing.

Future developments may include a second generation of LMWH (hypersulfate, affinity or chemically modified LMWH, heparin conjugates), or other antithrombins like hirudin, the principal anticoagulant of the medicinal leech, Hirudo medicinalis, which has been synthesized by recombinant DNA techniques; hirulogs, synthetic and highly specific analogues of hirudin that bind tightly to thrombin's active site; argatroban, a reversible competitive inhibitor of thrombin; δ-phenylalanyl-L-propyl-L-arginyl-chloromethyl ketone (PPACK), an irreversible serin protease inhibitor that, unlike heparin, prevents platelet deposition and thrombosis in dacron arterial grafts; and argipidine, another powerful antithrombin roughly comparable to hirudin in its activity.

Experiments in animal models of thrombosis have also been performed with specific inhibitors of factor Xa such as antistasin, and with activated protein C.

New pharmacological tools potentially applicable to the arterial side of the planet thrombosis include simple peptides containing the RGD sequence (arginine-glycine-aspartic acid), which competitively inhibit fibrinogen binding to platelet glycoprotein receptors (IIb/IIIa complex) or monoclonal antibody reacting with the same target (7E3 antibody). 7E3 has already been successfully administered to patients with unstable angina pectoris or myocardial infarction.

References


82. Frisbie JH, Suharah AA. Low-dose heparin prophylaxis for DVT in acute spinal cord injury patients: a controlled study.
Prophylaxis of venous thromboembolism

Venous thromboembolism is the most common cause of preventable in-hospital death. Prevention of the two cardinal manifestations of venous thromboembolism, deep vein thrombosis of the lower limbs and pulmonary embolism are substantially based on routine prophylaxis with anticoagulant agents. Once established both deep vein thrombosis and pulmonary embolism require prompt and efficient treatment in order to reduce the morbidity and mortality connected with the disease.

The current treatment of venous thromboembolism

Anticoagulation is currently the standard initial treatment in over 90% of patients with venous thromboembolism. Treatment with anticoagulants reduces mortality from pulmonary embolism, recurrence of pulmonary embolism and deep vein thrombosis and morbidity from these acute events. The role of thrombolytic treatment has not been completely defined; whatever the reasons and regardless of their appropriateness nowadays less than 10 percent of patients affected by venous thromboembolism receive thrombolytic treatment. Common practice is to start anticoagulant treatment with 5 to 10 days of unfractionated heparin, followed by oral anticoagulants for 3 months. This therapeutic approach is quite effective and safe. Actually when this approach is adopted the recurrence rate of venous thromboembolism and bleeding side effect rate has been estimated to be approximately 5%. Unfractionated heparin is usually administered by a continuous intravenous infusion or intermittent subcutaneous injections and the dose is adjusted on the basis of daily laboratory measurements. Provided that is given in a adequate starting dose and that its dose is properly adjusted, subcutaneous unfractionated heparin is as effective as intravenous unfractionated heparin. The administration of heparin by subcutaneous injection offers an advantage over the intravenous routes because of its ease of administration, facilitation of early patient mobilization and the potential for outpatient management. For intravenous administration, heparin should be given initially as a bolus of 2500 to 5000 units followed by a continuous intravenous infusion of 30,000 to 45,000 units/day and adjusted to maintain the activated partial thromboplastin time (APTT) 1.5 to 2.5 times the control level. For the subcutaneous administration of heparin, an initial intravenous bolus of 3000 to 5000 units should be administered at the same time that the first subcutaneous dose is given; thereafter, subcutaneous injections of 15,000 to 20,000 units should be given at 12-hourly intervals to maintain the mid-interval APTT at 1.5 to 2.5 times the control. Using this regimen, therapeutic plasma heparin concentrations and prolongation of the APTT are usually maintained throughout the 24-hour period.

The evidence for the requirement of heparin in the initial treatment of deep vein thrombosis comes from animal studies and from a recent clinical trial. Actually, in this double-blind trial patients with proximal deep vein thrombosis were randomised to either standard intravenous heparin plus oral anticoagulants or to oral anticoagulants alone. The frequency of asymptomatic extension of deep vein thrombosis or pulmonary embolism was significantly lower in patients who had received heparin plus...
oral anticoagulants compared with those who had received anticoagulants alone. The group receiving heparin plus oral anticoagulation also showed a lower incidence of symptomatic events. There was no significant difference in the frequency of bleeding side-effects between the treatment groups. In the treatment of established deep vein thrombosis there is evidence that insufficient doses of unfractionated heparin are associated with early recurrence of thrombosis.6,7

The duration of heparin therapy in the treatment of deep vein thrombosis and pulmonary embolism has conventionally been seven to ten days, followed, in absence of specific risk factors for recurrence, by secondary prophylaxis for three to six months with oral anticoagulant therapy. In patients with deep vein thrombosis an alternative to this approach is to commence heparin and oral anticoagulants at the same time and to discontinue heparin when the prothrombin time reaches the desired therapeutic level on the fourth to fifth day. This regimen is based on the results of two randomized trials comparing initial heparin plus early oral anticoagulants with standard seven to ten days heparin in the treatment of proximal vein thrombosis.8,9 The results of both studies showed that the discontinuation of heparin after five days is safe provided that oral anticoagulation, started at the same time or shortly after the start of heparin therapy, was in the therapeutic range for more than 24 hours. Thus, this approach of short duration heparin therapy with oral anticoagulants can be recommended for the majority of patients with proximal deep vein thrombosis. However, the findings of the two studies may not be applicable to patients with large iliofemoral vein thrombosis or major pulmonary embolism since these two classes of patients were excluded from one study8 and formed only a small proportion of patients in the second study.9

**Limitations of unfractionated heparin**

Although unfractionated heparin is both highly effective and relatively safe in the management of patients with venous thromboembolism this agent is far from being the ideal antithrombotic agent. The limitations of unfractionated heparin are based on its pharmacokinetic, biophysical and antihemostatic properties.

The pharmacokinetic limitations of unfractionated heparin are mainly due to its non-specific binding to plasma proteins and endothelial cells. This binding influences the anticoagulant effect of unfractionated heparin by competing with antithrombin III for heparin binding and by affecting the rate of heparin clearance. Unfractionated heparin presents a non-specific non-functional binding to plasma proteins such as fibrinogen, factor VIII, vitronectin and fibronectin.10 The plasma levels of these proteins are quite variable and, being acute phase reactants, have been reported to be increased in very sick patients and in patients with thromboembolic diseases. The elevated levels of these plasma proteins contribute to the heparin resistance observed in sick patients. The non-specific binding of unfractionated heparin to plasma proteins results in a complicated mechanism of clearance, limits its bioavailability at low doses and is responsible for marked interindividual variations in dose response. This last limitation is of critical clinical importance as it make a close laboratory monitoring necessary and thus preclude the out-of-the-hospital management of patients with deep vein thrombosis.

Furthermore due to the high concentration of these plasma proteins in the very sick patient therapeutic plasma levels of heparin are difficult to achieve in pulmonary embolism patients that usually require a high dose of unfractionated heparin. The biophysical limitations occur because the heparin/antithrombin III complex is unable to access and inactivate thrombin bound to fibrin11 or to the endothelial surface and factor Xa in the prothrombinase complex.12

The limitations due to the non-anticoagulant antihemostatic properties of unfractionated heparin are caused by a poorly defined effect on vessel wall permeability as well as by an inhibitory effect of heparin on platelet function. The limitations of unfractionated heparin related to the pharmacokinetic and antihemo-
static properties are not or are partially shared by the low molecular weight heparins, while the limitations caused by the lack of accessibility of the heparin/antithrombin III complex to fibrin-bound thrombin and factor Xa are overcome by several new classes of antithrombin III-independent thrombin and factor Xa inhibitors.

**Low molecular weight heparins**

Low molecular weight heparins are fractions of commercial heparin with a mean molecular weight of 4,000 to 5,000 produced by controlled depolymerization of unfractionated heparin. Low molecular weight heparins have anti-factor Xa activity similar to that of unfractionated heparin but have minor effect on aPTT and thrombin clotting time. Because of their superior pharmacokinetic properties and lack of plasma protein binding, low molecular weight heparins are effective for the prevention and treatment of venous thromboembolism when given once or twice daily without laboratory monitoring.

Clinical experience suggests that low molecular weight heparins may cause less bleeding than unfractionated heparin for an equivalent antithrombotic effect. The different bleeding potential is more evident when higher doses of anticoagulants are required as is the case for treatment of deep vein thrombosis or pulmonary embolism.

The pharmacokinetics of low molecular weight heparin are more predictable than those of standard heparin and their elimination half life is longer in comparison to standard heparin. Furthermore, as demonstrated by a recently published study, hemostatic activation in patients with deep vein thrombosis is controlled early by fixed doses of a low molecular weight heparin than by unfractionated heparin. These properties make low molecular weight heparins excellent candidates for the treatment of venous thromboembolism enabling weight adjusted, fixed-dose subcutaneous low molecular weight heparins to be used in the initial treatment of venous thromboembolism.

**Comparison of low molecular weight heparins and unfractionated heparin in the treatment of venous thromboembolism**

Most of the clinical experiences with low molecular weight heparins in the treatment of venous thromboembolism has been conducted in patients with deep vein thrombosis while the studies in patients with pulmonary embolism are still preliminary.

Several randomised studies have shown that low molecular weight heparins given either intravenously with dose adjustments, or subcutaneously in fixed doses, are more effective than continuous intravenous adjusted-dose unfractionated heparin in reducing size of venous thrombi assessed by repeated venography. Recently, two randomized trials used clinical endpoints during long-term follow-up to determine the relative safety and efficacy of low molecular weight heparin in the treatment of deep vein thrombosis. Both studies reported a lower incidence of recurrent venous thromboembolism and major bleeding complications in patients treated with fixed dose subcutaneous low-molecular weight heparin compared with patients treated with standard intravenous heparin adjusted to maintain the APTT at 1.5 to 2.0 times control. In addition, both trials reported a lower incidence of mortality due to non-venous thromboembolism related causes in the low molecular weight heparin treated groups.

The results of two meta-analyses of clinical trials on the comparison of low molecular weight heparins and unfractionated heparin in the treatment of deep vein thrombosis have been recently presented. In the meta-analysis performed by Lensing, 13 clinical trials comparing low molecular weight heparins and unfractionated heparin (subcutaneous injection and intravenous infusion) performed between 1985 and 1992 and involving a total of over 1700 patients have been identified. Unfractionated heparin was administered mainly intravenously. In all except one of the trials low molecular weight heparins were administered by a twice daily injections while in the remaining, that was the largest one, a once daily injection was adopted. Thrombus evolution as judged by venography
was adopted as the endpoint in most of the trials. Venography was repeated 5 to 10 days after the start of heparin treatment. In 6 studies results were reported of long-term clinical follow-up, however, in 2 of these follow-up was not conducted prospectively at the study center. The combined results of the studies that compared the post- with the pre-heparin treatment venograms show that a reduction of thrombus size occurred in 64% of low molecular weight heparins treated patients versus 50% in patients treated with unfractioned heparin, whereas an increase in thrombus mass was observed in 6% and 12% of patients, respectively. These differences are statistically significant (p<0.001). For the individual low molecular weight heparin preparations, a statistically significant reduction of the thrombus mass was observed for nadroparine (p<0.001) and enoxaparine (p<0.003).

Prospective long-term clinical follow-up was evaluated when available. The analysis of the pooled results of these studies revealed a statistically significant reduction in thromboembolic complications (61%; p<0.005): 12 (2.7%) of the 439 low molecular weight heparins treated patients versus 31 (7.0%) of the 443 unfractioned heparin treated patients. For the individual low molecular weight heparin preparations, the risk reduction was statistically significant only for nadroparine (61%; p<0.04). There was a strong trend (risk reduction, 60%; p=0.08) in favor of tinzaparine.

Enoxaparine has thus far been studied in a single study; none of the 67 enoxaparine treated patients developed a thromboembolic complication, whereas this occurred in only 1 of the 67 patients of the unfractioned heparin group. All the studies recorded the incidence of bleeding complications and enabled a distinction in major and minor bleeding. The analysis of the pooled results of all studies indicated that major bleeding occurred in 6 (0.9%) of the 652 low molecular weight heparin treated patients and in 21 (3.2%) of the 656 unfractionated heparin treated patients, for a risk reduction of 68% (p<0.005). The risk reduction for major bleeding observed for the individual low molecular weight heparins was statistically significant for tinzaparine (91%; p<0.01), and showed a trend in favour of nadroparine (62%; p=0.11). A comparable frequency of major bleeding was observed for dalteparine (2 of the 95 patients, 2.1%) and unfractioned heparin (2 of the 97 patients, 2.1%). The combined analysis of all individual low molecular weight heparin preparations demonstrate a mortality rate during long-term follow-up of 4.3% (19 of the 439 patients) in the low molecular weight heparin group versus 8.1% (36 of the 443 patients) in the unfractionated heparin group, for a risk reduction of 48% in favour of low molecular weight heparins (p<0.03). The risk reductions of the individual low molecular weight heparin preparations which contributed to this analysis were consistent, but none of them reached statistically significance.

The results of this meta-analysis indicated that low molecular weight heparins administered subcutaneously at fixed body-weight adjusted doses are more effective than adjusted-dose intravenous unfractionated heparin in the initial treatment of deep vein thrombosis with an observed risk reduction of thromboembolic complications of 61%. The findings of improved effectiveness are further supported by venographic observations that showed an important reduction of thrombus size in favour of low molecular weight heparins. These results were homogeneous among the individual low molecular weight heparin preparations. Major bleeding occurred statistically significantly less with low molecular weight heparins than with unfractionated heparin with an observed risk reduction for major bleeding of 68%. In the studies in which patients were prospectively followed up mortality rates were consistently in favour of low molecular weight heparins. The pooling of results of these studies revealed a surprising reduction of 48% (p<0.03) in favour of low molecular weight heparins, which was mainly associated with malignancy.

The efficacy and safety of different doses of a low molecular-weight heparin in the treatment of submassive pulmonary embolism has recently been compared with intravenous unfractionated heparin in a prospective randomized, dose finding study.²⁷ The primary outcome was
the evolution of pulmonary vascular obstruction; 101 patients were enrolled in four treatment groups: unfractionated heparin by continuous intravenous infusion and nadroparine subcutaneously at three increasing doses. Doses of nadroparine of 160 IU/Kg are as effective and safe as intravenous unfractionated heparin. Inclusions in groups treated with higher doses of nadroparine were stopped because of the high incidence of major bleeding.

**Conclusions**

The results obtained with low molecular weight heparins in the treatment of deep vein thrombosis are quite attractive. The simplified patient care with low molecular weight heparins raises the possibility of out-of-hospital treatment for many patients with deep venous thrombosis. However, before this approach is adopted for routine use, its safety and efficacy should be demonstrated in randomised trials. At least two large clinical trial aimed at evaluating the efficacy and safety of the home treatment of deep vein thrombosis are currently running. Should these trials confirm the value of home-treatment of deep vein thrombosis such a radical change from current practice will reduce costs and improve patient convenience.

**References**


Venous thromboembolism is a serious and potentially lethal complication of deep vein thrombosis (DVT). Effective prevention of pulmonary embolism (PE) can be achieved by using pharmacological prophylaxis in high risk patients and partial caval vein interruption in selected cases.

The standard medical treatment of DVT with anticoagulant and fibrinolytic drugs aims to avoid PE and the recurrence of thrombosis. An additional goal is represented by prevention of the postphlebitic syndrome. The role of surgery in the treatment of venous thromboembolism is controversial. Interruption of the vena cava is an established measure for proximal DVT when every anticoagulation is contraindicated or has proven ineffective. Current availability of vena cava filters has generated interest for alternative, less invasive methods than thrombectomy or venous interruption.

In our experience, the medical treatment of DVT does not always lead to satisfactory results. We have therefore started the evaluation of alternative approaches to the treatment of acute proximal DVT to be used in addition to the standard anticoagulant and fibrinolytic drugs, involving the adoption of temporary or definitive filters.

**Integrated endoluminal treatment of DVT**

A major prerequisite of the integrated approach is the obtainment of an instrumental diagnosis allowing visualization of the thrombus and of its characteristics, with respect to potential for embolization and susceptibility to lysis. In selected cases of ilio-femoral DVT, urokinase plus heparin or streptokinase may be then administered loco-regionally with the adoption of percutaneous transvenous catheterization, with the advantage of reducing dosages and duration of treatment and increasing the rate of thrombolysis.

Because thrombus fragmentation may occur with devastating consequences, vena cava protection must be ensured during infusion of the active drug. We have developed a protocol of thrombolysis with the use of catheters provided with filters for caval protection (Temporary Catheter-Filter, Thery®, Filcard®, Lysofilter®, Antheor®, PIC Emanuelli®).

Our experience is based on the positioning of 263 vena cava filters (238 definitive; 25 temporary) of different types from October 1984 to February 1994.

**Fibrinolytic treatment**

Thrombolytic drugs were infused either systemically or locoregionally on 53 occasions in 50 patients – 25 males, 25 females, aged 20-81 years, subdivided in 4 groups according to the type of filters and the modality of treatment (Table 1).

Some patients were subjected to more than one fibrinolytic treatment and occasionally to a single treatment with different types of filter (definitive and temporary).

For systemic fibrinolysis, urokinase was always administered in conjunction with sodium heparin (15,000-30,000 U/day) at dosages ranging from 2,200 to 4,400 IU/kg/hour for 12 to 48 hours; streptokinase was infused at a dose of 100,000 IU/hour for 12 to 24 hours.

For loco-regional fibrinolysis, dosages were similar or slightly reduced, but treatment dura-
Thrombolytic treatment and caval filters in venous thromboembolism

Fibrinolysis performed after placement of a definitive caval filter in patients with acute DVT (+ PE) to reduce the size of the thrombus

Fibrinolysis performed after placement of a temporary caval filter in patients with a acute DVT (+ PE) to reduce the size of the thrombus

Fibrinolysis performed at some distance from placement of a definitive caval filter because of filter-associated thrombosis or embolism

Fibrinolysis performed in patients with a temporary caval filter who had already received treatment but then developed a thrombotic obstruction requiring supplementary treatment before filter removal

Outcome of treatment

The outcome of fibrinolytic treatment in the 50 patients enrolled in this study is shown in Table 3. Some degree of lysis was consistently obtained in all patients. Complete recanalization of the venous segments involved by thrombosis was observed in 57% of the cases. After the initial infusion of fibrinolytic agents, two patients required the positioning of a definitive filter.

Follow-up and complications

Long term follow up of patients consisted of regular visits scheduled 3, 6 and 12 months from caval filter insertion and then once every year. On such occasions, patients underwent laboratory and instrumental evaluation, consisting of frontal and lateral abdominal radiograms during inspiration and of venous doppler examination (+color-doppler). Part of the patients were also subjected to cavography or computerized tomography for evaluation of proximal DVT and for the monitoring of short-term and long-term complications of caval filters, such as tilting or caval transfixion. Pulmonary perfusion scintigraphic scans were performed only in patients with previous PE.

A total of 11 complications were observed. Complications strictly related to the fibrinolytic treatment included the occurrence of one retroperitoneal hematoma, of hematomas at the site of catheter insertion and of one death for cerebral hemorrhage. Long-term complications included two episodes of pulmonary microem-
bolization (only observed with the LGM filter) and filter leg rupture (two cases with the Greenfield filter, one case with the Gunther filter and one case with the Filcard filter). Long-term follow-up of the patients is still ongoing, to permit evaluation of the functional integrity and duration of the definitive filters.

Conclusions

The venous percutaneous approach, when conducted by trained operators in specialized centers, allows the insertion of the filter even in high-risk patients ensuring adequate protection from embolism and satisfactory patency of the inferior vena cava.

The influence of definitive filters on the occurrence of moderate postphlebitic syndrome is of difficult evaluation. Caval filters must not be indiscriminately positioned, but careful selection of patients is required. Pulmonary embolism is a dangerous event also because of uncertainty about the best therapeutic choice.

We believe that it is important to balance accurately the selection of the currently available means of treatment to achieve optimal results.

References

5. Abstracts from the International Symposium of Vena Cava Filters (Poitiers-France October 9th-10th 1992).
SECONDARY PROPHYLAXIS OF VENOUS THROMBOEMBOLISM: RATIONAL USE OF ORAL ANTICOAGULANTS

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It is common practice to treat patients with deep venous thrombosis with heparin, followed by long-term oral anticoagulants to prevent recurrences. These recommendations are based on early evidence that patients treated with oral anticoagulants after discharge from hospital had a lower frequency of recurrent thromboembolic disease than a group that did not receive long-term anticoagulant therapy, and on subsequent more recent randomized studies.

Oral anticoagulants (warfarin and dicumarol) are vitamin K antagonists, which produce their anticoagulant effect by interfering with the cyclic interconversion of vitamin K and its 2,3-epoxide (vitamin K epoxide). Inhibition of this process leads to the depletion of vitamin KH2 and results in the production of hemostatically defective vitamin K-dependent coagulant proteins (prothrombin, factor VII, factor IX, and factor X). Oral anticoagulants are effective in the primary and secondary prevention of venous thromboembolism. Coumarins are indicated in patients with deep vein thrombosis, either proximal or distal, and shall be given for three to six months, although the optimal duration of oral anticoagulant therapy at present remains uncertain. Patients with venous thromboembolic disease have a high risk of recurrence if untreated, or if treated with subtherapeutic doses of anticoagulants. Recurrences would be reduced if anticoagulant therapy were continued indefinitely in all patients, but with this approach most patients would be exposed to the unnecessary inconvenience, risk, and expense of anticoagulant therapy.

Treatment with oral anticoagulants is indicated in patients with thromboembolic disease, after heparin therapy. Heparin should be administered intravenously as an initial bolus of 5,000 to 10,000 IU, followed by a continuous infusion of 1,000 to 1,500 IU/h. The rate of heparin infusion should be adjusted so that the activated partial thromboplastin time (aPTT) is approximately 2 to 2.5 times the control value. Heparin treatment should be maintained for at least 5 to 8 days.

Oral anticoagulants are administered during the first week of treatment with heparin and may be started as early as the first day of heparin treatment. Early introduction of warfarin on day 1 or 2 with a small loading dose (10 mg) will usually keep the total duration of heparin therapy at no more than seven days. It is important to overlap heparin treatment with oral anticoagulant therapy for at least 4 to 5 days. As with heparin, a minimum level of anticoagulation with oral anticoagulants seems necessary to achieve the antithrombotic state.

In the past, the suggested therapeutic range for oral anticoagulants was a prothrombin time prolongation between 1.5 and 2.5 times the baseline value (International Normalized Ratio (INR) of 3.0 to 7.0).

Evidence for multiple studies over the last decade indicates that an effective level of anticoagulation in DVT is reflected by a PT prolongation by an INR of 2.0 to 3.0 the baseline value, with marked reduction of the risk of bleeding (from 22.4% to 4.3%). Bleeding that occurs when the INR is less than 3.0, is frequently associated with an obvious underlying cause or an occult gastrointestinal or renal lesion.
Monitoring anticoagulant therapy

The prothrombin time test (PT) is the most common method used for monitoring oral anticoagulant therapy. The test is performed by adding calcium and thromboplastin to citrated plasma. The PT is responsive to depressions of three of the four vitamin K-dependent procoagulant clotting factors (prothrombin, factor VII and factor X) at a rate proportionate to their half-lives. During the first few days, the PT reflects primarily the depression of factor VII, which has a half-life of only six hours. Subsequently, the test is prolonged also by depression of prothrombin and factor X. Recently, there was concern that a more precise method was needed to assess the intensity of anticoagulation with oral anticoagulants. Commercial thromboplastins have different potencies and markedly affect the resulting PT. The INR method is gradually being adopted by hospital laboratories and clinicians. In this reporting method, the patient PT is compared to the mean PT for a group of normal individuals. The ratio is adjusted for the sensitivity of the laboratory’s thromboplastin determined by the International Sensitivity Index (ISI). Thus the INR=(PT\text{patient}/PT\text{normal})ISI. The use of INR permits physicians to obtain the appropriate level of anticoagulation independent of laboratory reagents and to follow published recommendations for intensity of anticoagulation.

PT monitoring is performed daily until the therapeutic range has been achieved, then twice or three times weekly for 1 to 2 weeks, then less often, depending upon the stability of PT results. If the PT response remain stable, the frequency of testing can be reduced to intervals as long as every 4 to 6 weeks. If adjustments to the dose are required, then the cycle of more frequent monitoring is repeated until a stable dose is again achieved. Unexpected fluctuations in dose response could be due to change in diet, inaccuracy in PT testing, undisclosed drug use, poor patient compliance, patient self-medication, or alcohol consumption. The observable anticoagulant effect that follows warfarin administration is delayed from 2 to 7 days, depending upon the dose administered. A practical approach is to give 10 mg of warfarin on day 1 and on day 2, to perform an INR test on day 3, and adjust the dose to 2.5-7.5 mg/day, according to PT, until INR is stable and in the therapeutic range. INR should be monitored three times in the first week of treatment, two times in the second week and then once every two to three weeks. If the PT response remains stable, the frequency of testing can be reduced to intervals as long as every 4 to 6 weeks.

Concurrent medication can influence the effect of warfarin on hemostasis by augmenting or inhibiting its anticoagulant effect or by interfering with platelet function. Table 1 reports drugs known to interfere with warfarin. In summary, a patient treated with warfarin should be receiving as few other drugs as possible, should use alcohol not at all or only moderately, and would be consuming a diet that contains a decreased but constant amount of vitamin K.

Table 1. Substances that interfere with warfarin.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Known to potentiate anticoagulant effects</strong></td>
<td></td>
</tr>
<tr>
<td>Allopurinol</td>
<td>Hypermetabolic states</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>Impaired vitamin K absorption</td>
</tr>
<tr>
<td>Anabolic steroids</td>
<td>Liver disease</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>Low intake of vitamin K</td>
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<tr>
<td>Chloramphenicol</td>
<td></td>
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<td>Clotrimazole</td>
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<td>Erythromycin</td>
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<td>Fluconazole</td>
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<td>Isoniazide</td>
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<td>Ketoconazole</td>
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<td>Metronidazole</td>
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<td>Omeprazole</td>
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<td>Phenylbutazone</td>
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<td>Phenytoin</td>
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<td>Pimozide</td>
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<td>Quinidine</td>
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<td>Sulfapyridine</td>
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<tr>
<td>Tamoxifen</td>
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<tr>
<td>Thiosemicarbazone</td>
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<tr>
<td>Tolbutamide</td>
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<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td></td>
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<tr>
<td>Vitamin E (high dose)</td>
<td></td>
</tr>
<tr>
<td><strong>Known to inhibit anticoagulant effects</strong></td>
<td></td>
</tr>
<tr>
<td>Barbiturates</td>
<td>Alcohol</td>
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<tr>
<td>Carbamazepine</td>
<td>Increased intake of vitamin K</td>
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<tr>
<td>Cholestyramine</td>
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<td>Clofibrate</td>
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<td>Vitamin E (high dose)</td>
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</table>
Oral anticoagulant in the treatment of deep vein thrombosis

The first study that established the need for oral anticoagulants in patients with deep vein thrombosis or pulmonary embolism was reported by Coon and Willis. The study was retrospective, and utilized clinical parameters only for the diagnosis of initial and recurrent thromboembolism, nevertheless it formed the basis for continuing anticoagulant therapy after hospital discharge. In the past, the suggested therapeutic range for anticoagulation was a PT prolongation between 1.5 and 2.5 times the baseline value, utilizing rabbit brain thromboplastin as reagent (INR of 3.0 to 7.0).

Evidence from multiple studies over the last decade indicates that an effective level of anticoagulation in venous thromboembolism is reflected by a PT prolongation by an INR of 2.0 to 3.0 the baseline value.4,7,14-16 The findings of all of these trials showed that less intense anticoagulation with warfarin results in few bleeding complications yet gives adequate protection against recurrent thromboembolism. The optimal duration of OAC therapy for patients with DVT is therefore unclear. Patients with a first episode of DVT often receive 3 months of warfarin after initial heparin therapy.

Patients who develop recurrent venous thrombosis without provocation after a 3 months course of adequate anticoagulant therapy have a high risk of recurrence unless their recurrent episode is treated with warfarin long-term. The duration of anticoagulation for venous thromboembolism should probably be tailored to the individual patient. Patients with known, slowing resolving risk factors (prolonged immobilization) should be treated for at least three months; patients with malignancy, antithrombin III, protein C or S deficiency, resistance to activated protein C, homocystinuria, lupus-like anticoagulant, or recurrent venous thromboembolism should be treated indefinitely. Attempts to reduce OAC therapy duration to 4 weeks, based on the hypothesis that early normalization of impedance plethysmography defines a group of patients at low risk for recurrent DVT, were unsuccessful.17

Complications

Complications of warfarin therapy are not uncommon. The most frequent problem is hemorrhage, which usually develops as an extension of warfarin’s physiologic effect.8,19 Hemorrhage is related to prolongation of the PT. There is now evidence that bleeding is reduced when the therapeutic range is reduced from an INR of 3.0 to 4.5 to an INR of 2.0 to 3.0. Is now accepted that anticoagulation above 3.0 is unnecessary.

For an individual patient, the cumulative risk of bleeding is directly related to the length of anticoagulant therapy. It is likely, however, that the risk of bleeding varies during the course of anticoagulant therapy.21 Two studies reported higher frequencies of bleeding early in the course of therapy.23,24 In one of these studies, the frequency of major bleeding was reported to be 3.0% during the first month, and it decreased to 0.8%/month during the rest of the first year of therapy, and to 0.3%/year thereafter.23 If clinically indicated, warfarin’s effects can be reversed within 24h by parenteral administration of vitamin K. If bleeding is very serious, it can be treated with fresh frozen plasma. Warfarin-induced bleeding is increased by concomitant aspirin use, which both impairs platelet-function and produces gastric erosions; by age over 65; by a history of stroke or gastrointestinal bleeding. Table 2 summarizes the risk of bleeding related to anticoagulant therapy intensity.25

Another well known but uncommon complication of oral anticoagulant therapy is a vascular purpura that causes skin necrosis and occurs predominantly in women during the first weeks of treatment.26-28 It affects areas of abundant fatty tissue, such as in the breasts, buttocks, or thighs. This complication has been associated with protein C deficiency and malignancy.29-31

Coumarin therapy is implicated in the genesis of fetal malformations if given in the first trimester.22,31 Warfarin crosses the placenta and its administration should also be avoided during the last 3 weeks of gestation because of the danger of fetal and maternal hemorrhage at delivery, or soon thereafter. Therefore, oral anticoagulants should not be administered during pregnancy. The therapy of choice in these cases should be heparin, given subcutaneously,
in an adjusted dose to prolong aPTT beyond 1.5 times control. Warfarin could be given in the post-partum period, since it is not excreted in breast milk. 

Patient in chronic warfarin treatment may require surgery or invasive procedures. This represents a problem with two possible approaches. The first is to lower the dosage of warfarin to keep at an INR of approximately 1.5. The second is to reverse the warfarin effect with a small dose (1-2 mg) of vitamin K by subcutaneous injection, and to operate under a prophylactic cover of low dose heparin, 5,000 I.U. 2 hours preoperatively, and then every 12 hours, postoperatively. Warfarin can be reintroduced in the post-operative period. An assessment of the risk of recurrent thrombosis, if anticoagulants are stopped, versus the risk of excessive bleeding, if they are not stopped, should be made in every case.

**Conclusive remarks**

As final practical consideration, we suggest to begin oral anticoagulant therapy early during heparin administration, with a small loading dose of about twice the average maintenance dose. Heparin is discontinued when the INR is in the therapeutic range (2.0-3.0). A small reduction in the INR should be anticipated when heparin is discontinued, as heparin is often responsible of a prolongation of the INR. INR is monitored daily for the first 5-6 days, then twice weekly for two weeks, then less often, depending on the stability of PT results. If INR remains stable, the frequency of testing can be reduced to every 3 to 5 weeks. One approach is to treat all patients having a first episode of proximal venous thrombosis with an initial course of heparin, and then to continue oral anticoagulants for three months in the absence of continuing risk factors. It is common practice to treat the first recurrence with a 6-12 month course of anticoagulants and subsequent recurrences with anticoagulants for an indefinite period of time. Oral anticoagulant therapy is continued indefinitely: a) in patients who have more than one recurrent episode of venous thromboembolism; b) in patients with venous thromboembolism who have a continuing risk factor such as a deficiency of ATIII, protein C or protein S, a circulating antiphospholipid antibody or malignant disease; c) in patients with thromboembolic pulmonary hypertension.

**References**

The clinical effectiveness of oral anticoagulant drugs for a variety of indications has been established in well-designed studies. Oral anticoagulants are effective in the primary and secondary prevention of venous thromboembolism, and in preventing systemic arterial embolism in patients with atrial fibrillation or with tissue or mechanical prosthetic heart valves. It has been shown that myocardial infarction can be prevented by oral anticoagulants in patients with peripheral arterial disease. In patients with myocardial infarction, oral anticoagulants have proven effective in reducing death or recurrence of vascular events both in-hospital and for a long period of time after the event. Patients with valvular heart disease or dilated cardiomyopathy may also benefit from anticoagulant therapy.

In all these indications, effectiveness emerges in spite of the burden of severe bleeding complications which are the major side effect of anticoagulant treatment. The attempt to lower the incidence of bleeding still represent the major challenge of basic and clinical investigators working in the field of anticoagulant drugs. Because of the clear relationship existing between the prolongation of the prothrombin time induced by vitamin K antagonists and the occurrence of intracranial hemorrhage, increasing attention has been given to the central role of laboratory monitoring of anticoagulant treatment. A major progress was represented in the early eighties by the introduction of the model of reagent calibration known as the International Normalized Ratio (INR) system. The model is based on the linear relation between the logarithm of the prothrombin time ratios obtained with reference and test thromboplastins; the degree of anticoagulation is reported by converting the prothrombin time ratio measured with any thromboplastin into an INR that would be obtained if the WHO reference thromboplastin were used to assess the prothrombin time. The INR is calculated by raising the prothrombin time ratio to an exponent value which is characteristic of the thromboplastin reagent used and measures its responsiveness to reduction of the vitamin K-dependent clotting factors (International Sensitivity Index, ISI). By definition, the WHO reference thromboplastin has an ISI of 1.0. Since the introduction of the INR system, confidence in the safety and efficacy of oral anticoagulant treatment has grown because of the feeling of good comparability of the degree of anticoagulation reported by different laboratories when measuring the clotting time prolongation of the same plasma samples. During the last decade, the number of patients on oral anticoagulant treatment has steadily increased, in parallel with the new indications for treatment emerging from the literature. The rational basis for transformation of the prothrombin time into the international normalized ratio is sound and represents a relevant achievement of classical standardization strategies. An exercise conducted in the Emilia region has shown that between laboratory comparability of results was significantly improved by substitution of prothrombin activity with INR values. It should however be kept in mind that the clinical efficacy of this procedure in reducing the undesired side effect of bleeding while maintaining effective antithrombotic activity was never proven.

In this paper, the problems related to the implementation of the INR system in the monito-
ring of oral anticoagulation will be critically reviewed and future perspectives regarding more sensible tools for the detection of side effects and potentially useful laboratory tests will be outlined.

**Pitfalls of the INR system**

Uncertainty about the actual intensity of anticoagulation achieved with any prescribed prothrombin time ratio may expose patients to unnecessary risks of bleeding or thromboembolism. The effect of this uncertainty on the benefits and risks of lifelong anticoagulation in patients with prosthetic heart was recently explored by decision analysis. In that paper, the average annual bleeding rate as a function of the INR was extrapolated from the rates of bleeding episodes observed in randomized studies which had explicitly examined the risk of bleeding with different intensities of oral anticoagulant therapy and had involved a total of more than 1,000 patient\-years. Because in none of these studies was there a statistically significant difference between the two groups tested in the incidence of thromboembolic events, the relation between the intensity of anticoagulation and a weighted average annual rate of thromboembolic events was deduced from studies in which the patients did or did not receive anticoagulant therapy, assuming a declining exponential shape for the curve with a flat tail beyond the mean INR of 5.9 (Figure 1). The authors’ conclusions are to recommend that international normalized ratios are reported in the monitoring of anticoagulant treatment or that alternatively the same standard thromboplastin is used in all laboratories. From the formulas and correction factors provided by Eckman et al for the calculation of the risks of bleeding and embolization, the nadir of the curve relating permanent neurologic impairment and death from either cause (hemorrhagic or embolic) to the degree of anticoagulation is predicted at

![Figure 1. Relationship of the occurrence of major bleeding (open circles) and all thromboembolic episodes (closed circles, panel A) and of either death or stroke by bleeding (open circles) or thromboembolism (closed circles, panel B) with INR values in patients on oral anticoagulant treatment according to the metaanalysis of Eckman et al. (37). The continuous curves with no symbols indicate the cumulative incidence of events by bleeding and thromboembolism. The nadir of such curves occur respectively at mean INR of 2.0 and 3.0 with corresponding annual rates per year of 10% and 29%. Assumptions regarding hemorrhage were that about 20% of all bleeding episodes are major, that approximately 5% of major bleeding events result in permanent neurologic impairment and that roughly 7% of major bleeding episodes are fatal. Assumptions regarding thromboembolism were that the annual rate of thromboembolic events is 18% for patients not on anticoagulant therapy and 1.4% for those receiving anticoagulant therapy and that 18% and 30% of all thromboembolic events result in either death or permanent cerebral sequelae.](image-url)
an INR of 3.1 with a cumulative event rate close to 3.0 percent per year (Figure 1). Ironically, the results of a multicenter trial which appeared only two weeks before in the same prestigious journal\textsuperscript{38} were quite different. In this randomized, double-blind, placebo-controlled trial, the efficacy and safety of adding low-dose aspirin daily to warfarin treatment (target INR 3.0-4.5) was assessed in 370 patients with mechanical heart valves or with tissue valves plus atrial fibrillation or a history of thromboembolism. The mean INR observed in the group treated with warfarin only was 3.1, a value predictably associated with the lower attainable incidence of major adverse events.\textsuperscript{37} Nevertheless, major systemic embolism, intracranial hemorrhage or death from hemorrhage or vascular causes occurred at a rate of 10 percent per year, a figure expected for INRs lower than 1.2 or higher than 4.8 based on the analysis of Eckman et al.\textsuperscript{37} (Figure 2). When considering single-centre studies, the incidences of death or stroke with permanent sequelae are highly variable;\textsuperscript{39-42} at variance with the Canadian trial, data obtained in one center for a comparable number of patient-years indicate a much lower incidence of major adverse effects (0.15 percent per year) at a mean INR of 3.7.\textsuperscript{41} Comparison of two target INR levels in a randomized trial may be inappropriate to test the intensity of anticoagulation which offers the optimum result in terms of balance between effect and side-effect; studies evaluating the risk of untoward events at different achieved intensities may better look for optimum effect. Since INRs vary considerably over time in individual patients, a method of analysis based on time-spent-at-each-INR has been advocated\textsuperscript{41} and its adoption may be at least partly responsible for the different mean INR value at which nadir rates of death or stroke with permanent sequelae were observed in two of the studies reported in Figure 2.\textsuperscript{40,41}

However, there are few other reasons which may explain why simple adoption of INRs does not result in satisfactory control of anticoagulant treatment. A significant imprecision in the estimate of the true INR is anticipated on mathematical grounds for thromboplastins with high ISI, as it is the case with most of the reagents used in North America.\textsuperscript{44} In addition, the thromboplastin sensitivity may change significantly depending on the equipment used for clot detection,\textsuperscript{45} a phenomenon also observed – albeit to a lesser extent – with activated partial thromboplastin reagents.\textsuperscript{46} To overcome these problems, manufacturers have been urged to provide ISI values adapted to the method used for clot detection. Still, in an exercise conducted in 1992 among 52 Italian anticoagulation clinics affiliated of the Federazione Centri Sorveglianza Anticoagulati, the INRs measured on a lyophilized pooled plasma taken from patients on warfarin ranged from 1.8 to 4.8 (mean±2 SD), indicating that either an increase or a decrease in the dosage of warfarin would have been recommended to the same patient depending on the laboratory involved (Figure 3). Similar observations of discrepant commercial ISI assignment leading to failure of the INR system to generate consistent results and calling for improvements

![Figure 2. Annual rates of death and stroke with sequelae in patients with prosthetic heart valves according to the mean INR. The dashed line represents the rate expected by the metaanalysis of Eckman et al (37). Annual rates range from 0.3% to 9.9. The reported studies are ref. 41 (open circles), ref. 42 (closed circle), ref. 39 (open triangles), ref. 40 (closed triangles), ref. 38 (placebo group, open square; aspirin group, closed square). When INR were not reported an average ISI of 2.1 was assumed (44).](image-url)
Oral anticoagulants

in the reporting of the prothrombin time have appeared in the literature. At the present time, the suggestion to use the same standard thromboplastin in all laboratories would seem more justified.

Although variable, the incidence of death or stroke with permanent sequelae in single centre studies is clearly lower than in the Canadian multicenter trial, indicating a beneficial effect of anticoagulation clinics irrespective of interlaboratory comparability of prothrombin time determinations. This beneficial effect has been recently demonstrated in an Italian study comparing the incidence of severe bleeding or thromboembolism in patients on oral anticoagulant treatment with prosthetic heart valves before and after referral to the anticoagulation clinic. Five-fold and ten-fold reductions in the rates of major bleeding and of clinically apparent thromboembolism were observed after referral.

In our centre, where monitoring of oral anticoagulant treatment is performed in over 500 patients, the rates of adverse events observed over the last two years were 15.5% for all bleeding episodes (0.5% for major bleeding) and 0.7% for thromboembolic episodes at a mean INR of 2.6. The quality of individual oral anticoagulant treatment – defined on the basis of the percentage of INR determinations falling within the therapeutic range – was evaluated in patients on stabilised lifelong treatment, resulting optimal or good (75% or more of INRs within the desired range) in 43% of patients and poor (<50% INRs within the desired range) in 21%. Since both acenocoumarol and warfarin are administered to our patients, direct comparison of the quality of treatment obtained with the two drugs could be performed (Table 1). Similar to our colleagues from Parma, we found that the quality of treatment was superior with warfarin – a drug with a longer lasting activity – than with acenocoumarol. Judging from the observation of INR values both above and below the therapeutic range (Figure 4), a greater incidence of bleeding and thromboembolic complications would be anticipated with acenocoumarol; however, it is not known whether adverse effects do actually occur more frequently in patients receiving the latter drug. Another matter of confusion is represented by the pattern of seasonal variation in INR values, because conflicting reports have been published. Once again, it is not known whether such variations in the response of the liver to vitamin K-antagonists are relevant to the occurrence of clinical complications.

Because bleeding events – at least minor episodes – are relatively frequent and influence

Table 1. Quality of individual treatment in patients on lifelong oral anticoagulant therapy referred to the anticoagulation clinic of the S. Raffaele Hospital according to the anticoagulant drug administered. The percentage of patients falling within each quality level is reported.

<table>
<thead>
<tr>
<th>Quality of treatment* (% of INR values within therapeutic range)</th>
<th>Warfarin (n=86)</th>
<th>Acenocoumarol (n=98)</th>
<th>p</th>
</tr>
</thead>
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<tr>
<td>optimal (&gt; 90%)</td>
<td>33%</td>
<td>6%</td>
<td>&lt;.0001</td>
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<tr>
<td>good (76%-89%)</td>
<td>27%</td>
<td>23%</td>
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<tr>
<td>adequate (51%-75%)</td>
<td>30%</td>
<td>42%</td>
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<tr>
<td>poor (&lt; 50%)</td>
<td>10%</td>
<td>30%</td>
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*defined as in reference 42. Average dosages (weekly pills) of warfarin and acenocoumarol were significantly different in the two groups of patients (warfarin, 6.2 ± 2.8 vs acenocoumarol, 4.0 ± 2.8, p<.0001).
perception of health in patients on lifelong treatment, there has been lately a tendency to reduce the target INR for all the indications of oral anticoagulant therapy.\textsuperscript{55} Mini doses of warfarin can lower the plasma levels of critical vitamin K-dependent clotting factors,\textsuperscript{56} a rationale for their effectiveness in preventing thromboembolism after major surgery or during chemotherapy for metastatic cancer.\textsuperscript{53} There is no doubt that bleeding complications occur rarely with warfarin dosages scarcely prolonging the prothrombin time.\textsuperscript{57} However, failure of mini-doses to protect from postoperative deep vein thrombosis has been observed in high risk settings,\textsuperscript{58,59} indicating the need for very low to very high intensities of anticoagulation depending on the thrombogenic stimulus.

Tests reflecting the \textit{in vivo} clotting tendency may permit tailoring of treatment schedules to the individual patients, a goal simply not achievable with the INR system.

**Prothrombin fragment 1+2: a potential tool in the monitoring of anticoagulant therapy**

To the best of our knowledge, anticoagulation exerts beneficial effects through the inhibition of \textit{in vivo} thrombin formation. Specific assays are available permitting early detection of increased \textit{in vivo} thrombin formation or activity.\textsuperscript{60} Prothrombin fragment 1+2 (F1+2) is a large gamma-carboxylated glutamic acid containing peptide (> 20 kD) released from the aminoterminal end of prothrombin when the zymogen is activated to thrombin by factor Xa. Fibrinopeptide A is a small peptide (< 1.5 kD) cleaved from the alpha-chain of fibrinogen by thrombin. The two peptides have different half-lives and their measurement is differently affected by pre-analytical variables. As a tool to monitor the intensity of anticoagulation, measurement of F1+2 should be preferable to fibrinopeptide A for a number of reasons. Firstly, F1+2 levels are directly related to prothrombin activation and are not dependent on the mechanisms of inhibition of thrombin activity within the circulation. Secondly, the very short half-life of fibrinopeptide A (few minutes) makes its measurement scarcely suitable to evaluate a chronic
anticoagulant effect. Thirdly, there is a large body of evidence indicating that reliable measurement of F1+2 is relatively insensitive to preanalytical artifacts like the quality of venipuncture and does not require adoption of sophisticated (and expensive) anticoagulant mixtures.

To evaluate the potential of F1+2 as a marker of the in vivo activity of anticoagulant drugs, we performed studies both in the early and the stable phase of oral anticoagulant treatment. The changes in INR and in the plasma levels of prothrombin fragment 1+2 and vitamin K-dependent clotting factors II, X and VII were measured in patients starting warfarin on the first postoperative day after heart valve replacement. Despite the early attainment of INR values greater than 2.0, the originally elevated F1+2 levels did not decrease until day 3 of therapy with normalization on day 6. Qualitatively similar results were obtained in healthy volunteers taking warfarin, with a delay of 3 days between attainment of therapeutic INR values and decrease of F1+2 levels below the normal range. These data show that during the early phase of treatment, oral anticoagulants do not prevent in vivo prothrombin activation until after the drop of factor II. The prolonged lag-phase before effective inhibition of prothrombin activation may explain failure of acenocoumarol alone to prevent symptomatic extension or recurrence of venous thromboembolism in the initial treatment of proximal-vein thrombosis, suggesting that in patients requiring prompt and effective antithrombotic treatment, overlap of full-dose heparin with warfarin should be continued for at least 72-96 hours after the achievement of therapeutic INR values.

At the present time, three companies manufacture F1+2 ELISA kits, which differ in several respects (Table 2). Quite obviously, an acceptable degree of comparability should be observed with kits from different companies before measurement of F1+2 levels is recommended for monitoring purposes. F1+2 levels were measured in citrated and heparinized plasma from apparently healthy subjects and in citrated plasma from 90 subjects on stabilized oral anticoagulant treatment (INR values ranging from 1.3 to 9.0), with the kits manufactured by Baxter, Behring and Organon Teknika. Total assay imprecision ranged from 6.9% to 12.8%. From the data reported in Figures 5 and 6 it is apparent that with the different commercial kits there are large differences in the absolute values of F1&2 and in their distribution; however, in patients on oral anticoagulants, the F1+2 values determined with the various assays were well correlated and independent of the kit - there was a
consistent inverse relationship between INR and F1+2 levels (Figure 6).

Poor comparability of the Baxter and Behring ELISA methods has been recently reported partly due to the anticoagulant mixtures, but mainly dependent on quantitative and qualitative differences in the standards supplied for calibration by the manufacturers. The authors conclude that standardization of the F1+2 assays cannot be achieved easily simply by using a common standard.

We have also observed in heparinized plasma F1+2 values markedly higher than the corresponding values in citrated plasma (Figure 5), a phenomenon originally reported by Bauer et al. However, by omitting outliers from analysis, the slope of the regression of F1+2 values determined in heparinized and in citrated plasma deviated from equality by a value entirely explained by the dilution of plasma with citrate. In our experience, performance of the Baxter and Behring calibrators in the two ELISA systems is quantitatively but not qualitatively different. A common reference preparation to calibrate in-house standards seems at present applicable to F1+2 standardization; a collaborative study is currently in progress within the frame of the International Federation of Clinical Chemistry aimed to the harmonization of the different commercial ELISA methods for the purpose of their utilization in the monitoring of oral anticoagulant treatment.

Figure 6. Relationship of prothrombin fragment 1+2 with INR levels in patients (n=99) on stable oral anticoagulant treatment. All determinations were performed in citrated plasma. The control population is the one reported in Figure 5. Upper panels: Enzygnost F1+2, Behring; lower panels: F1.2 Elisa, Baxter. Patients on oral anticoagulant treatment were randomly selected with INR values below 2.0 (n=33), between 2.0 and 3.0 (n=33) and above 3.0 (n=33). Least square regression analysis was performed after reciprocal transformation of data.

\[
\begin{align*}
F1+2, \text{nmol/L (Behring)} & \quad r = -0.603, p<0.001 \\
F1+2, \text{nmol/L (Baxter)} & \quad r = -0.360, p<0.001
\end{align*}
\]
Ultimate demonstration of the usefulness of F1+2 measurements in patients on oral anticoagulant treatment will require carefully conducted studies. Only randomized, prospective studies may show whether a moderate intensity of anticoagulation – as based on INR values – would protect from recurrence, provided F1+2 levels are depressed. In such studies detection of adverse effects should however be more accurately than it has been until now.

Pitfalls in the direction of adverse effects of anticoagulants therapy

In all reported efficacy studies, diagnosis of bleeding and embolic events has been clinical. Whereas bleeding events may be accurately recorded by the clinician, sensitivity and specificity of the clinical diagnosis of embolism are unknown. Uncertainty about the true incidence of embolic events may bias the conclusions of efficacy studies aimed to identify the optimal intensity of anticoagulation.

We have recently evaluated by nuclear magnetic resonance imaging (NMR) the presence of (high probability) ischemic cerebral lesions in patients with mechanical heart valve prostheses, in the aortic or mitral position, followed over a total of 100 patient-years. Clinically, episodes of suspected thromboembolism (TIA-like) had occurred at a rate of 2% in this series. By NMR, ischemic lesions – often multiple – were found in about half of the patients, with a higher prevalence of lesions in those with mitral valve prosthesis. In view of the high incidence of silent thromboembolism (> 80% patient/years), the clinical diagnosis markedly underestimates the true incidence of embolic complications in patients with mechanical prosthetic heart valves. Clearly, the uncertainty of the clinical diagnosis of thromboembolism may be at least partly responsible for the variable efficacy of oral anticoagulants in preventing embolic events in different studies.

Despite the clearcut demonstration of efficacy in all patients with myocardial infarction, a commentary to the ASPECT study still favours the use of aspirin over oral anticoagulants, mainly because of the increased rate of major cerebral hemorrhage and the much greater cost and complexity of oral anticoagulants. A series of studies is currently in progress evaluating the efficacy of the association of low-dose warfarin with aspirin, with the aim of reducing the bleeding risk at more conservative INR values. However, it is doubtful that a favourable outcome of these studies may convince cardiologists to put their patients on oral anticoagulant treatment unless an effective network of anticoagulation clinics is available and the safety of anticoagulant treatment is further improved. Better laboratory tests, diagnostic tools and careful follow-up of patients are required to improve the efficacy of long-term oral anticoagulation.

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The blood coagulation system is regulated by a number of anticoagulant proteins present in plasma or on the surface of endothelial cells. Under physiological conditions, pro- and anti-coagulant mechanisms are delicately balanced and disturbances result in either bleeding or thromboembolic disorders. An important natural anticoagulant system has been elucidated in recent years. Protein C, which is a key component in the system, is a vitamin K-dependent plasma protein which, after its activation on endothelial cells by the thrombin-thrombomodulin complex, selectively degrades coagulation factors Va and VIIIa.

The functional importance of protein C is illustrated by the life-threatening disease that develops already in the neonatal period in individuals with homozygous protein C deficiency. The disorder is described as purpura fulminans and is characterized by generalized microvascular thrombosis. A milder form, characterized by an increased risk of venous thrombosis in young and middle-aged adults, is associated with heterozygous protein C deficiency. Protein S, which is another vitamin K-dependent plasma protein, functions as cofactor to activated protein C (APC). Protein S appears in two forms in plasma, as free protein and in complex with the C4b-binding protein (C4BP), which is a regulatory protein of the classical complement pathway. The free form accounts for approximately 30-40% of total protein S and only the free form is functioning as APC cofactor. The function of protein S is not fully understood and the effect of protein S as cofactor to APC is weak in *in vitro* experiments using purified component. However, the physiological importance of protein S as an anticoagulant protein is underscored by the association between heterozygous protein S deficiency and thromboembolic events.

Thromboembolic disease has also been found in association with other inherited defects of the hemostatic process such as antithrombin deficiency, plasminogen deficiency and congenital dysfibrinogenemia. The presence of lupus anticoagulants, i.e. antibodies against phospholipid, may also predispose to thrombosis. Patients with thromboembolic disease occurring at a relatively young age frequently have family histories of thrombosis. As heterozygous deficiencies of protein C, protein S, antithrombin or plasminogen, are only found in a minority of cases it is suggested that there are other genetic factors predisposing for thrombosis.

A novel mechanism for familial thrombophilia characterized by resistance to activated protein C

A serendipitous observation initiated the work that led to the identification of inherited resistance to activated protein C as a major
cause of thrombosis. A technician in our laboratory performed a functional protein C assay on a sample from a thrombosis patient and noticed that different sample dilutions gave different results. When the assay was performed at low dilutions the results suggested that the patient had functional protein C deficiency. Normal values were obtained when the sample was tested at higher dilutions. This observation initiated the search for the possible cause and also led to the development of a new coagulation based assay, the APC-resistance test.

The sample came from a man born in 1942. At the age of 19 years, he experienced an episode of deep venous thrombosis in one leg and between 1980 and 1987 he suffered from multiple episodes of deep venous thrombosis. Several members of the proband’s family had similar histories of recurrent venous thrombosis suggesting an inherited cause of the disease. Values for antithrombin, protein C, protein S, plasminogen, fibrinogen, thrombin/reptilase-times, and routine coagulation variables were normal.

The observation which initiated the work, i.e. that low dilutions of the patient plasma yielded low values in the functional protein C assay, prompted us to evaluate whether a factor in the patient plasma influenced the anticoagulant response to APC. For this purpose, the APC-resistance test, which is based on an activated partial thromboplastin time (APTT) reaction, was developed. This assay measures the anticoagulant response in plasma to added APC. So far, assays based on measurements of the anticoagulant activity of APC have been devised to identify individuals with protein C or protein S deficiency. Usually these assays include protein C- or protein S-deficient plasma, and the plasma to be tested contributes the missing factor. The APC-resistance test was not designed to measure proteins C or S, but rather the anticoagulant response in patient plasma to added purified APC. It was based on the hypothesis that a poor response to APC could predispose for thrombosis. The anticoagulant response of proband’s plasma to APC was consistently found to be much smaller than that of control plasma (Figure 1).

A poor anticoagulant response to APC could be due to any of a number of mechanisms, some known to exist and others being hypothetical. They include the presence of autoantibodies against protein C, a lupus antibody inhibiting the APC function, a fast acting protease inhibitors to APC, functional protein S deficiency, mutations in the genes for factors VIII or V in the genomic parts encoding the APC-cleavage sites (this would create APC-resistant factors VIIIa or Va), and finally the involvement of previously unrecognized mechanisms or APC-cofactors. These possibilities were investigated in the propositus and in his family members.

To determine whether the APC-resistance was inherited, plasmas from family members were tested with the APC-resistance test. The results were either expressed as prolongations of clotting time or as APC-ratios. The APC-ratio is the clotting time measured in the presence of APC divided with the APT-time obtained in the absence of APC. Several of the family members manifested APC-resistance, suggesting the underlying biochemical defect to be inherited (Figures 2 and 3). A 50% transmission of the poor anticoagulant response to APC between generations III and IV suggested an autosomal dominant mode of inheritance, which is also compatible with results of more recent family
Figure 2. Pedigree of a family with thrombophilia. The propositus is indicated with an asterisk. Females are denoted with circles and males with squares. Diagonal lines indicate deceased family members. Filled symbols denote subjects with a history of thrombosis (modified from ref. #13).

Figure 3. APC-resistance in family members. The APC-resistance test was performed on plasmas from controls (C) and family members (F). Each individual is represented by a circle. A, graphic presentation of the data after logarithmic transformation (Ln, natural logarithm); B, linear representation of the APC-ratios (modified from ref. #13).
studies.\textsuperscript{14} The range of values (both when expressed as prolongations of clotting time and as APC-ratios) of controls was wide and the distribution was skewed, but became essentially normal after logarithmic transformation.

The inherited nature of the APC-resistance made it unlikely that it was due to an inhibitor of immunoglobulin type. However, to exclude the possibility, the proband’s plasma was again tested in the APC-resistance test when depleted first of IgG, then IgA and finally IgM. Despite the complete removal of the respective immunoglobulin, the proband’s plasma still was resistant to APC, as compared to control plasma treated in the same way arguing against an APC autoantibody. It was theoretically possible that the resistance to APC could be due to a mutation in the gene of one of the plasma protease inhibitors, creating an efficient APC-inhibitor. The presence of a fast-acting protease inhibitor reacting with APC was excluded because APC was inhibited at normal rate in the proband’s plasma.\textsuperscript{13}

To further elucidate the possibility of an inhibitor causing the apparent APC-resistance, mixtures of control and proband plasma were tested in the APC-resistance test using two different APC-concentrations. When plotted against the percentage of proband plasma, the two curves were exponential, but were essentially linear and parallel after logarithmic transformation of the data.\textsuperscript{13} These results argued against the presence in the proband’s plasma of an APC-inhibitor. Moreover, normal values for protein C in proband plasma, as measured by Coatest Protein C, an assay which measures the amidolytic activity of protein C after activation with a snake venom, made an inhibitor blocking the active site of APC less likely.

To elucidate the possibility of APC-resistance being caused by a functional protein S deficiency, because the APC-protein S interaction is species-specific. Several lines of experimental results suggest that the APC-resistance test does not detect protein S deficiency.\textsuperscript{13,14} Thus, patients with known inherited protein S deficiency may manifest normal response to APC. Furthermore, immunodepletion of protein S of normal human plasma results in only an approximately 50% reduction of the APC-dependent prolongation of clotting time in the APC-resistance test.

APC exerts its anticoagulant effect through proteolytic degradation of factors Va and VIIIa. Mutation in the factor VIII gene (located on the X-chromosome) is associated with hemophilia and it was hypothetically possible that mutations in or close to the APC-cleavage sites could yield factor VIII molecules resistant to APC. However, this was excluded by DNA-linkage analysis which demonstrated the proband and his affected brothers to have different factor VIII genotypes. Moreover, PCR-amplification and nucleotide sequencing of the factor VIII exons encoding the APC-cleavage sites revealed normal sequence. Factor VIII in plasma circulates in complex with von Willebrand factor. The possibility that the poor APC-response was linked to the von Willebrand factor gene was also excluded on basis of DNA-polymorphism analysis.\textsuperscript{13}

The anticoagulant response to APC in factor IXa- and factor Xa-based clotting assays was investigated to further elucidate the nature of APC-resistance. The factor IXa-based assay was almost as efficient as the APTT-based assay in distinguishing family members from normals. In the factor Xa-based assay, the family members manifested significantly poorer anticoagulant response to APC than controls, although the difference was less pronounced than in the other two assays. The coefficient of correlation between the factor IXa- and APTT-based assays was 0.7, that between the factor Xa- and APTT-based assays 0.4, and that between the factor IXa- and Xa-based assays 0.7.\textsuperscript{13} The combined results argued against the possibility of the APC-resistance being caused by a mutation in the factor V gene resulting in APC-resistant factor Va. The results did however not exclude
mutations in other parts of the factor V gene. As will be disclosed below, our recent data unexpectedly suggest that mutations in the factor V gene indeed appear to be the cause of APC-resistance. However, these mutation do not create APC-resistant factor Va molecules. The molecular background for the APC-resistance is instead related to the unexpected finding that factor V is a cofactor to APC and that factor V gene mutations lead to loss of this anticoagulant function.

The inherited poor anticoagulant response to APC could not be explained by the currently accepted scheme of the protein C anticoagulant system. Based on results presented in our first paper on APC-resistance, the presence in normal plasma of a previously unrecognized cofactor to APC was predicted. The resistance to APC appeared to be best explained by an inherited deficiency of such a cofactor.

Many of the coagulation factors were first postulated on the basis of observations made in patients with bleeding problems. The proteins involved in the protein C anticoagulant system were found via a different route. Proteins C and S were initially described as vitamin K-dependent proteins with unknown functions. Their anticoagulant properties were delineated a few years later. As deficiencies of these proteins would hypothetically lead to a thrombotic tendency, immunological assays were used to investigate patients with thrombosis. The association between deficiencies of protein C or protein S and thrombosis is now well established. The novel cofactor to APC is the first anticoagulant factor to be proposed on basis of observations made in an individual patient with thrombosis.

**Anticoagulant cofactor activity found to be a novel property of factor V corrects inherited resistance to activated protein C**

The hypothesis that APC-resistance was caused by deficiency of an APC-cofactor was soon experimentally proven. A fraction obtained from normal plasma corrected the defect of APC-resistant plasma, whereas corresponding fraction from APC-resistant plasma was inactive. This told us that it would be possible to obtain the novel APC-cofactor in purified form which was a prerequisite for its detailed characterization and elucidation of its functions. Our work led to the unexpected discovery that the new APC-cofactor was identical to one of the old coagulation factors, namely factor V.

Thus, factor V is an important component of the protein C anticoagulant system functioning as cofactor to APC, as well as being the precursor to procoagulant factor Va.

Factor V is a single chain high molecular weight glycoprotein (Mr=330,000) present in plasma. During its activation by thrombin, three peptide bonds are cleaved, factor Va being the resulting complex between the 105 kDa heavy chain and the 74 kDa light chain (Figure 4). The function of the two large activation peptides, derived from the central portion of factor V, is unknown.

The first step in the purification of the new
APC-cofactor was barium-citrate absorption, which removed protein C, protein S and the other vitamin K-dependent proteins. After fractionated PEG 6000 precipitation, the APC-cofactor activity was found to be present in the 8% PEG supernatant. It was further purified by anion exchange chromatography on a column with Q-Sepharose and then by a gelfiltration on Sephacryl S-300. This purification protocol was similar to a procedure we had previously designed for the purification of coagulation factor V, and factor V was indeed found to be present in the same fractions as APC-cofactor activity (Figure 5). The protein in the S-300 pool manifested characteristics previously reported for factor V. Additional efforts to separate the two activities using several other chromatographic principles were unsuccessful and APC-cofactor activity was in fact found to purify together with factor V on every chromatographic support we tried.

Even though the APC-cofactor activity manifested the same chromatographic behaviour as factor V it was still possible that the two activities were properties of different molecules. To elucidate this further we decided to make monoclonal antibodies and to use them in an effort to separate factor V from the APC-cofactor activity. The protein in the S-300 pool was used as antigen in the production of monoclonal antibodies. Seventeen antibodies were obtained, and they were all found to react with factor V.

Figure 5. Elution patterns of factor V and APC-cofactor activity on Q-Sepharose and Sephacryl S-300 chromatographies. The elution profiles of APC-cofactor activity (upper sections) coincided with that of factor V (middle sections). APC-cofactor activity was associated with an APC-dependent prolongation of clotting time of APC-resistant plasma, whereas factor V gave a shortening of clotting time of factor V-deficient plasma (modified from #15).
One of the antibodies was used for affinity chromatography. The protein that bound to the column was eluted and found to have both factor V and APC-cofactor activities, which demonstrated that the two activities were closely linked and presumably properties of the same molecule. The purified protein was also applied to columns with immobilized polyclonal antibodies against human factor V or against bovine factor Va fragments. Both APC-cofactor and factor V activities were retained on the columns, but the denaturing conditions required to elute the bound protein resulted in loss of both biological activities.

The affinity purified factor V was added to APC-resistant plasma and the anticoagulant response to APC tested. A dose-dependent increase in anticoagulant response to APC was observed. Approximately 25 mg/L was required to correct the poor APC-response of APC-resistant plasma (Figure 6). This is in the same order of magnitude as the normal plasma concentration of factor V.

In our first report, we concluded that inherited APC-resistance was not due to factor V or VIII gene mutations resulting in APC-resistant molecules. Such mutations would preferably have been located in regions encoding the APC-cleavage sites. However, our data did not exclude the possibility of mutations in other parts of the factor V gene being the cause of APC-resistance. Possible linkage of APC-resistance to the factor V gene was not investigated, as no DNA-polymorphisms linked to the factor V gene were known at the time. We have found individuals with APC-resistance to have normal levels of factor V procoagulant activity. This suggests APC-resistance to be caused by mutations in the factor V gene yielding selective loss of the anticoagulant properties of factor V without affecting the procoagulant properties of the factor V molecule.

As different parts of the factor V molecule appear to be important for its pro- and anticoagulant activities, it follows that one or the other activity should be possible to modify by monoclonal antibodies. We found two of our monoclonal antibodies against factor V to partially inhibit the APC-cofactor activity directly in plasma, whereas they did not affect the procoagulant activity of factor V. Interestingly, a patient with a severe thrombotic disease has been described as having an acquired autoantibody against factor V. The patient antibody presumably specifically inhibited the APC-cofactor activity of factor V, which explains the apparent paradox that an antibody against factor V is associated with thrombosis rather than with a bleeding diathesis.

Many studies aimed at the elucidation of the function of APC and its possible cofactors have used degradation of factor Va (measured with factor V assays) as means of monitoring APC-activity. This approach was successful for the identification of protein S as cofactor to APC, but it is obviously inadequate to identify factor V as an APC-cofactor. The identification of factor V as an APC-cofactor was dependent on the availability of plasma from an individual with inherited APC-resistance.

The molecular mechanisms of the anticoagulant properties of factor V remain to be elucidated. There are known properties of factor V which probably are important. Factor V, like
Inherited resistance to activated protein C

factor Va, binds to phospholipids on platelets and endothelial cells, and both forms interact with APC and with protein S. It is conceivable that phospholipid-bound factor V forms a complex with protein S and APC, a complex which efficiently degrades factors VIIIa and Va.

At first, it came as a surprise to us that factor V was the novel cofactor to APC. Upon closer consideration, it is obvious that nature has devised an appropriate, unique and ingenious means of regulating blood coagulation. The ability of factor V to function as an APC-cofactor may be instrumental in regulating the amounts of factors VIIIa and Va on the surface of unactivated platelets and endothelial cells. As activation of factor V by thrombin result in the expression of potent procoagulant activity, and possibly to the loss of APC-factor activity, a new aspect of the relationship between pro- and anti-coagulant mechanisms is suggested (Figure 7). Thrombin was the first procoagulant enzyme shown to express anticoagulant properties, when bound to thrombomodulin.

Factor V is the first coagulation cofactor demonstrated to express anticoagulant properties as well as being the precursor to a procoagulant cofactor, and our results emphasize the importance of the balance between pro- and anti-coagulant properties of the coagulation factors.

Inherited APC-resistance as a basis for venous thrombosis

It has been obvious for people working in the field that genetic factors play an important role in thrombophilia and that a thrombotic episode should be looked upon as a sign of a possible familial thrombotic tendency. As discussed above, deficiencies of anticoagulant proteins like protein C, protein S and antithrombin are only been found in a minority of thrombosis patients. Other genetic factors being involved in the pathogenesis of familial thrombophilia have remained elusive and DNA linkage studies have been proposed as a possible means to identify them. To elucidate the prevalence of APC-resistance in thrombosis patients we studied a
well characterized cohort of patients with thromboembolic disease. The patients were referred to the coagulation laboratory at Malmö General Hospital between September 1991 and December 1992. They represent a selected cohort, which however was similar to a previously studied cohort in which the prevalences of other inherited deficiencies of anticoagulant proteins had been determined. \(^1\)

The study population included 72 women and 32 men. \(^2\) The thrombotic events were deep venous thrombosis in a leg (n=83), pulmonary embolism (n=17) or thrombosis in cerebral vessels (n=4). In 31% of the patients, histories of more than one thrombotic event were found. In 60% of cases, predisposing factors for thrombosis were identified, the most common being pregnancy and the use of oral contraceptives. Family histories of thrombosis were found in 45% of cases. The laboratory investigation was performed at least three months after the last thrombotic episode. The APC-resistance test was used to measure the anticoagulant response to APC, the results being expressed as APC-ratios. Patients on oral anticoagulation were excluded as APC-dependent prolongations of clotting time were excessive in individuals on oral anticoagulant therapy. Protein C deficiency was found in two patients and protein S deficiency in three. The three protein S-deficient individuals manifested normal APC-response. No antithrombin deficiency was identified, and none of the patients manifested signs of lupus anticoagulants.

The patients' APC-ratios were significantly lower than those of controls (Figure 8). Few patients had high APC ratios, and values manifested a distinctly bimodal distribution. Results of a family study demonstrated an APC-ratio of <2.0 to be associated with thrombosis. Approximately 40% of all thrombosis patients were below this limit, whereas corresponding value in patients with positive family history was 50%. This demonstrates APC-resistance to be the major risk factor for familial thrombophilia. Approximately 7% of controls had

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**Figure 8. APC-resistance in controls, thrombosis patients and relatives.** The results of the APC-resistance test were plotted as APC-ratios, each individual being represented by a circle. The 5th, 25th, 50th, 75th and 95th percentiles are indicated. The difference in APC-ratios between thrombosis patients and blood donors was significant (p<0.0001). The 34 propositi were not included among the relatives; (modified from 14)
APC-ratio below 2, suggesting inherited APC-resistance to be prevalent in the general population.\(^\text{14}\)

New samples were requested from patients manifesting APC-resistance and low or borderline APC-ratios were found in all of them. APC-ratios in control subjects were also found to be reproducible, and the correlation between APC-ratios measured in samples drawn on two different occasions was very high.\(^\text{14}\) To elucidate whether APC-resistant plasmas from different patients were deficient in the same anticoagulant factor, they were retested in 1:1 mixture with plasma with pronounced APC-resistance. In none of them did the APC-ratio increase to any significant extent, demonstrating APC-resistant individuals from different families to have mutations in the same gene.

We found the mean APT-time to be significantly shorter in patients than in controls and the APC-dependent prolongation of clotting time correlated with the APT-time.\(^\text{14}\) This indicated APC-resistant individuals to have shorter APT-times than individuals with high APC-response. It is noteworthy that a short APT-time has been reported to be a significant risk factor for postoperative thrombosis.\(^\text{20}\) From this it can be concluded that APC-resistance is an important risk factor for postoperative thrombosis. As the genetic defect resulting in APC-resistance appears to be present in around 5% of the population it may be worthwhile to include the APC-resistance test in the preoperative evaluation. This may be important not only in young individuals but also in older people, as APC-resistance is a genetic defect associated with a life-long increased risk of thrombosis.

The APC-resistance test is considered to be a satisfactory screening test. There are important methodological considerations regarding the test. As it is based on the addition of APC directly in an APTT reaction, careful standardization is of the utmost importance. The APC-response is affected by the level of APC-activity, the APTT-reagent, the instrumentation, and by sample handling.\(^\text{14,21}\) Provided that these variables are carefully controlled, the results are consistent and reproducible also in samples drawn on different occasions. Each analytical batch included a control, and as the results were reproducible, we did not include a calibrator in the assay. However, future studies comparing inter-laboratory variation of the APC-resistance test will require the establishment of an international calibrator as well as appropriate control plasmas.

Several protein S deficient individuals did not exhibit APC-resistance.\(^\text{14}\) Protein S does not affect the APC-resistance test to any major degree and the plasma levels of protein S does not correlate with the APC-response, which may appear surprising. On the other hand, defects in the APC-cofactor activity of factor V appear to influence available functional assays for protein S, as suggested by Faioni and colleagues.\(^\text{22}\) Nine families in which they had pre-
viously suspected functional protein S deficiency were reevaluated among other things with the APC-resistance test. They came to the conclusion that these families did not have functional protein S deficiency but rather APC-resistance. The influence of factor V-dependent APC-cofactor activity in the protein S assay was the cause of the improper classification. It can be concluded that the APC-resistance test from a practical point of view is useful as a means of screening patients for genetic defects in the factor V molecule resulting in APC-resistance. In most cases the influence of protein S in the assay can be disregarded.

A weak inverse correlation was found between the APC-ratio and the prothrombin complex level, suggesting the APC-response to be higher at low levels of vitamin K-dependent protein concentrations. This was consistent with high APC-ratios observed in individuals on oral anticoagulant therapy. In our study we found no significant difference between men and women in APC-ratio, and no correlation to age, weight or height.

Family studies were initiated in 45 families in which the propositus had APC-resistance and thrombosis. In 34 (76%) of the families, APC-resistance was found in at least one first degree relative. In all, 211 individuals (123 women and 88 men) were included. In addition to the 34 propositi, 15 relatives from 13 families had a history of thrombosis. Thus, a total of 49 family members had suffered from thrombosis, and 45 of them had APC-ratios <2.0. Using an APC-ratio <2.0, the odds ratio for thrombosis was 10.4. An APC-ratio <2.0 was found in approximately 45% of the 177 relatives which was consistent with an autosomal dominant mode of inheritance. Protein S deficiency was found in two individuals with histories of thrombosis (both from the same family). As their APC-ratios were normal it was obvious that protein S deficiency was not linked to APC-resistance. This is also what is to be expected as the protein S gene is on chromosome 3, whereas the factor V gene is on chromosome 1.

The thrombosis-free survival curves suggested the probability of an APC-resistant individual in these families being free of thrombosis at the age of 45 to be around 60% (Figure 9). The corresponding value for relatives without APC-resistance was 97%. The inclusion of the index cases introduced a bias into the analysis. After excluding the 34 index cases with APC-resistance together with the two protein S deficient cases, the difference in survival curves was still significant (p=0.0015), suggesting individuals with APC-resistance to be at higher risk of thrombosis than those without the defect.

APC-resistance, which is highly prevalent in a cohort of patients with thromboembolic disease, is the major risk factor for venous thrombosis. Our findings have been confirmed in reports from other laboratories. Griffin et al. found a 52-64% prevalence of APC-resistance in patients with juvenile and/or recurrent venous thromboembolism unexplained by other causes. In a study of unselected, consecutive thrombosis patients, Koster and colleagues found APC-resistance in 21% of the patients and in 3% of controls. Faioni and colleagues reported approximately 33% of their thrombosis patients to have APC-resistance. The explanation for the different prevalencies in different studies is presumably to be found in the selection criteria of patients.

APC-resistance is inherited as an autosomal dominant trait. The identification of factor V as the anticoagulant cofactor which corrects APC-resistance suggests mutations in the factor V gene to cause APC-resistance and we have recently found that APC-resistance is indeed linked to the factor V gene. It appears valid to conclude that APC-resistance is caused by mutations in the factor V gene yielding selective loss of the APC-cofactor activity of factor V. It is conceivable that individuals with severe APC-resistance are compound heterozygotes for the genetic defect. In this respect it is noteworthy that we in several families have found both parents to be APC-resistant, e.g. in a young man with pronounced APC-resistance who died of pulmonary embolism after by pass surgery due to claudication. If the prevalence in the general population of APC-resistance is 1/20, the prevalence of compound heterozygosity is 1/1600 individuals. Moreover, it is interesting to note that the risk for one individual with APC-
resistance to marry a person with APC-resistance is 5%.

To sum up, our results suggest mutations in the factor V gene to be highly prevalent in patients with venous thrombosis. Such mutations result in resistance to APC due to selective loss of APC-cofactor activity of factor V. APC-resistance is at least 10 times more common than any of the other inherited deficiencies of anticoagulant proteins in patients with thromboembolic disease and is the major cause of familial thrombophilia.

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AUTOIMMUNE PROTEIN S DEFICIENCY: A DISORDER PREDISPOSING TO THROMBOSIS

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The occurrence of autoantibodies to blood coagulation factors – mainly factor VIII and less frequently FIX and von Willebrand factor – is a well established condition with important implications concerning clinical manifestations and treatment strategies. The microheterogeneity of factor VIII, a considerably large molecule, may be in part responsible for the formation of antibodies to factor VIII in patients with severe hemophilia A. Thus, infusion of exogenous factor VIII or IX in patients with no factor VIII or IX cross-reacting material may reasonably be assumed as the main stimulus leading to antibodies production in predisposed patients. At variance, the appearance of so called spontaneous antibodies to factor VIII, von Willebrand factor, factor IX, factor V and prothrombin in apparently normal subjects or in patients with disorders affecting the immune system has not yet found a sound explanation, although they are not exceedingly rare and may lead to life-threatening bleeding manifestations. Screening coagulation tests performed on whole plasma can easily substantiate the clinical suspicion of an acquired inhibitor because they would be markedly prolonged and would fail to normalize upon mixing with normal plasma. Although the lupus anticoagulant is by far the antibody most commonly responsible for such a laboratory picture it is very rarely associated with a bleeding diathesis; in addition, the dependency of the lupus anticoagulant – but not of acquired inhibitors specifically directed to clotting factors – on phospholipid is a tool to discriminate between the different types of antibody. Whenever an inhibitor to a single clotting factor is present, specific laboratory tests are available to quantitate its levels, with relevant implications regarding the treatment of bleeding episodes.

Antibodies directed to components of the natural anticoagulant systems may represent a cause of unexplained thromboembolic disease. The natural anticoagulant systems control the degree of intravascular coagulation mainly at the level of the microvasculature. The tissue factor pathway inhibitor (TFPI) inhibits the activity of the factor VIIa-tissue factor complex by forming an irreversible, inactive complex with factor Xa which in turn blocks the activity of FVIIa without affecting its interaction with tissue factor. Antithrombin III and heparin cofactor II are two serine protease inhibitors which form inactive, irreversible complexes with the critical enzymes of the coagulation cascade, i.e. factor Xa and thrombin. Their activity is dramatically potentiated by heparin and by diverse glycosaminoglycans exposed on the surface of endothelial cells. Finally, the protein C system is a highly sophisticated assembly of membrane receptors, enzymes and cofactors which neutralizes the activity of the activated coagulation cofactors V and VIII. The three above mentioned systems represent the major natural mechanisms of defense against uncontrolled coagulation leading to thrombosis. Congenital quantitative or qualitative deficiencies of antithrombin III, heparin cofactor II, protein C, protein S and of a newly described activa-
Autoimmune protein S deficiency have been reported in families with an increased tendency to thromboembolic disease.

Autoantibodies directed to TFPI, antithrombin III and heparin cofactor-II have not been reported in the literature. Although it could be speculated that limited testing is responsible for inhibitors to TFPI and heparin cofactor-II to go unrecognized, this is certainly not the case for antithrombin III inhibitors, because reliable and accurate assays measuring antithrombin III activity are available since a long time and there is little doubt that antithrombin III is among the most largely and perhaps unduly tested plasma protease inhibitors in general laboratories of clinical chemistry. It is therefore remarkable that autoantibodies predisposing to thrombosis have been only found targeted to the major components of the protein C system.

Autoantibodies to protein C, have been described so far on two occasions. In the first case a history of recurrent thrombosis was present in a patient with multiple myeloma, whereas in the other case a lifelong history of thrombosis eventually leading to death in an otherwise healthy subject was observed. In both cases, the presence of an inhibitor was suspected on the basis of the discrepancy between reduced activity and normal antigen levels of protein C. In neither report was direct evidence provided of the interaction of the antibody with protein C.

We have recently identified the presence of spontaneous antibodies to protein S in two children following viral or bacterial infections.

Case reports

Case 1. A painful enlargement of the left testis was observed in an eleven year-old boy during recovery from chickenpox, diagnosed 12 days earlier. The patient was febrile (38.2°C), had elevated ESR, C-reactive protein and lactate dehydrogenase but no evidence of lymphadenopathy or hepatosplenomegaly. Liver- and kidney-function tests were normal. Red blood cell and platelet counts were normal, but there was leukocytosis with marked neutrophilia. The serum electrophoretic profile and complement components C3 and C4 were normal. There was a slightly positive response to anti-smooth muscle cell and anticardiolipin antibodies (26 GPL U/mL).

At surgery, there was extensive hemorrhagic necrosis of the didymis and epididymis with thrombosis of the pampiniform plexus and of the lower third of the spermatic vein, but no evidence of torsion of the spermatic cord. In spite of calcium heparin prophylaxis (5,000 U t.i.d.), the patient developed distal deep vein thrombosis of the left leg on the third post-operative day. Sodium heparin was given intravenously (25,000-31,000 U per day) aiming at an activated partial thromboplastin time ratio of 1.8-2.5. During the night of the fifth postoperative day, the boy developed symptoms of pulmonary embolism which was confirmed by ventilation-perfusion scanning. The heparin infusion was increased to 40,000 U per day, resulting in maintenance of the APTT ratio between 2.0 and 2.5. Venography demonstrated thrombus extension into the left iliac veins and the inferior caval vein, with initial backward progression into the right common iliac vein. A temporary caval filter was positioned and locoregional r-tPA infusion was started and continued for 4 days with maintenance of intravenous heparin, resulting in substantial resolution of thrombosis in the inferior caval and proximal iliac veins. In spite of effective heparin treatment – according to the APTT, thrombosis of the right subclavian and axillary vein followed removal of the temporary filter. Three weeks after orchiectomy, administration of oral anticoagulants was started and heparin infusion interrupted after 5 days. Anticoagulation was continued for one year (target International Normalized Ratio value = 2.5). At this time, venography had shown substantial recanalization of the femoropopliteal deep vein axis of the inferior limb.

Coagulation data. A complete laboratory picture was obtained on the third postoperative day, while the boy was on intravenous heparin infusion. The APTT ratio was 1.60, the PT ratio 1.19, the Reptilase time ratio was 1.02, the fibrinogen level 238 mg/dL and the platelet count 194,000/µL. Antithrombin III (101% of nor-
mal), plasminogen (131%), heparin cofactor II (102%), and protein C activity levels (101%) were normal. Protein S levels were markedly reduced (14% total protein S antigen, < 6% free protein S antigen and anticoagulant activity) with increased C4b-binding protein (140%). Vitamin K-dependent factors VII (84%), IX (100%), X (98%) and II (104%) were normal.

The boy’s total protein S antigen levels returned gradually to normal by the 19th postoperative day (84%), when plasma C4b-binding protein was 190% and free protein S antigen 17% of normal.

No protein S deficiency was detected in any of the propositus’ family members investigated and there was no family history of thrombosis.

Case 2. A previously healthy, eight year old boy presented with painful, symmetrical, pretibial indurations on both legs five days after the onset of fever, pharyngitis and cervical lymph nodes enlargement. Laboratory data revealed thrombocytopenia, mild leukocytosis with relative neutrophilia, elevated C-reactive protein and consumption of complement components C4, C3 and of CH50. There were no signs of shock. The antistreptolysine titre – initially negative – increased rapidly to > 1000 IU/mL. Antibodies directed to protein S were searched by a variety of methods. Under physiological conditions, protein S (MW 70 kd) circulates in plasma as free protein S and in reversible complex with C4b-binding protein (MW 570 kd), a polymorphic regulatory protein of the complement system. The presence of two protein S forms in plasma with different molecular weight and electric charge was anticipated to complicate identification of protein S-antibody complexes.

Identification of anti-protein antibodies

Antibodies directed to protein S were searched by a variety of methods. Under physiological conditions, protein S (MW 70 kd) circulates in plasma as free protein S and in reversible complex with C4b-binding protein (MW 570 kd), a polymorphic regulatory protein of the complement system. The presence of two protein S forms in plasma with different molecular weight and electric charge was anticipated to complicate identification of protein S-antibody complexes.

We first tried to identify an additional precipitin arc upon addition of purified human protein S to the patient plasma followed by crossed-immunoelectrophoresis. A third slow-moving peak was actually observed at a final protein S concentration of 20 µg/mL; the area of the additional precipitin arc apparently increased upon immunodepletion of plasma C4b-binding protein.

The gel-filtration profile of radiolabeled protein S added to the patient plasma was also investigated (Figure 1). Two distinct peaks of radioactivity were eluted with the patient plasma. This profile of protein S filtration was not changed upon immunodepletion of C4b-binding protein. Although two peaks were still present 3 months after the thrombotic event (case #1),
the higher molecular weight peak was reduced, with a corresponding increase in the area of the lower molecular weight peak (Figure 1, left panel). A similar chromatographic profile was obtained when labelled and cold protein S were added to normal plasma immunodepleted of C4b-binding protein and incubated with increasing concentrations of mouse monoclonal antibodies to human protein S. The higher molecular weight peak increased with increasing antibody concentration with a corresponding decrease in the lower molecular weight peak (Figure 1, right panel).

Albeit suggestive of an abnormal protein S binding factor in plasma, these experiments could not be taken as confirmatory of the presence of an antibody to protein S in the patients’ plasma. Direct evidence of an autoantibody was obtained by immunoblotting and isoelectric focusing analysis.

Binding of IgG antibody to protein S was demonstrated by transferring purified protein S onto nitrocellulose membranes and incubating the membranes with the patients’ plasma or IgG fraction. The IgG bound to blotted protein S was detectable by the addition of rabbit anti-human IgG or radiolabeled protein S. Polyacrylamide isoelectric focusing of the patients’ plasma proteins permitted the identification of IgG bands which were absent in normal pooled plasma. Interestingly, while in case 1 only two discrete IgG bands migrating in the pH range of 6.8-7.2 were found – indicating the mono-oligoclonal origin of the antibody, mul-

Figure 1. Gel filtration analysis of plasma samples supplemented with radiolabelled (125I) protein S.

Left panel. Closed circles: patient plasma (case 1) at diagnosis incubated with purified unlabelled protein S (20 μg/ml, final concentration) and with 125I-labelled protein S (1 μg/ml, final concentration). Open circles: patient plasma (case 1) at diagnosis incubated of C4b-binding protein (residual C4b-binding protein <20% of pre-incubation value) by incubation with an immobilized mouse monoclonal antibody to C4b-binding protein (DS) for 30 min at 37°C. After removal of C4b-binding protein cold (20 μg/ml) and labelled protein S (1 μg/ml) were added. Closed triangles: patient plasma (case 1) 3 months after diagnosis when protein S levels had returned to normal (85%). Only labelled protein S was added to plasma (1 μg/ml).

Right Panel. Open circles: normal pooled plasma immunodepleted of both protein S and C4b-binding protein and incubated with cold (20 μg/ml) and labelled protein S (1 μg/ml). Open triangles: normal pooled plasma immunodepleted of both protein S and C4b-binding protein and incubated with cold (20 μg/ml) and labelled protein S (1 μg/ml) and with a mouse monoclonal antibody to human protein S (HPS2, 20 μg/ml, final concentration). Open inverse triangles: normal pooled plasma immunodepleted of both protein S and C4b-binding protein and incubated with cold (20 μg/ml) and labelled protein S (1 μg/ml) and with a mouse monoclonal antibody to human protein S (HPS2, 40 μg/ml, final concentration). Virtually identical protein S filtration profiles were observed with another anti-protein S monoclonal antibody (HPS10/32).

All samples (100 μl) were applied after a 2 hour incubation at 37°C to a TSK G-3000 column (30 x 0.5 cm, LKB-Pharmacia) equilibrated in 0.1 M NaCl, 13 mM sodium citrate, 5 mM benzamidine-HCl, 20 mM Tris HCl, pH 7.2. Flow rate was 0.3 ml/min and 0.2 ml fractions were collected. The FPLC apparatus was Ultrochrom gTi, LKB-Pharmacia, Sweden. Counts per minute (CPM) were normalized for the total radioactivity recovered in each run.
Multiple IgG bands were observed in case #2, covering the pH range from 6.8 to 9.0, consistent with the presence of a polyclonal autoantibody to protein S.

To monitor the changes in antibody titer, a direct enzyme-linked immunosorbent assay (ELISA) was set up using protein S-coated microwell plates, the patients' plasma and rabbit antihuman IgG. Serial plasma dilutions were added to the wells and incubated for one hour. After washing, rabbit antihuman IgG was added, and the plates were incubated for one hour. After repeated washings, peroxidase-labeled, affinity-purified goat antibody to total rabbit IgG was added, followed by incubation, washing and addition of the peroxidase substrate. Because false positivities are quite common with protein S coated to the plastic, we felt necessary to confirm specificity of the ELISA detection of anti-protein S IgG by displacement of the antibody from solid-phase protein S after the addition of purified protein S to the patients' plasma.

In addition to monitoring the changes in the antibody titer, depending on the specificity of the rabbit anti-human IgG – anti-kappa chain, anti-lambda chain – the ELISA could also be exploited to determine the preponderance of a single light chain class in oligoclonal IgG anti-protein S antibodies (Figure 2). In both patients, the decline in the antibody titer was accompanied by the return of plasma protein S to normal levels.

**Discussion**

Thrombotic complications in children are rare and frequently secondary to congenital abnormalities of the coagulation system. Among associated conditions favoring thrombotic episodes, infection was prospectively detected in 7.3% of the cases in the Canadian Registry. The observation of autoantibodies to protein S associated with infectious diseases in two children suggests that at least part of infection-related thrombosis in young boys may be due to a transient state of selective protein S deficiency, possibly due to rapid clearance of circulating protein S-antibody complexes. Animal studies have shown that deficiency of free protein S exacerbates the disseminated intravascular coagulative response to sublethal endotoxinemia and eventually causes death. Our observations represent a clinical counterpart of the studies in baboons, demonstrating that immune-mediated disturbances of the protein C system predi-
spose to severe thrombotic manifestations. Interestingly, a polyclonal antibody to protein S, inducing a more severe deficiency of the protein and presumably of its activity, was associated with purpura fulminans, a condition recognizing homozygous protein C or protein S deficiency as a major pathogenic mechanism. According to a recently proposed classification, purpura fulminans may also happen in individuals with acute, current severe infection usually caused by gram negative bacteria (acute infectious purpura fulminans), and in individuals without known abnormalities of the protein C system or acute sepsis (idiopathic purpura fulminans). Idiopathic purpura fulminans usually occurs in young children and is frequently characterized by a history of an antecedent preparatory disease, most commonly a bacterial or viral infection. Among the cases reviewed by Francis, varicella and pharyngitis (streptococcal/staphylococcal) represented by far the most commonly observed preparatory diseases, representing 55% of total cases. In acute infectious purpura fulminans, protein S is invariably less reduced than protein C. At variance, transient but severe deficiencies of total protein S have been reported in subjects with idiopathic purpura fulminans and previous varicella or febrile enlargement of cervical lymph nodes. Probably, autoimmune protein S deficiency is responsible for most cases of idiopathic purpura fulminans.

Two questions remain unanswered. First, there is no clear explanation for the production of anti-protein S antibody. One possibility is represented by cross-reactivity of protein S with viral or bacterial structures. In spite of the frequent association of transient protein S deficiency with chickenpox, the anti-protein S antibody of our case 1 did not cross-react with antigens of the varicella-zoster virus. The presence in both our cases of anti-phospholipid IgG and other autoantibodies suggest that occurrence of anti-protein S antibody may be part of a generalized abnormality of the immune response. The relationship of anti-protein S antibodies to antiphospholipid antibodies is intriguing, also in view of the very high affinity of protein S for negatively charged phospholipid surfaces. It has been suggested that anticardiolipin antibodies – which can be transiently detected after viral infections – may be the pathogenic anti-protein S autoantibodies. Identity of antiphospholipid and anti-protein S antibodies is however unlikely. Anti-phospholipid antibodies, defined on the basis of either lupus anticoagulant activity or anticardiolipin reactivity, are a heterogeneous family of antibodies with varying affinities for protein-phospholipid complexes. In a series of patients with antiphospholipid antibodies and fetal wastage reduced levels of free protein S were found, but no anti-protein S antibodies could be detected by immunoblotting. At variance with the high frequency of falsely positive results by ELISA, we did not observe antibodies directed to protein S in a large number of patients with lupus anticoagulant or antiphospholipid antibodies when using the immunoblotting technique. We also failed to observe anti-protein S antibodies in subjects with HIV infection, a condition very frequently associated with the presence of antiphospholipid antibodies and reduced protein S levels. On the other hand, anti-protein S antibodies were detected by immunoblotting in 10 out of 19 patients with lupus anticoagulant and in 29% of patients with HIV infection and low free protein S levels. The reason for these discrepancies – which may underline methodological problems – is not clear at the present time. It should be pointed out that our data are not in contrast with the finding, in patients with antiphospholipid antibodies of IgG inhibiting protein S – and to a lesser extent protein C – activity, because such antibodies may only recognize phospholipid-bound protein S.

As shown in Figure 3, anti-protein S antibodies may represent a minor population of antiphospholipid antibodies capable of binding to protein S even in the absence of phospholipid, similar to what has been shown to occur for prothrombin. The protein S structures representing the antibody target(s) are presently unknown.

A second question with relevant clinical implications relates to the therapeutic approach to patients with anti-protein S antibodies. In the presence of thrombosis, a relative resistance to heparin therapy may be present, and there mi-
might be an indication for the infusion of protein S or activated protein C concentrates as a useful adjunct to anticoagulant treatment. On the other hand, anti-protein S antibodies may also be found in the absence of thrombosis; danazol (but not prednisone) has proven effective in restoring normal protein S levels in a patient with mixed connective tissue disease, antiphospholipid syndrome and possibly autoimmune protein S deficiency. More experience is needed before guidelines for the treatment of autoimmune protein S deficiency can be recommended.

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Antiphospholipid antibodies (APA) are a family of immunoglobulins which were originally thought to be specific for phospholipids. Among the antibodies included in this family are: lupus anticoagulants (LA), anticardiolipin antibodies (ACA), and reagin. These antibodies are heterogeneous and have been arbitrarily separated on the basis of relatively simple laboratory tests. The first of these antibodies, reagin, was identified by serologic tests for syphilis. Subsequently, LA was described in systemic lupus erythematosus (SLE) patients who had prolonged coagulation tests and an apparent lack of clinical bleeding. More recently using a solid phase radioimmunoassay and/or ELISA assay, ACA have been quantitated.

The association of APAs and both arterial and venous thromboembolic events was first reported by Bowie and colleagues in 1963. Their report focused attention to the diversity of thrombotic sites including major arterial and venous locations as well as small vessels of the dermis (livedo reticularis). Following the initial report, many case studies and series have emphasized the apparent association between positive laboratory tests for APA and a clinical history of thrombosis. Much of the literature correlating APAs and thrombotic events has been based on retrospective analysis. More recently, several prospective studies have confirmed the association of APA and thromboembolic events.

Methods of detection

Lupus anticoagulants are immunoglobulins (IgG, IgM, or IgA) which interfere with one or more of the in vitro phospholipid dependent tests (e.g. activated partial thromboplastin time [APTT], dilute Russell Viper Venom time [dRVVT], Kaolin Clotting Time [KCT]) of coagulation. There is no consensus with regard to minimal laboratory criteria necessary for the diagnosis of LA. The Scientific and Standardization Committee (SSC), subcommittee for the standardization of lupus anticoagulants has recently published recommended criteria. Nevertheless, many contemporary articles describing LA do not follow these recommendations. As a result, analysis of the literature is fraught with marked inconsistencies regarding the incidence of LA in various patient groups.

The diagnosis of LA requires three sequential steps: 1) demonstration of an abnormal phospholipid dependent screening coagulation test, 2) proof the abnormality of the screening test is due to the presence of a circulating inhibitor (synonym: anticoagulant), 3) proof the inhibitor is phospholipid dependent. A variety of tests have been used to screen for the presence of LA. These tests include: APTT, dRVVT, KCT, plasma clotting time, and Textarin time. Clearly, there is no single test which will detect all of the patients with LA. Consequently, it is very important for laboratories to utilize more than one test to screen for the presence of LA. If the APTT is used as the primary screening procedure, a sensitive reagent should be employ-
ed. There is wide variability of APTT reagent sensitivity to the presence of LA.\textsuperscript{15,16}

Demonstration that the prolonged screening test is due to the presence of an inhibitor requires mixing studies using variable ratios of patient to normal plasma. In the case of moderately to markedly prolonged (i.e. greater than 10 seconds above the upper limit of normal) APTT results, a mixture of 1 part patient to 1 part normal plasma is employed. However, in the setting of a minimally prolonged APTT (i.e. less than 10 seconds above the upper limit of normal), a ratio of 4 parts patient to 1 part normal plasma is used. Failure to correct upon mixing with normal plasma is diagnostic of the presence of an inhibitor. It is important for the laboratory to rule out specific inhibitors (e.g. factor VIII) which may result in clinical bleeding.

The third step in the diagnosis of LA requires demonstration of phospholipid sensitivity. This step allows the laboratory to separate lupus anticoagulants from other inhibitors. A number of different test systems have been employed to demonstrate phospholipid dependence. At least four different approaches have been utilized: 1) decreasing the amount of phospholipid to enhance the inhibitor effect, 2) increasing the amount of phospholipid to bypass or neutralize LA, 3) the use of specific configurational changes in phospholipids (hexagonal phase), 4) employing snake venoms in a ratio of phospholipid dependent time to phospholipid independent time.\textsuperscript{17,18} Of the various confirmatory tests, the platelet neutralization procedure is the most widely employed.\textsuperscript{19}

Anticardiolipin antibodies were originally detected using a solid phase radioimmunoassay.\textsuperscript{6} Subsequently, an enzyme-linked immunosorbant assay (ELISA) was introduced.\textsuperscript{20} Cardiolipin was chosen as the target antigen because of its presence in the original antigen used for the VDRL test system (a mixture of cardiolipin, cholesterol, and phosphatidylcholine). In addition to cardiolipin, other anionic phospholipids have been utilized in ELISA assay systems. Recently, it has been demonstrated that ACAs react with cardiolipin only in the presence of a plasma protein: $\beta_2$ glycoprotein I ($\beta_2$GPI).\textsuperscript{21} $\beta_2$GPI is also known as apolipoprotein H. Based on recent studies, it appears the antibodies detected by the ACA ELISA assay, in fact, recognize a complex of $\beta_2$GPI and cardiolipin.\textsuperscript{22} ACA can be quantitated and also identified by isotype (IgG, IgM, and IgA). There are available reference sera for each of these isotypes. The results of the ACA ELISA have been reported utilizing various formats. It is not widely appreciated that the distribution of normal values is logarithmic rather than Gaussian. As a results, the upper limit of normal given by many clinical laboratories is too low. This may account for the rather high incidence of minimally positive tests.

**Relationship between lupus anticoagulant and ACA**

Initially, LA and ACA were thought to be the same antibody detected utilizing different laboratory test systems. However, with the increased incidence in APA testing, it soon became clear these two tests detected different antibodies in many cases.\textsuperscript{23} Many patients with the so-called *primary antiphospholipid antibody syndrome* may have both antibodies (approximately 60%). However, in the remaining 40% of patients, one antibody may be present and the other absent. Some plasma samples which have demonstrable LA activity require the presence of $\beta_2$GPI whereas others appear to require prothrombin.\textsuperscript{21,24}

**Relationship to thrombosis**

Approximately one-third of patients with APA have a history of at least one thrombotic episode. When comparing patients with SLE who are APA positive (e.g. either LA or ACA or both) with SLE patients who are APA negative, there is approximately three- to four-fold increase in the incidence of thrombotic events in the APA positive group.\textsuperscript{25} These retrospective studies together with other recent prospective studies identify the presence of APA as a marker of a patient population at increased risk of thrombosis. Many laboratories currently test for the presence of APA in the setting of thrombophilia. Perhaps the only other current laboratory test with a higher frequency of positivity in
thrombophilia studies are patients with activated protein C resistance.\textsuperscript{26}

**Clinical manifestations of thrombotic events**

Deep vein thrombosis is the most common venous event.\textsuperscript{27,28} Approximately one-third of patients with DVT also have accompanying pulmonary emboli.\textsuperscript{29} Some cases of pulmonary hypertension may be secondary to recurrent embolic events. Venous thrombosis also occurs in other anatomical locations including the superior vena cava, cerebral venous sinuses, inferior vena cava, and hepatic veins. When hepatic veins are thrombosed, a typical Budd-Chiari Syndrome occurs.

On the arterial side of the circulation, the cerebral arteries are most frequently involved.\textsuperscript{30} Transient ischemic attacks (TIA), amaurosis fugax, and stroke, particularly in young adults, have been linked to the presence of APA.\textsuperscript{31} The combination of cerebrovascular disease and livedo reticularis is known as Sneddon’s syndrome. Other major arteries may be affected including coronary vessels, large peripheral arteries, and mesenteric arteries.

Small vessels may also be the site of thrombotic events. Depending upon the anatomic location, this may result in multi-infarct dementia, renal thrombotic microangiopathy, retinal ischemia and infarction, superficial skin necrosis, livedo reticularis or gangrene.

**Pathophysiology of APA-related thrombosis**

Given the heterogeneity of APAs both within and between patients, it is likely that there are multiple potential pathogenic mechanisms. The major focus of initial studies was the endothelial-platelet axis. Carreras and colleagues were the first to suggest an alteration in the prostacyclin/thromboxane A\textsubscript{2} balance.\textsuperscript{32} Using animal aortic rings, they demonstrated the ability of certain patient plasmas to inhibit prostacyclin (PGI\textsubscript{2}) production. This effect could be bypassed by adding arachidonic acid to the experiment. They concluded that LAs were capable of preventing mobilization of arachidonic acid from endothelial cell membranes. The PGI\textsubscript{2} production would thus tip the balance to a prothrombotic state due to a relative excess of thromboxane A\textsubscript{2}. Following these initial studies, a number of investigators have attempted to repeat these experiments.\textsuperscript{33,34,35} The findings have been inconsistent.

The protein C anticoagulant system would seem to be an ideal candidate to explain the prothrombotic effect of APAs.\textsuperscript{36} Both proteins C and S are vitamin K dependent proteins which are bound to negatively charged phospholipid surfaces in the presence of calcium ions. Thus, potentially, APAs could interfere with surface binding of these proteins with resulting loss of the regulatory effects of this anticoagulant pathway. There are two phospholipid dependent steps in the protein C system: activation of protein C by the thrombin-thrombomodulin complex on the surface of endothelial cells and the down-regulation of factor Va and factor VIIIa on cell surfaces by activated protein C (APC). In individual patients, impairment of both of these steps has been demonstrated.\textsuperscript{37,38} More recently, Dahlback has demonstrated a lack of response in certain patient plasmas to the addition of activated protein C.\textsuperscript{26} He postulated the presence of another cofactor in addition to protein S. Recent work would suggest that factor V is the second cofactor.\textsuperscript{26}

Other abnormalities of regulatory pathways have been reported including impaired fibrinolysis and altered antithrombin III activity. Other suggested mechanisms include the presence of antiendothelial antibodies (AECA) in some patients with APA.\textsuperscript{39} These antibodies potentially could down-regulate the anticoagulant properties of endothelial cells (e.g. thrombomodulin or prostacyclin production) and up-regulate procoagulant properties (e.g. tissue factor). The platelet has also been suggested as a target for APA.\textsuperscript{40} Thrombocytopenia is often reported in patients with SLE and associated APA.

**Treatment**

The treatment of patients with APAs and a history of thrombosis is similar to other patient groups with comparable thromboembolic events. APA positive patients should be treated
as a high risk group with appropriate long-term oral anticoagulant therapy. The addition of antiplatelet medication may also be indicated. The intensity of oral anticoagulant therapy should be greater than the routine patient with no underlying disease and an idiopathic thromboembolic event. Individuals maintained with an International Normalized Ratio (INR) of 2.0-3.0 appear to suffer a high incidence of recurrence. Current thought suggests an appropriate INR of 3.0 to 3.5.

In some patients it may also be necessary to treat the underlying autoimmune disease (e.g. SLE) with anti-inflammatory agents and immunosuppression. In patients with the catastrophic antiphospholipid antibody syndrome, it may also be necessary to use plasmapheresis and intravenous immunoglobulin as well as immunosuppression.

References

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The term antiphospholipid antibodies (aPL) refers to a large group of immunoglobulins that are detected by means of test systems employing phospholipids (PL). Early in this century Wasserman, and one year later Michaelis set up a complement fixation test and a flocculation test, respectively, to identify reagin in the sera of patients suffering from syphilis (Table 1). In 1941, Pangborn identified the antigen towards which these antibodies were directed, i.e. a phospholipid extracted from bovine heart, and thus named cardiolipin (CL). In the '50s, Conley and Hartmann, and subsequently Frick reported a coagulation inhibitor in patient plasmas positive for the presence of reagin as ascertained by the flocculation test (VDRL). This inhibitor was detected frequently in patients with LES, and was thus termed lupus anticoagulant (LA) by Feinstein and Rapaport. LA was rarely associated with bleeding disorders, but it was often present in patients with thromboembolic disease, as reported in 1956 by Laurell and Nilsson, and in 1965 by Bowie in a series of 8 patients. In 1965 the association between a circulating anticoagulant and fetal loss was also described. In 1980 Thiagarajan and coworkers demonstrated for the first time that when isolated human IgM monoclonal antibodies with LA activity were tested in double immunodiffusion against PL, they were able to precipitate anionic, but not zwitterionic phospholipids. In 1983, Harris set up a radioimmunoassay to detect aPL by means of CL adsorbed onto plastic surfaces, and two years later Loizou described the popular aCL ELISA.

In vitro mechanism of action
To elucidate the mechanism of action of aCL-IgG, we purified these immunoglobulins by means of cardiolipin-liposome and protein A affinity chromatography. Like purified IgM monoclonal antibodies, purified polyclonal aCL-IgG bind anionic but not zwitterionic PL in ELISA plates, and possess LA activity. The binding of these antibodies to procoagulant PL in coagulation assays could sterically impede vitamin K coagulation protein assembly on the PL surface, and thus delay thrombin formation. In fact, when phosphatidyl-serine coated plates were incubated with I25I prothrombin in the presence of Ca++, labeled protein binding was impaired in the presence of aCL-IgG. From these experiments, we concluded that the general mechanism of action of aPL in vitro was the inhibition of vitamin K dependent coagulation factor binding onto a PL surface.

Search for an in vivo target
The in vitro prolongation of PL dependent coagulation tests is rarely associated with bleeding. Indeed, when activated platelets are substituted for PL, prolonged coagulation times normalize, thus demonstrating that LA is an exquisitely in vitro phenomenon, with no corresponding in vivo delay in thrombin formation but paradoxically associated with the occurrence of thromboembolic events. To explain thrombosis, vascular endothelium could constitute an in vivo target for aPL. By incubating cultured bovine endothelial cells with total IgG from a patients with LA and thrombosis, in 1981 Carreras and coworkers were able to...
demonstrate a reduction in endothelium-released PGI2. To relate this effect to aPL, we set up a system to verify the binding of aCL to endothelial cells. Human umbilical vein endothelial cells were grown to confluence onto plastic plates, and then incubated with 80 µg/mL purified aCL-IgG from 7 positive patient plasmas. Bound IgGs were detected by means of 125I-monoclonal anti human IgG antibody reactive against the Fc portion of human IgG; the results were expressed as endothelial cell bound molecules, calculated on the basis of monoclonal antibody specific activity and 1:1 binding of monoclonal antibody to human IgG. A human IgG (16 µg/mL) directed against HLA and normal IgG served as positive and negative control. As shown in Figure 1, binding was around 300,000 molecules/cell for the positive control, and less than 10,000 molecules/cell when aCL-IgG or control IgG were used.

The identification of aCL cofactor

In the meantime, three groups independently showed that a plasma cofactor, β2-glycoprotein I (β2GPI), was necessary for autoimmune antiphospholipid antibodies to bind anionic PL. As low as 1 µg/mL β2GPI was sufficient to determine aPL binding. The characteristics of this protein are shown in Table 2. β2GPI is a single chain 50KD molecular weight glycoprotein, which presents a microheterogeneity that can be ascribed to the carbohydrate moiety. This carbohydrate moiety (19%) is represented by 5 oligosaccharide residues attached to asparagin. The mean plasmatic concentration of β2GPI is 0.2 mg/mL, 65% free and 35% bound to lipoproteins. The β2GPI is composed of 326 amino-acids, with a high prevalence of cysteine and proline. Its synthesis is encoded in chromosome 17. It is heat resistant, perchloric acid-soluble, and interacts irreversibly with an air-water interfaces. β2GPI binds to anionic PL, DNA, heparin, and mitochondria, determining their agglutination. Among its biological activities, β2GPI inhibits ADP-induced platelet aggregation by stimulating adenylate cyclase, and determines anticoagulant activity by inhibiting contact activation of
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Intrinsic blood coagulation, or prothrombinase activity. Finally, it possesses a lipoprotein lipase activity that clears triglyceride-rich lipoprotein from the circulation.

The way in which this glycoprotein mediates aCL binding to CL is not known. This plasma protein cofactor could be the true antigen; however the fact that aCL binding is allowed when $\mu\mathrm{GPI}$ is bound to anionic PL but not to heparin could indicate that both aCL and $\mu\mathrm{GPI}$ comprise the antigen towards which aPL are directed. Either the protein or PL could undergo conformational changes and thus induce aPL binding.

**Attempts at ex vivo aPL purification**

If both PL and $\mu\mathrm{GPI}$ constitute the target antigen for aPL it should be possible to purify aPL from an in vivo structure containing both molecules. We tried to elute aPL from gel filtered platelets, in which in vitro platelet activation and $\mu\mathrm{GPI}$ binding are thus avoided, but did not succeed; we concluded that in our study patients, aPL were not bound to the platelet membrane, nor were the platelets the in vivo target for these antibodies. As approximately 35% of $\mu\mathrm{GPI}$ is bound to lipoproteins and thus to a PL matrix, it is possible that $\mu\mathrm{GPI}$-bearing lipoproteins could constitute the in vivo target for aPL. To test this hypothesis, we isolated the various lipoprotein classes from the plasma of two middle age, female patients with high aPL titre and thrombosis. Both patients had a history of venous thromboembolic disease, and were on oral anticoagulant treatment. Both had IgG aCL (70 and 56 GPL units respectively), no IgM aCL, and a strong LA activity (2.87 and 2.27 respectively using the dilute Russell viper venom time method and equal volumes of patient and control plasma). Lipoprotein density classes were separated by sequential ultracentrifugation, and the lipoprotein fractions (VLDL+IDL, LDL, HDL2, HDL3) were delipidated using ethyl ether/ethanol.

Both patients had mild hypercholesterolemia and hypertriglyceridemia, and one had a high level of Lp(a) (37 mg/dL). $\mu\mathrm{GPI}$ was identified by SDS-PAGE in VLDL+IDL, LDL, HDL2, and HDL3 fractions of one patient, and only in the VLDL+IDL fraction of the other. The protein content of the isolated lipoproteins did not contain aCL or LA activity. Based on our findings in these two patients, we concluded that $\mu\mathrm{GPI}$-containing lipoprotein classes do not bear aPL or LA activity, and these lipoproteins do not appear to be the in vivo target of aPL.

**Conclusions**

At this time, we can only state that the in vitro target for autoimmune aPL is a complex antigen comprising PL and $\mu\mathrm{GPI}$. Indeed, these antibodies recognize $\mu\mathrm{GPI}$ bound to PL but not to heparin. However, when we tested 8 aCL and LA positive plasmas in an anti-$\mu\mathrm{GPI}$ ELISA, all 8 plasmas showed high positivity, thus raising the possibility that aCL in fact might be antibodies...
directed against $\beta_2$GPI. In any case, the in vivo target for these antibodies should be a structure containing both PL and $\beta_2$GPI. However, our attempts to elute aCL from the platelet membrane or the $\beta_2$GPI-containing lipoprotein fractions were not successful. Therefore, the in vivo target of these antibodies, as well as the mechanism of thrombosis in patients with the aPL syndrome, remain uncertain.

References
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Antiphospholipid antibodies (aPL) are a heterogeneous population of antibodies which are detected by immunological assays (anticardiolipin antibodies) and/or clotting assays (lupus anticoagulant). Recent reports described lupus anticoagulant (LA) as directed against a prothrombin-phospholipid complex, while anticardiolipin antibodies (aCL) seem to be directed against a β2-glycoprotein-phospholipid complex.

aPL were characteristically found in patients with systemic lupus erythematosus (SLE) or related autoimmune disorders, although aPL were described during infectious diseases, neoplastic disorders, liver cirrhosis, in otherwise apparently healthy subjects or following drug exposure. In these settings aPL may appear transiently or persist for years.

Forty-fifty percent of SLE patients with aPL have a clinical history complicated by thrombosis, Venous thrombosis is the most frequent event that commonly occurs in the deep venous system of the legs and is often recurrent. Portal vein, renal vein, retinal vein involvement and pulmonary hypertension were also described in aPL positive patients. Arterial thrombosis most commonly affects cerebral arteries, but may also involve the coronary, mesenteric, renal and retinal arteries.

The prevalence of a previous thrombosis in SLE patients was 3-4 fold higher in LA positive and 2-4 fold higher in aCL positive cases in comparison to aPL negative ones. A recent cross-sectional study of consecutive SLE patients, confirmed the significant association of aPL with previous thrombosis. However, prospective trials indicating that aPL increase the risk for thrombosis are still lacking.

The prevalence of previous thrombosis in aPL positive (+) patients with non-SLE disorders was reported lower (22%) than in SLE patients (42%), while in most series, drug-related aPL positivity was not linked to a history of thrombosis. Finally, in young patients with thrombosis but without an underlying disease, the prevalence of aPL was studied extensively.

Among young patients with venous thrombosis, stroke, transient ischemic attack (TIA) and myocardial infarction, aPL were more frequently found than in normal controls or patients with non-thrombotic disorders, but the clinical significance of aPL positivity in these patients is still unknown. Some studies evaluated if these aPL(+) subjects have a higher risk of recurrence of thrombosis, but the results are conflicting.

Interestingly, a recent study analyzed the clinical course of 70 aPL(+) patients after the first thrombotic event. The study showed that the site of the first event (arterial or venous) predicted (91%) the site of subsequent events. It is noteworthy that the strength of the association between aPL and previous thrombosis was increased by using multiple assays to detect LA and by determining the presence of persistently positive aPL, by repeating the assays at least 3 months apart.

The presence of a group of patients in whom aPL and thrombosis coexist without clinical features of SLE has lead to the notion of primary antiphospholipid syndrome (PAPS). The present criteria for the syndrome include one or more features among thrombosis, thrombocytopenia and spontaneous abortions together.
with aPL positivity measured on two occasions.\textsuperscript{19,20} Other proposed features of PAPS are autoimmune hemolytic anemia, valvular heart disease, livedo reticularis and choreoathetosis.\textsuperscript{14} It is noteworthy that in a 1989 study of 70 patients, there was a lower female to male (2:1) ratio than in SLE (9:1) and interestingly, at follow-up none of these patients developed SLE.\textsuperscript{21}

**Deep venous thrombosis**

The occurrence of deep venous thrombosis (DVT) has been studied in many retrospective studies, analyzing consecutive aPL(+) patients or consecutive SLE patients with or without aPL. A study of 500 SLE patients found a relative risk of 2.1 for a first DVT and a relative risk of 10.5 for recurrent DVT in subjects with high-titer IgG aCL.\textsuperscript{12} In a recent review, the occurrence of DVT in aPL(+) patients, was three times as common (42%), in aPL(–) patients with SLE (12%).\textsuperscript{7}

Recently, in a prospective, nested case-control study, healthy adult men with high-titer of circulating aCL showed a significantly higher risk for DVT or pulmonary embolism than aCL(–) men, independently of age and smoking status. Interestingly, the increased risk was strongly significant above an aCL titer of 33 GPL Units, corresponding to the 95th percentile of the values; the relative risk rose with increasing aCL levels. The estimated risk for DVT or pulmonary embolism in aCL(+) subjects was similar to other well-established risk factors for DVT, such as estrogen oral contraceptive use, smoking, hypertension and obesity.\textsuperscript{12} Venous thrombosis most commonly occurs in the deep veins, but any part of the venous system can be affected. In a recent study, 56% of consecutive liver cirrhosis patients with splanchnic venous thrombosis (SVT) showed aPL positivity indicating that aPL may be a risk factor;\textsuperscript{23} this suggestion is supported by a previous study, showing that aPL may favor hepatic veno-occlusive disease or SVT.\textsuperscript{24}

**Cerebral ischemia**

The most common neurologic syndrome associated with aPL is ischemic stroke. The other neurological manifestations of ischemia include TIA, ischemic optic neuropathy, retinal venous thrombosis, cerebral venous thrombosis and multi-infarct dementia.\textsuperscript{25} In retrospective studies cerebrovascular ischemia and aPL positivity were commonly associated with SLE or antiphospholipid syndrome.\textsuperscript{26,27} A prospective study confirmed that aPL may be an important risk factor for ischemic stroke in SLE patients, but not in healthy adult men.\textsuperscript{22}

Different results have been obtained by analyzing the prevalence of aPL in young patients (<50 years) with cerebral ischemia. In a series of stroke registries recently reviewed, 2-7% of brain infarcts in young adults have been attributed to hematological causes; among these aPL positivity was the acquired prothrombotic condition most frequently detected.\textsuperscript{28}

In 1984, Hart showed LA positivity in 4% of a young stroke population.\textsuperscript{29} In the last years, probably due to the use of more sensitive tests, a stronger association seems to be emerging.\textsuperscript{30} In a recent study, LA and aCL were detected in 18% and 24% respectively of consecutive young patients with stroke. It is noteworthy that only a minority of these patients had SLE; moreover aPL(+) subjects were younger and with a history of frequently recurrent cerebral ischemic episodes.\textsuperscript{31}

**Coronary artery disease**

In SLE myocardial infarction occurs in 2-8% of patients;\textsuperscript{32} aPL were suggested as possible risk factor, together with early atherosclerosis related to long-term steroid administration and coronary arteritis.\textsuperscript{33} Actually, the relationship between aPL and arterial thrombosis in the general population was estimated in approximately 5% of aPL(+) subjects, particularly in young (<45 years) patients, where atherosclerosis was infrequent.\textsuperscript{34} A previous study showed that young survivors of myocardial infarction with high aCL levels have a significantly higher risk of recurrent cardiovascular events.\textsuperscript{14} This observation was not confirmed by three subsequent studies, where anticephalin antibodies and aCL were
not predictive for mortality, reinfarction, non-hemorrhagic stroke and venous thromboembolism during 12-24 months of follow-up.\textsuperscript{15,35,36}

Otherwise, another report described a significant association between the preoperative aCL levels and the incidence of late graft failure in patients with coronary artery bypass graft surgery.\textsuperscript{37}

The lack of standardization of aPL assays, the criteria for aCL positivity, the presence of transiently aPL(+) patients and the small number of events may explain these conflicting results.

**Fetal loss**

In prospective studies performed in SLE women, the incidence of fetal loss was 4 to 10 times higher in aPL positive cases; aPL(+) women had a 60% risk of fetal loss.\textsuperscript{1} In some studies the risk was dependent on the titer and the IgG type of aCL.\textsuperscript{38,39} Recently, it has been shown that a more significant association between aPL and fetal loss in SLE patients can be shown by performing multiple LA tests and by repeating aCL assays on more than one occasion to identify persistently aPL(+) subjects.\textsuperscript{17}

On the contrary, aPL positivity does not predict fetal loss in women without apparent underlying diseases and without a history of fetal loss;\textsuperscript{40} however, this study excluded all patients with previous pregnancy loss and the aPL study was performed once by using only two different screening tests for LA.

On the contrary aPL are more commonly found in non-SLE women with repeated fetal loss compared to women without such a history;\textsuperscript{41} but until now prospective studies have not been carried out and the predictive value of aPL in non-SLE women is still to be defined.

**Antiphospholipid antibodies and thrombosis: physiopathological aspects**

Several components of the hemostatic system have been explored to explain the relationship between lupus anticoagulant and thrombosis, such as coagulation and fibrinolytic systems, platelet and endothelial cell.\textsuperscript{11,42} Activation of clotting system in LA positive patients is a mechanism potentially accounting for the association between LA and thrombosis. This possibility has been recently demonstrated by two studies\textsuperscript{43,44} showing that patients with systemic lupus erythematosus and antiphospholipid antibodies have high circulating levels of the fragment F1+2, a cleavage product forming through the prothrombin conversion to thrombin, which is considered a marker of in vivo thrombin generation.\textsuperscript{45} F1+2 plasma levels were elevated overall in patients with positivity for lupus anticoagulant while there was not a strong association between high levels of F1+2 and anticardiolipin antibodies.\textsuperscript{44} This ongoing prothrombotic state may be explained by endothelium-mediated lupus anticoagulant interaction with the coagulation system. LA has been shown to inhibit, on endothelial cells, the interaction between thrombin and thrombomodulin,\textsuperscript{46} so reducing the activation of protein C, an inhibitor of factor Va and factor VIIIa. An in vitro study, failed to demonstrate that LA is able to activate the clotting system on endothelial surface.\textsuperscript{47} Conversely, this effect was evident when lupus anticoagulant was combined with tumor necrosis factor (TNF), a cytokine which induces endothelial damage and shifts endothelial function toward procoagulant activity.\textsuperscript{48} These findings seem to suggest that LA may activate the coagulation cascade only in presence of endothelial damage.

Previous studies demonstrated that LA positive patients have an imbalance between profibrinolytic and antifibrinolytic activity in the peripheral circulation.\textsuperscript{49} Thus, an increased activity of tissue-plasminogen inhibitor activity has been reported in SLE patients positive for LA.\textsuperscript{50}

The interaction between LA and eicosanoids is another mechanism potentially explaining the association with thrombosis. LA has been reported to inhibit in vitro the endothelial production of prostacyclin,\textsuperscript{51} which is a potent inhibitor of platelet aggregation and vasodilatant substance. This possibility has been confuted by other authors who failed to observe that LA is capable of inhibiting PGI\textsubscript{2} synthesis.\textsuperscript{52} Also, the measurement of urinary excretion of 2,3-dinor-6-keto-PGF\textsubscript{1-α}, a stable metabolite of PGI\textsubscript{2}, showed that SLE patients positive for
LA have increased urinary excretion of PG12.

The relationship between LA and platelet function has been extensively studied but failed to give conclusive results. Overall, there is no clear evidence that LA per se activates platelet function. Recent in vitro experiments suggest that LA may enhance thrombin-induced platelet activation.

Conversely two recent studies showed that in patients with LA there is an increased urinary excretion of 11-dehydro-thromboxane-B2, a stable metabolite of thromboxane A2. This observation is a result of in vivo platelet activation since the administration of aspirin, which inhibits platelet PGG/PGH synthase, significantly reduced the urinary excretion of 11-dehydro-TxB2.

In conclusion in vivo studies have shown that patients with aPL have an ongoing prothrombotic state, documented by high values of F1+2, and platelet activation. While the mechanism accounting for these findings have to be elucidated, hyperreactibility of the clotting system and platelet provides plausible explanation for the association between aPL and thrombosis.

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Basic, Laboratory and Clinical Aspects of Venous Thromboembolism
Foreword

From March 9th to March 12th, 1994, a bunch of friend scientists collected in Cortina d’Ampezzo, Italy to held the First Winter Meeting on Coagulation, dedicated to Basic, Laboratory and Clinical Aspects of Venous Thromboembolism.

The meeting was characterized by the remarkable level of the presentations, by the mutual exchange of themes and experiences from different settings and different disciplines, and by the particular atmosphere of the place. Authors released original informations, subsequently confirmed in the literature, and most presentations were the subject of extensive, profitable and friendly discussion.

This supplement of Haematologica reports the proceedings of this fascinating meeting, and is the results of the combined efforts of the authors and of the editors, who succeeded in releasing it with no financial support.

We hope that our work be rewarded by our readers’ interest, and that this meeting be the first of a long series.

Co-editors

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Antonio Girolami
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