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3. Lusher JM. Blood Coag Fibrinolysis 2006; 17(suppl 1): S45-49
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Currently, there are several lines of evidence of the interplay between coagulation and inflammation in the propagation of various disease processes, including venous thromboembolism (VTE) and inflammatory diseases. The vitamin K antagonists (VKAs) such as warfarin have been the standard treatment of thromboembolic diseases for over 60 years. However, the level of anticoagulation with VKAs is frequently outside the therapeutic range, potentially compromising safety and efficacy because of the food/drug interactions, and other variables. These limitations have prompted development of new oral anticoagulants that target Factor IIa or Factor Xa and that are given in fixed doses without routine anticoagulation monitoring. Major advances in the development of oral anticoagulants have resulted in considerable progress towards the goal of safe and effective oral anticoagulation that does not require frequent monitoring or dose adjustment, and has minimal food/drug interactions. Indirect inhibitors of factor Xa and factor IIa such as low-molecular-weight heparin (LMWH) and the pentasaccharide fondaparinux represent improvements to unfractionated heparin for acute treatment of VTE, constituting a more targeted anticoagulant approach, with predictable pharmacokinetic profiles and no requirement for monitoring. The VKAs, with their inherent limitations in terms of multiple food and drug interactions and frequent need for monitoring, remain the only oral anticoagulant approved for long-term secondary thromboprophylaxis in VTE. Newer anticoagulant drugs include oral direct thrombin inhibitors (dabigatran), oral direct factor Xa inhibitors (rivaroxaban, apixaban, and others), and tissue factor/factor VIIa complex inhibitors that have been “tailor-made” to target specific pro-coagulant complexes, and have the potential to greatly expand oral antithrombotic targets for both acute and long-term treatment of VTE, and for the prevention of stroke in atrial fibrillation patients.

Inflammation plays a key role in triggering a prothrombotic state through the activation of platelets, induction of coagulation, and vascular insult. Venous thromboembolism (VTE) continues to be a major cause of morbidity and mortality in the western world.1 VTE represents 1 in 10 hospital deaths, and post-thrombotic syndrome and pulmonary hypertension occur in 10% of deep-vein thrombosis (DVT) and 5% of pulmonary embolism (PE) patients, respectively.2 For more than 50 years, traditional drugs such as unfractionated heparin (UFH) were used parenterally for acute treatment, followed by oral vitamin K antagonists (VKAs) such as warfarin for long-term treatment (Figure 1). These drugs exert their antithrombotic effects by inhibiting multiple steps of the coagulation cascade, but there are inherent limitations for each drug.

For acute VTE treatment, the limitations of UFH include a less than predictable anticoagulant response with the need for frequent monitoring, and the potential for severe toxicity, especially heparin-induced thrombocytopenia (HIT) in up to 3% of patients.3 Over the past fifteen years, the use of LMWH, and more recently, the synthetically-derived pentasaccha-
ride fondaparinux has improved the acute management of VTE. A more targeted approach to pro-coagulant complex inhibition, predictable pharmacodynamic characteristics, and improved safety profiles have enabled complete treatment of VTE on an outpatient basis for selected patients without the need for anticoagulant monitoring. Other parenteral drugs, such as the direct thrombin inhibitors (DTIs) lepirudin and argatroban, have achieved only limited use in acute VTE treatment, namely in thrombosis associated with HIT.

Optimal long-term treatment of VTE is defined by the limitations of VKAs, the only oral anticoagulants currently approved for use. These limitations include a slow onset of action and the need for bridging anticoagulants with a parenteral drug in the acute setting, multiple food and drug interactions, and a narrow therapeutic window, necessitating frequent coagulation monitoring and dose adjustment. Additionally, some patient subgroups cannot tolerate VKA, such as pregnant patients requiring anticoagulation, in whom VKA is associated with a risk of teratogenicity, or patients in whom VKA is associated with higher risks of recurrent thromboembolism and major bleeding, such as those with active cancer. In both of these patient groups, emerging data support the use of long-term lowmolecular weight heparin (LMWH), with limited parenteral use.

An improved understanding of the molecular mechanisms of coagulation and thrombosis, and the potential to apply this knowledge at the clinical level to different patient subgroups has led to the development of newer antithrombotic drugs for use in VTE treatment. Many of these drugs are orally active, synthetically derived, and target specific pro-coagulant complexes within the coagulation cascade. These drugs can broadly be categorized as interfering with the initiation of coagulation [tissue factor/factor VIIa (TF/FVIIa) complex], propagation of coagulation [indirect and direct inhibitors of activated factor X (FXa) or FIXa], and thrombin activity (DTIs). This review will focus on these investigational drugs for VTE treatment, with an emphasis on those undergoing or that have recently completed phase II or III clinical studies (Table 1). The role of anticoagulants on stroke in atrial fibrillation patients will also be discussed. Atrial fibrillation (AF) is an epidemic, affecting 11.5% of the population in the developed world. Its significance lies predominantly in that AF patients have a five-fold increased risk of stroke. Stroke associated with AF is usually more severe and confers increased risk of morbidity and mortality, and poor functional outcome. Despite advances in the development of promising experimental approaches for selected patients with acute stroke, primary prevention with pharmacological agents remains the best approach to reducing the burden of stroke.

**Figure 1.** Advances in anticoagulants in the United States. There have been important advances in injectable anticoagulants in the last decade, but no new class of oral anticoagulant has been introduced since 1954 in the United States. Vitamin K antagonists such as warfarin are the only currently available oral anticoagulants. As outlined in the review, a number of very promising oral anti-Xa and oral anti-IIa (direct thrombin inhibitors or DTIs) are approved in the European and other worldwide markets and awaiting FDA approval in the United States.
2. Anticoagulant Targets That Inhibit the Initiation Phase

2.1 TF/FVIIa Complex Inhibitors

The TF/FVIIa complex, as part of the extrinsic system of the coagulation cascade, is considered to be the key system for the initiation of coagulation. In the venous vascular system, exposure of TF in orthopedic surgery and in subsets of cancer patients is believed to be responsible for the high risk of VTE in these patient groups, making pharmacological inhibition of the TF/FVIIa complex important. The function of TF can be blocked using several approaches, such as antibodies that prevent the binding of FVIIa to TF, active-site-inhibition of FVIIa, small molecules or antibodies that block TF/FVIIa complex function, and molecules that inhibit the active site of FVIIa in the TF/FVIIa complex after binding to FXa. Moreover, TF pathway inhibitor (TFPI), a naturally occurring inhibitor, forms a neutralizing complex with TF/FVIIa and FXa. Thus TFPI, either by up-regulation of endogenous TFPI pools or exogenous administration of recombinant TFPI (rTFPI), represents an attractive anticoagulant that acts by blocking the pathological impact of TF/VIIa and Xa in coagulation and beyond.

Nematode Anticoagulant Proteins:

The hematophagous hookworm *Ancylostoma caninum* produces a family of small, disulfide-linked protein anticoagulants (75-84 amino acid residues) referred to as nematode anticoagulant proteins (NAPs), which have been the focus of antithrombotic drug development efforts due to their ability to inhibit the TF/FVIIa complex. One of these nematode anticoagulant proteins, NAP5, inhibits the amidolytic activity of FXa with a Ki=43 pM, and is the most potent natural FXa inhibitor identified to date. NAP5 does not inhibit FVIIa in the TF/FVIIa complex. Rather, it either binds to FXa alone, or, as is the case for its family member NAPc2, in combination with a protein exosite, resulting in potent inhibition of the TF/FVIIa complex. NAPc2 has been tested subcutaneously for VTE prophylaxis in a phase I/II clinical trial using mandatory unilateral venography in 293 patients undergoing total knee replacement surgery. At a dose of 3 μg/kg administered within one hour after surgery, NAPc2 was associated with an overall DVT rate of 12.2%, a proximal DVT rate of 1.3%, and a major bleed rate of 2.3%. Further clinical trials with NAPc2 are in progress.

Recombinant NAPc2, like other inhibitors of TF/FVIIa, including TFPI and active site-blocked FVIIa (ASIS, FFR-rFVIIa or FVIIai), have a promising role in the prevention and treatment of venous and arterial thrombosis, and could potentially be efficacious in the management of disseminated intravascular coagulopathies, due to their ability to selectively inhibit TF/FVIIa.

**Anti-TF/VIIa:**

FVIIa is a key serine protease involved in the initiation of the coagulation cascade. It is a glycosylated disulfide-linked heterodimer comprised of a heavy chain and a light chain. The light chain contains an amineterminal gamma-carboxyglutamatic acid-rich (Gla) domain and two epidermal growth factor (EGF)-like domains, and there is a chymotrypsin-like serine protease domain in the heavy chain. TF, a membrane bound protein, is an essential cofactor of FVIIa that is required for maximal activity towards its biological substrates (FX, FIX and FFVII). As such, the TF/FVIIa complex plays an important role in normal physiology as well as in thrombotic diseases such as unstable angina (UA), disseminated intravascular coagulation (DIC), and DVT. In addition to its function as an initiator of coagulation, TF/FVIIa plays an important role in inflammation and angiogenesis. A wide array of strategic approaches to inhibiting the biochemical and biological functions of the TF/FVIIa complex has been pursued. These have been greatly enhanced by elucidation of the structures of TF, FVII, FVIIa, and the TF/FVIIa complex, resulting in inhibitors that are directed specifically towards either FVIIa or TF. Antagonists of the TF/FVIIa complex include active site inhibited FVIIa, TF mutants, anti-TF antibodies, anti-FVII/FVIIa antibodies, naturally occurring protein inhibitors, peptide exosite inhibitors, and protein and small molecule active site inhibitors. These antagonists can inhibit catalysis directly at the active site as well as impair function by binding to exosites that interfere with substrate, membrane, or cofactor binding. Several different small molecule potent inhibitors of TF/FVIIa have been shown to reduce thrombus weight in animal models and decrease the level of interleukin-6 (IL-6) in a LPS-stimulated mouse model of endotoxemia. A study designed to evaluate the antithrombotic efficacy and bleeding propensity of a selective, small-molecule inhibitor of TF/VIIa in comparison to small-molecule, selective inhibitors of factor Xa and thrombin in a nonhuman primate model of thrombosis was reported by Suleymanov et al (2003). The data indicated that TF/VIIa inhibition effectively prevents arterial thrombosis, with less impact on bleeding parameters than equivalent doses of factor Xa and thrombin inhibitors.
Tissue Factor Pathway Inhibitor (TFPI):

The anticoagulant effects of TFPI in animals and the role of TFPI release in secondary anticoagulant mechanisms of LMWH action in humans have been demonstrated. To date, rTFPI has been tested only in experimental models. TFPI is a natural (that is, endogenous) inhibitor of TF coagulant and signaling activities. TFPI exerts anti-angiogenic and anti-metastatic effects in vitro and in vivo. In animal models of experimental metastasis, circulating and tumor cell-associated TFPI significantly reduce tumor cell-induced coagulation activation and lung metastasis. Heparins and heparin derivatives, which induce the release of TFPI from the vascular endothelium, also exhibit antitumor effects, and TFPI likely contributes significantly to these effects. Recently, a non-anticoagulant LMWH with intact TFPI-releasing capacity was shown to have significant anti-metastatic effects in an experimental mouse model. Evidence of dual inhibitory functions of TFPI on TF-driven coagulation and signaling strengthen the rationale for considering TFPI a potential anticancer agent.

TFPI-2, a member of the Kunitz-type serine protease inhibitor family, is a structural homologue of TFPI. The expression of TFPI-2 in tumors is inversely related to the degree of malignancy, suggesting a role for TFPI-2 in the maintenance of tumor stability and inhibition of neoplastic growth. TFPI-2 inhibits the TF/VIIa complex and a wide variety of serine proteinases, including plasmin, plasma kallikrein, FXIa, trypsin, and chymotrypsin. Aberrant methylation of TFPI-2 promoter cytosine-phosphorothioate-guanine (CpG) islands in human cancers and cancer cell lines results in decreased expression of TFPI-2 mRNA and decreased synthesis of TFPI-2 protein during cancer progression. TFPI-2 has been shown to induce apoptosis and inhibit angiogenesis, thereby potentially contributing significantly to inhibition of tumor growth. Restoration of TFPI-2 expression in tumor tissue inhibits invasion, tumor growth, and metastasis, lending support to the use of TFPI-2-targeted therapeutics as novel treatments for cancer.

3. Inhibitors of Coagulation Propagation

It is widely accepted that FXa, as part of a prothrombinase complex with FVas, has a central role in clot formation, given that it is generated by the extrinsic and intrinsic pathways of coagulation as they converge into the final common pathway. In addition, within the prothrombinase complex, one molecule of FXa can exponentially generate 138 molecules of thrombin per minute. Theoretically, FXa inhibitors might have an advantage over thrombin inhibitors by preventing the activation of coagulation amplification mechanisms as well as thrombin generation in both platelet-rich arterial thrombosis and fibrin-rich venous thrombosis, making FXa a prime target for anticoagulant drug design. However, in animal models of thrombosis, selective FXa inhibition was less potent than direct thrombin inhibition in arterial and venous models of thrombosis, and associated with increased global clotting times, indicating that inhibition of FXa is less effective than direct thrombin inhibition in controlling thrombin formation.

The possibility that selective inhibition of FXa upstream could result in a safer bleeding profile cannot be entirely ruled out; in the absence of thrombin inhibition, small amounts of thrombin would escape neutralization and facilitate hemostasis.

Inhibitors of FXa include both indirect (anti-thrombin-mediated) and direct antithrombin (AT)-independent selective inhibitors. Other possible targets of coagulation propagation, through the prothrombinase complex or other routes, include FXIa inhibitors, FVIIIa and FVa inhibitors, activated protein C, or soluble thrombomodulin.

3.1. Indirect FXa Inhibitors

The synthetically derived pentasaccharide fondaparinux and idraparinux represent the most advanced selective indirect FXa inhibitors. These agents exert their action through high-affinity binding and activation of AT, which then inhibits free FXa. Fondaparinux contains the pentasaccharide sequence of heparin, and selectively binds to and induces a conformational change in AT, increasing the anti-Xa activity of AT nearly 300-fold in a catalytic fashion. Fondaparinux has a linear pharmacokinetic profile and predictable anticoagulant response, with a plasma half-life of approximately 18 hours and >95% bioavailability after intravenous or subcutaneous injection, allowing non-monitored once daily subcutaneous dosing. In addition, it does not bind to platelet factor 4, and has not been associated with drug-induced thrombocytopenia. Fondaparinux is currently approved for acute treatment of DVT and PE based on the recently completed MATISSE studies in VTE. As such, fondaparinux represents the first of a class of selective indirect FXa inhibitors to provide proof of concept that FXa inhibitors can be used to treat thrombosis in the acute stage as efficaciously as drugs with established AT activity. Fondaparinux is also the first of a new class of antithrombotic drugs designed specifically to inhibit a single target or procoagulant complex in the coagulation cascade.
ORAL ANTICOAGULANTS

3.1. Idraparinux (Sanofi-Aventis)

Idraparinux is a hypermethylated, long-acting pentasaccharide that can be administered with once-weekly dosing. Idraparinux sodium is a second generation pentasaccharide with sulfated side chains, which results in a 30-fold higher binding affinity to AT as compared to fondaparinux, and a 120 hour elimination half-life, allowing once-weekly administration. It has similar clinical properties as fondaparinux, with 100% bioavailability after parenteral administration, linear pharmacokinetics, a predictable anticoagulant response with no need for monitoring, does not induce platelet aggregation, has no effects on platelet factor 4, and no evidence of induction of thrombocytopenia. The major drawback, as for fondaparinux, has been the lack of an antidote, although the importance of an antidote in clinical practice is controversial. However, it should be noted that biotinylated idraparinux, discussed in detail later, does have an antidote.

The PERSIST study, a randomized phase II, dose-ranging study, compared idraparinux with warfarin over a 12-week course of treatment for DVT (after initial studies of enoxaparin with idraparinux demonstrated efficacy at all doses that was similar to warfarin). No clear dose-response relationship for efficacy was shown with idraparinux, but a significant dose-response relationship for major bleeding was shown. A large phase III trial comparing the efficacy and safety of idraparinux with heparin or fondaparinux and dose-adjusted warfarin in both acute and long-term treatment of DVT and PE has recently been completed.

3.1.2. SSR126517E (biotinylated idraparinux)

This synthetic pentasaccharide being developed by Sanofi-Aventis exerts antithrombotic properties through AT-mediated inhibition of FXa activity. It is identical to idraparinux with the exception of a biotin moiety covalently affixed through a linker to the pentasaccharide structure so that anti-FXa activity can be neutralized in vivo by avidin. In vitro studies revealed that SSR126517E binds with high-affinity (Kd=10⁻⁹ mol/l) to human AT and inhibits FXa in a concentration-dependent manner. It does not inhibit platelet aggregation or cross-react with antibodies from sera of patients with HIT. In phase I studies, the median time to reach maximum concentration was four hours, with an absolute bioavailability of 100% and a half-life of approximately 200 hours. Exposure of SSR126517E to avidin resulted in a rapid decrease of anti-Xa activity and no serious adverse events.

3.2. Selective Direct Factor Xa Inhibitors (Table 1)

Advantages of direct FXa inhibitors include the absence of intermediary molecules such as AT that may potentially contribute to inconsistent anticoagulation, particularly in acute or inflammatory states. DX-9065a was the first of a class of small, synthesized, selective direct FXa inhibitors to undergo phase II clinical trials in arterial thrombosis. Efforts to develop orally available selective FXa inhibitors for VTE and prevention of stroke in AF patients are underway (Table 1).

3.2.1. Razaxaban

Razaxaban (BMS-561389), developed by Bristol-Myers Squibb (formerly DuPont) is a small molecule oral direct FXa inhibitor that does not require anticoagulant monitoring. In phase I trials involving young and elderly volunteers, it was well tolerated and well absorbed, with only nuisance bleeding reported; FXa inhibition and dose-dependent anticoagulation were noted. Razaxaban was investigated for proof of principle DVT prevention in patients undergoing total knee replacement. In phase IIb trials, Razaxaban at doses of 25, 50, 75, or 100 mg twice daily administering starting 8 hours after surgery was compared to enoxaparin at a dose of 30 mg twice daily initiated 12-24 hours after surgery. The study revealed efficacy but an unacceptable risk/benefit profile at higher doses. Razaxaban was discontinued for further development in March 2005 in lieu of apixaban, an oral FXa inhibitor under development by the same company with a more favorable safety profile.

3.2.2. Apixaban

Apixaban (formerly BMS-562247 or DPC-AG0023) is an orally active, small-molecule direct FXa inhibitor being developed by Bristol-Myers Squibb with a more favorable safety profile than Razaxaban. It is a highly potent inhibitor of human FXa, with a Ki of 0.08 ± 0.01 nM, and binds to serum proteins at a rate of 87%. It has a consistent oral absorption profile and linear pharmacokinetics, with a maximal plasma concentration achieved within 3 hours and an effective half-life of 9 hours for twice daily administration, and 14 hours for once daily administration. It is eliminated via both hepatic and renal routes. Apixaban had only modest effects on two traditional markers of anticoagulation, International Normalized Ratio (INR) and activated partial thromboplastin time (aPTT). Phase I clinical studies revealed mild bleeding and prolonged bleeding time, with no evidence of elevated transaminases (alanine transaminase or aspartate transaminase ≥ 5 times the upper limit of the normal range). The safety...
profile of apixaban remains to be determined in phase II/III trials, and it is presently undergoing phase III clinical studies in elective total knee replacement surgery patients and patients with acute DVT. The efficacy and safety of apixaban as a thromboprophylaxis in patients following total knee replacement was determined. Apixaban in doses of 2.5 mg b.i.d. or 5 mg q.d. exhibited a promising benefit-risk profile as compared to current standards of care following total knee replacement.

Apixaban is also in Phase III trials studying the prevention of stroke and other thromboembolic events in patients with AF. The AF program consists of two trials. The ARISTOTLE trial is investigating apixaban compared to warfarin in approximately 15,000 patients with AF. The AVERROES trial is investigating apixaban compared to aspirin in approximately 5,600 patients with atrial fibrillation who are ineligible for VKA treatment or haven’t tolerated previous VKA treatment. The VTE treatment program consists of two trials. The AMPLIFY trial is a 6-month trial investigating apixaban compared to enoxaparin in approximately 4,800 patients with acute DVT or PE. The AMPLIFY-EXT trial is a 12-month trial investigating apixaban compared to placebo for extended treatment to prevent recurrent VTE in approximately 2,400 patients who have completed 6 to 12 months of treatment VTE.

3.2.3. Rivaroxaban (Bayer HealthCare AG and J&J/Scios, Inc.)

Rivaroxaban (BAY 59-7939) is a small molecule, selective oral direct FXa inhibitor developed by Bayer for the prevention and treatment of thrombosis. Rivaroxaban is an oxazolidinone derivative optimized for inhibiting both free Factor Xa and Factor Xa bound in the prothrombinase complex. It is already marketed in a number of countries by Bayer as Xarelto and if approved by the United States FDA, it will be marketed by Ortho-McNeil Pharmaceutical. It is the first available orally active direct inhibitor of coagulation Factor Xa. Oral rivaroxaban may be given in fixed once-daily doses, with potentially no coagulation monitoring. These properties, along with results from preclinical and clinical studies, suggest that the use of rivaroxaban might be more advantageous than current treatments. Studies in arterial and venous animal models have demonstrated that rivaroxaban has potent antithrombotic properties without prolonging bleeding times. In healthy subjects, rivaroxaban was well tolerated with a predictable pharmacological profile and a low propensity for clinically relevant drug interactions. In preclinical studies, endogenously generated FXa was inhibited with an IC50 of 21±1 nM. This antithrombotic effect was demonstrated in different thrombosis models at doses of 0.6-10 mg/kg, depending on the model and species, administered orally. In dogs, bioavailability was determined to range from 60-86%.

In phase I studies, rivaroxaban was rapidly absorbed (maximum concentration [Cmax] reached after 30 minutes) and well tolerated (up to 80 mg single dose in healthy people). Elimination occurred with a terminal half-life of 4.86-9.15 hours (steady state). Prothrombin time (PT), aPTT, and HepTest were prolonged in a dose-dependent manner, and there was no effect on bleeding time. In elderly men and women (≥60 years), mean area under the concentration-time curve and Cmax tended to be about 20% higher. There were no drug-drug interactions or induction of major cytochrome P450 (CYP) isoforms, with the exception of strong CYP3A4 inhibitors, and no prolongation of QTc.

Four large dose-ranging studies of rivaroxaban (ODIXa-HIP, ODIXa-HIP2, ODIXa-KNEE, and ODIXa-OD-HIP) have been completed, covering a 12-fold increase in dose, from 2.5 to 30 mg b.i.d. and 5 to 40 mg once daily (q.d.) for VTE prevention in major orthopedic surgery. The open-label phase IIa ODIXa-HIP trial using mandatory venography confirmed proof of principle for rivaroxaban use for VTE prophylaxis. Other studies have confirmed the efficacy and safety of rivaroxaban as compared to enoxaparin under double-blind, double-dummy conditions for VTE prevention in patients undergoing orthopedic surgery. Phase II studies of rivaroxaban for the prevention of VTE after major orthopedic surgery support these findings, and suggest that a total daily dose range of 5 to 20 mg rivaroxaban has similar efficacy and safety as enoxaparin, with 10 mg rivaroxaban once daily the optimal dose.

The drug development plan for rivaroxaban is aggressive, with a program of simultaneous investigation of multiple indications rather than a sequential approach. At present, over 24,000 patients (12,000 are predominantly post-operative orthopedic patients) have been evaluated in completed phase II and III trials of rivaroxaban for thromboprophylaxis and treatment of DVT. By the time all the currently enrolling trials have concluded, more than 50,000 patients will have been evaluated in randomized controlled trials of rivaroxaban. The advantages of rivaroxaban include the potential for once daily dosing for all indications, no required dose-adjustment for...
body-weight, no known interactions with common cardiovascular medications, a relatively safe pharmacodynamic profile with respect to bleeding risk and hepatotoxicity, no clinically significant interaction with aspirin, and the ability to bridge with LMWH when necessary. On the other hand, rivaroxaban is partially renally cleared and will require dose-adjustment in those with grade III chronic kidney disease, and is not being studied in patients with a creatinine clearance less than 30 mL per minute. In addition, since rivaroxaban metabolism is affected by potent cyp P450 3A4 inhibitors such as ketoconazole and clarithromycin and protease inhibitors, use will be restricted in certain special populations. Nonetheless, after extensive phase II, and now emerging phase III trial data, it appears that rivaroxaban is effective in preventing and treating VTE with a bleeding risk comparable to other anticoagulants. The results of randomized trials evaluating rivaroxaban for the prevention of stroke and non-central nervous system (CNS) embolism in AF and secondary prevention of acute coronary syndromes are currently ongoing.

While there are several other oral anticoagulants in development, none have been evaluated as extensively or in as many patients as rivaroxaban. Rivaroxaban has the advantage that it can be dosed once a day, which has been shown to improve patient compliance and outcomes. Despite once daily dosing, rivaroxaban has a half-life that is considerably shorter than other oral FXa inhibitors, which is advantageous in the event of bleeding or an urgent need to discontinue anticoagulation. Unlike the direct thrombin inhibitor dabigatran, the bioavailability of rivaroxaban is very good, and there is low risk of drug-drug interactions, including with medications that alter gastric pH, which are taken chronically by 3% of the US population. Perhaps more importantly, rivaroxaban has been shown to have no effect on platelet aggregation, and its pharmacokinetic profile is unaffected by aspirin and other notable cardiovascular medications such as digoxin. Finally, after clinical investigation in thousands of patients, rivaroxaban appears to have no significant hepatotoxicity and a bleeding risk comparable to other conventional anticoagulants.

Rivaroxaban could potentially be used in several clinical indications and disease states, including VTE prophylaxis, long term treatment of DVT, PE, and the prevention of stroke and non-CNS embolism in patients with AF and possibly acute coronary syndromes. In order to understand the efficacy and safety of such a wide range of patient populations, several large simultaneous studies evaluating rivaroxaban in both venous and arterial thromboembolism are under way. The greatest therapeutic impact of rivaroxaban might be in providing a much-needed and attractive alternative to warfarin. While formal cost-effective analyses are not yet available, avoiding the intense, costly, and frequent monitoring required with VKAs, as well as the potential to reduced adverse vascular events precipitated by the narrow therapeutic window of VKAs, will most likely translate into a significant improvement in quality-of-life and cost-savings. While further data (especially large phase III trials) and caution is required, there is reason for optimism. Rivaroxaban may very well be the long-awaited alternative to warfarin.

The RECORD4 study concluded that rivaroxaban was significantly more effective in reducing the occurrence of venous blood clots following knee replacement surgery than enoxaparin. In addition, rivaroxaban is being studied in phase III clinical trials for stroke prevention in non-valvular AF (ROCKET-AF), prevention of VTE in hospitalized medically ill patients (MAGELLAN), treatment and secondary prevention of VTE (EINSTEIN), and secondary prevention of major cardiovascular events in patients with acute coronary syndrome (ACS) (ATLAS ACS TIMI 51).

3.2.4. YM-150
Astellas (formerly Yamanouchi) has developed YM-150, an oral selective FXa inhibitor for DVT prevention. The compound has an immediate antithrombotic effect after oral administration, with a dose-dependent response and prolongation of PT; no significant food interactions have been noted. In a phase II dose-escalation study in patients undergoing elective primary hip replacement surgery, YM-150 (3, 10, 30, or 60 mg PO q.d.) given 6-10 hours after surgery for 7-10 days was compared to enoxaparin administered at a dose of 40 mg SQ q.d. 12 hours before surgery. There was no major bleeding, and the median incidence of VTE ranged from 52% in the 3-mg group to 19% in the 60-mg group. Overall, the drug appears to be safe and well tolerated. A dose escalation study of YM150 in the prevention of VTE in elective primary hip replacement surgery was also carried out. YM150, administered at doses of 10-60 mg daily starting 6-10 hours after primary hip replacement was shown to be safe, well tolerated and effective.

3.2.5. DU-176b
Daiichi Sankyo is developing DU-176b, an oral FXa inhibitor for the treatment of thrombotic disorders.
### Table 1. Oral anticoagulants in phase II/III trials.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Target</th>
<th>Dosing</th>
<th>Adjustment and Interactions</th>
<th>Indications</th>
</tr>
</thead>
</table>
| Rivaroxaban (Bayer Schering) | Factor Xa  | Once or Twice daily | · Avoid or monitor with CrCl <30 ml/min  
· Dose reduction for CrCl 30-49 ml/min  
· Avoid or monitor with strong CYP450 3A4 inhibitors | · Phase III for VTE prophylaxis after joint replacement completed (n=2531).  
· Phase II for secondary prevention after ACS enrolling (n=1350).  
· Phase II for treatment of DVT completed, phase III enrolling (n=2900).  
· Phase III for prevention of stroke in non valvular AF enrolling (n=14,000).  
· Phase III for treatment of PE enrolling (n=3,300). |
| Apixaban (Bristol-Myers Squibb and Pfizer) | Factor Xa  | Twice daily  | · Avoid or monitor with strong CYP450 3A4 inhibitors                                      | · Phase II trial for treatment of acute DVT completed.  
· Phase III trials for VTE prevention after joint replacement enrolling (n=7258).  
· Phase III trial for prevention of VTE in medical patients (n=6524).  
· Phase III trial for prevention of stroke in non valvular AF enrolling (n=15,000). |
| PRT054021 (Portola Pharmaceuticals) | Factor Xa  | Twice daily  | · Undefined yet                                                                        | · Phase II trial for VTE prophylaxis after joint replacement completed. |
| LY517717 (Eli Lilly)          | Factor Xa  | Once daily   | · Undefined yet                                                                        | · Phase II trial for VTE prophylaxis after joint replacement completed. |
| Dabigatran (Boehringer Ingelheim) | Factor IIa (thrombin) | Once or Twice daily | · Avoid or monitor with proton pump inhibitors                                           | · Phase III trial for VTE prevention after joint replacement completed.  
· Phase III trial for treatment of VTE enrolling (n=2554).  
· Phase III trial for prevention of stroke in non valvular AF enrolling (n=15,000). |

*Additional oral anticoagulants in preclinical or early clinical developments are not listed.

Preclinical data in mouse models revealed potent antithrombotic effects in AT-positive and AT-deficient mice. In rat models, DU-176b at doses of 0.05-1.25 mg/kg/hour prevented arterial and venous thrombosis.

In terms of clinical studies, DU-1766 in a single 60 mg dose was given to healthy males. The drug inhibited FXa activity, reduced thrombin generation, prolonged PT, aPTT and INR, and reduced venous and arterial thrombosis by 28% and 26%, respectively, in a Badimon chamber. Further studies are planned.

#### 3.2.6. LY-517717

LY-517717, an indol-6-yl-carbonyl derivative, is the lead in a series of oral selective FXa inhibitors being developed by Eli Lilly as part of a research collaboration with other partners. It is 1000-fold more selective as an FXa inhibitor than other serine proteases, with a Ki of 5 nM. The oral bioavailability of LY-517717 is approximately 25-82%, with a plasma half-life of 7-10 hours. In a rat atrioventricular shunt model, the median effective dose was 5-10 mg/kg PO, and absorption in dogs indicated no bleeding issues. In a phase I study, LY-517717 was found to be well tolerated and suitable for once daily administration. In a dose-escalating study, 511 patients undergoing hip or knee replacement surgery were randomized to receive one of six oral doses of LY-517717 (25, 50, 75, 100, 125, or 150 mg), or enoxaparin (40 mg SQ daily), started preoperatively, for 6-10 doses. The 100, 125, and 150 mg dose groups were non-inferior to enoxaparin in the incidence of symptomatic or venographically proven DVT or PE. The compound produced a dose-dependent prolongation of PT and was well tolerated, and there were no differences in bleeding risk as compared to enoxaparin.

#### 3.3. Selective, Direct Factor IXa Inhibitors

Although the development of direct FIXa inhibitors is at an earlier phase than direct FXa inhibitors, the theoretical advantages are similar. TTP 889, manufactured by Trans Tech Pharma, is an oral, direct Factor IXa inhibitor with a half-life of 20 hours, enabling once daily dosing. A phase II proof of principle study for
VTE prevention in hip fracture surgery, the FIXIT trial, recently completed enrollment of 206 patients who received standard in-hospital thromboprophylaxis. Efficacy and safety are being compared between patients randomized to receive TTP 889 versus placebo for up to 3 weeks post-discharge.53

3.4. Factor XIIa inhibitors
Development of FXIIa inhibitors is currently at the preclinical level. BMS-262084 is an irreversible and selective small molecule inhibitor of FXIIa with an IC50 of 2.8 nM against human factor XIIa. The effect of inhibiting activated blood coagulation FXIIa with BMS-262084 has been determined in rat models of thrombosis and hemostasis.54 BMS-262084 doubled aPTT in human and rat plasma at concentrations of 0.14 and 2.2 µM, respectively. Consistent with FXIIa inhibition, the PT was unaffected at concentrations up to 100 µM. BMS-262084 administered by intravenous loading with sustained infusion was effective against FeCl (2)-induced thrombosis in both the vena cava and carotid artery. In contrast, doses of up to 24 mg/kg+24 mg/kg/h had no effect on TF-induced venous thrombosis or ex vivo PT. Doses of up to 24 mg/kg+24 mg/kg/h did not significantly prolong bleeding time provoked by puncture of small mesenteric blood vessels, template incision of the renal cortex, or cuticle incision. These results demonstrate that pharmacologic inhibition of FXIIa achieves antithrombotic efficacy with minimal effects on provoked bleeding.54

4. Inhibitors of Thrombin Activity
Thrombin is the central serine protease in hemostasis. The mechanisms of action of thrombin involve coagulation, platelet activation, fibrinolysis, and vascular cell biology. In addition to its major role in fibrin formation and activation of FXIII, which cross-links fibrin, thrombin is essential for feedback activation of other coagulation factors such as FV, FVIII, and FIX.55 Thrombin is involved in platelet activation and subsequent aggregation,52 and can act as an anticoagulant by binding to thrombomodulin, which converts protein C to its active form, inactivating FVa and FVIIIa. Thrombomodulin-bound thrombin regulates coagulation through activation of thrombin-activatable fibrinolysis inhibitor (TAFI) and subsequent down-regulation of fibrinolysis. Predictions that thrombin inhibitors would be more effective than FXa inhibitors in arterial thromboembolic disease (where thrombin has a key role in platelet activation) and less effective in arterial thromboembolic disease (where thrombin has a key role in platelet activation) and less effective in arterial thromboembolic disease (where thrombin has a key role in platelet activation) and less effective in arterial thromboembolic disease (where thrombin has a key role in platelet activation) and less effective in arterial thromboembolic disease (where thrombin has a key role in platelet activation) and less effective in arterial thromboembolic disease (where thrombin has a key role in platelet activation) and less effective in arterial thromboembolic disease (where thrombin has a key role in platelet activation) and less effective in arterial thromboembolic disease (where thrombin has a key role in platelet activation) and less effective in arterial thromboembolic disease (where thrombin has a key role in platelet activation) and less effective in arterial thromboembolic disease (where thrombin has a key role in platelet activation). Given its central role in the coagulation cascade, inhibitors of thrombin activity—whether mediated by AT or acting directly on the active site—represent an important class of anticoagulant drugs in our armamentarium.

4.1. Indirect Thrombin Inhibitors
SNAC/Heparin
SNAC (sodium N-[8(2-hydroxybenzoyl)amino] caprylate), developed by Emisphere Technologies, enables macromolecule delivery of the large negatively charged and poorly absorbed heparin molecule through a non-covalent complex with heparin, allowing passive, transcellular absorption. SNAC itself has no pharmacological activity. Phase I studies revealed that in doses of up to 10.5 g SNAC/150,000 U heparin, the compound is well tolerated, with nausea being the only significant adverse event observed. Dose-dependent increases in aPTT and anti-FXa levels were also observed, suggesting that both AT-mediated thrombin and FXa inhibition play a role in the anticoagulant effects of the drug.56 In addition, there was an apparent food and diurnal effect, but no age effect.

The PROTECT study was a large phase III study in 2,264 hip-replacement patients with two SNAC treatment arms (low-dose SNAC and high-dose SNAC) for 30 days versus 10 days of enoxaparin at a dose of 30 mg SC every 12 hours.57 Mandatory venography on days 27-30 revealed an overall VTE rate of 31.8% in the low-dose SNAC group, 29.7% in the high-dose group, and 26.1% in the enoxaparin group. The rates of proximal DVT/PE were 18.6% in the low-dose group and 13.8% in the high-dose group, both of which were significantly higher than in the enoxaparin group. There was overall poor compliance in 22.1% of the patients on the low-dose regimen and in 31.4% of the patients on the high-dose regimen, suggesting that substance compliance was a key factor in failure to achieve proof of principle for the use of SNAC/heparin in VTE prevention as an indirect FIHa/Xa inhibitor. Improved formulations of heparin in solid dosage form are currently under clinical investigation.58,59 An orally administrable chemical conjugate of heparin and hydrophobic deoxycholic acid (DOCA), referred to as LHD, has also been developed. LHD was pre-formulated with dimethyl sulfoxide (DMSO) as solubilizer to further improve oral bioavailability (9.1% in monkeys). LHD was absorbed mainly in the jejunum and ileum of the small intestine, although it is in the ileum that absorption was most notable. Mechanistic studies of LHD absorption using Caco-2 cell monolayers, which mimic the intestine, demonstrated that the high permeability of LHD is

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mediated by passive diffusion through the transcellular route, and permeation is affected in part by bile acid transporters. These results demonstrated the feasibility of using chemically modified heparin for long-term oral administration as an effective therapy for VTE.\textsuperscript{58} A more recent clinical study determined the true PK for injectable versus oral heparin.\textsuperscript{59}

4.2. Direct Thrombin Inhibitors (DTIs) (Table 1)
The development of DTIs was driven by three major factors: increasing recognition of immune thrombocytopenia as a potentially severe complication of heparin use,\textsuperscript{60} the notion that heparin-AT inhibition of thrombin produces only weak inhibition of cell-surface- or clot-bound thrombin, which is active and can be released during fibrinolysis,\textsuperscript{61} and the non-specific binding properties of heparin, necessitating frequent monitoring. Hence, non-AT-based thrombin inhibitors with improved safety profiles over heparin, the ability to inhibit surface- or clot-bound thrombin, and predictable dose-responses would be advantageous in the clinical setting. Furthermore, oral formulations of these drugs would be a major advantage. DTIs could be ideal drugs for the treatment of HIT, as this condition is characterized by the generation of large amounts of thrombin. A theoretical concern about DTIs is that they could inhibit the anticoagulant properties of thrombin, namely inhibition of the thrombin-thrombomodulin-mediated negative feedback mechanism of the protein C system, with the possibility of rebound hyper-coagulability.\textsuperscript{62}

Four parenteral DTIs have emerged: lepirudin, bivalirudin, argatroban, and melagatran, the first three of which have been approved for clinical use. Lepirudin is a naturally occurring bivalent DTI indicated for thromboprophylaxis of HIT. Argatroban is a prototype non-covalent, reversible, small molecule DTI indicated for thromboprophylaxis or treatment of HIT. Melagatran is the active form of the oral, prodrug, small molecule DTI ximelagatran, discussed below. All of these drugs have limitations in terms of parenteral use, need for frequent monitoring, and high cost.

4.3. Selective Oral DTIs

4.3.1. Ximelagatran
Ximelagatran, developed by AstraZeneca, represents the first of a new class of orally active, small-molecule DTIs to reach late-stage development with limited clinical indications for VTE prevention. Ximelagatran is a hydrophilic prodrug that is converted by a cytochrome P450-independent liver enzyme system to its active form melagatran. Bioavailability of ximelagatran is approximately 20%, and the half-life is 4-5 hours in patients. It can be administered twice daily and does not require anticoagulant monitoring or dose adjustment. Ximelagatran was studied extensively in a large phase III trial for VTE prevention and treatment and was found to be either superior or equivalent to warfarin in terms of efficacy.\textsuperscript{63-68} However, initial long-term data with ximelagatran revealed elevated liver enzymes (approximately 6%). Based upon this and other considerations, it was not approved by the US FDA. It was, however, approved in other countries for short-term, post-orthopedic thromboprophylaxis. In February 2006, AstraZeneca withdrew ximelagatran from the world market due to continuing concerns about severe liver toxicity with long-term use.

4.3.2. Dabigatran
Dabigatran etexilate is another small molecule, orally active, prodrug DTI developed by Boehringer Ingelheim that has reached late-stage clinical development. It is being studied for various clinical indications and may replace warfarin as the preferred anticoagulant in many cases. It is orally administered and is currently marketed as Pradaxa since April 2008 in European countries and Pradax in Canada. It is rapidly absorbed and converted to the active form, dabigatran. It has linear characteristics in terms of concentration and global coagulation parameters, including thrombin clotting time, INR, and ecarin clotting time. Dabigatran has a Ki of 4.5±0.2 nmol/l, peak plasma concentration of two hours post-dose, and a half-life of approximately 14-17 hours after multiple dose administration.\textsuperscript{69} It is metabolized mainly (80-85%) by renal excretion.

The BISTRO II study was a multicenter, parallel group, double-blind, dose-finding study for VTE prevention in 1,949 patients undergoing total hip or knee replacement.\textsuperscript{70} Patients were randomized to receive dabigatran (50 mg, 150 mg, or 225 mg b.i.d., or 300 mg q.d.) starting 14 hours after surgery. The comparator was enoxaparin (40 mg q.d.) initiated 12 hours prior to surgery. A significant dose-dependent decrease in DVT was observed with increasing doses of dabigatran (\textit{P}>.001). Compared to enoxaparin, DVT was significantly lower in patients receiving dabigatran 150 mg b.i.d. (odds ratio [OR] 0.47, \textit{P}=.0007), 300 mg q.d. (OR 0.61, \textit{P}=.02), and 225 mg b.i.d. (OR 0.47, \textit{P}=.0007). Major bleeding was lower with the low dose of 50 mg b.i.d. (0.3% vs. 2.0%, \textit{P}=.047), but elevated at higher doses, with trends almost reaching statistical significance in those receiving the 300 mg dabigatran...
oral anticoagulants

A randomized, double-blind, non-inferiority trial was conducted comparing dabigatran etexilate to enoxaparin for prevention of VTE after total hip replacement.71 Patients (3494 total) undergoing total hip placement. Patients (3494 total) undergoing total hip replacement were randomized into treatment for 28-35 days with dabigatran etexilate 220 mg (n=1157) or 150 mg (n=1174) once daily, starting with a half-dose 1-4 hours after surgery, or subcutaneous enoxaparin (40 mg) once daily (n=1162), starting the evening before surgery. The primary efficacy outcome was the composite of total VTE (venographic or symptomatic) and death from all causes during treatment. On the basis of the absolute difference in rates of VTE with enoxaparin versus placebo, the non-inferiority margin for the difference in rates of thromboembolism was defined as 7.7%. Both doses of dabigatran were non-inferior as compared to enoxaparin. There was no significant difference in major bleeding rates with either dose of dabigatran etexilate as compared with enoxaparin.71 The frequency of increased liver enzyme concentrations and acute coronary events during the study did not differ significantly between the groups. The study concluded that oral dabigatran etexilate was as effective as enoxaparin as compared to the development of new agents designed to target specific pro-coagulant complexes in the coagulation pathway, inhibiting coagulation initiation, coagulation propagation, or thrombin activity. With respect to efficacy, during acute treatment of VTE, newer antithrombotic agents must exhibit at least non-inferiority in a methodologically sound study as compared to the existing parenteral agent of choice, LMWH, and the emerging agent fondaparinux. This is true particularly in high-risk venous thromboses, such as ileo-femoral VTE, PE, or VTE associated with cancer. For long-term VTE treatment, there is a need to improve upon existing oral anticoagulants, namely, VKAs. Target-selective oral agents must exhibit an improved safety profile (especially as it pertains to major or clinically significant bleeding), ease-of-use, and tolerability as compared to VKAs. If successful, emerging oral anticoagulants could negate the traditional distinction of acute versus long-term treatment of VTE, as they could potentially be used throughout the spectrum of disease, without the need for overlap with parenteral therapies.53 Lastly, any new long-term anticoagulant

less often with dabigatran 110 mg than warfarin; dabigatran 150 mg had similar bleeding to warfarin.72 A large (2539 patients), randomized, double-blind trial by the RE-COVER study group demonstrated non-inferiority of dabigatran when compared to warfarin in the treatment of acute VTE, with a similar rate of major bleeding and a lower rate of combined major plus non-major bleeding. Patients randomized to dabigatran had fewer minor bleeds but more dyspepsia and more drug discontinuation. Dabigatran-treated patients did not require coagulation testing.73

4.3.3. TGN-167

TGN-167 (TRI-50c-04) is an oral thrombin inhibitor being developed by Trigen Holdings for the potential treatment of thrombosis. A controlled-release formulation of the drug is also being developed with Eurand for long-term treatment of thrombosis. The compound produces a marked increase in thrombin clotting time, with minimal effects on aPTT. A double-blind, phase I, dose-escalation study with 20 volunteers showed the drug to be well tolerated, with no significant adverse events reported.74 At 600 mg, all dosed subjects achieved effective anticoagulant activity in vitro and phase II studies are being planned.

5. Expert Opinion and Conclusions

The antithrombotic management of VTE will undergo significant changes in the next 5-10 years. Limitations of existing parenteral and oral anticoagulants have led to the development of new agents designed to target specific pro-coagulant complexes in the coagulation pathway, inhibiting coagulation initiation, coagulation propagation, or thrombin activity. With respect to efficacy, during acute treatment of VTE, newer antithrombotic agents must exhibit at least non-inferiority in a methodologically sound study as compared to the existing parenteral agent of choice, LMWH, and the emerging agent fondaparinux. This is true particularly in high-risk venous thromboses, such as ileo-femoral VTE, PE, or VTE associated with cancer. For long-term VTE treatment, there is a need to improve upon existing oral anticoagulants, namely, VKAs. Target-selective oral agents must exhibit an improved safety profile (especially as it pertains to major or clinically significant bleeding), ease-of-use, and tolerability as compared to VKAs. If successful, emerging oral anticoagulants could negate the traditional distinction of acute versus long-term treatment of VTE, as they could potentially be used throughout the spectrum of disease, without the need for overlap with parenteral therapies.53
must be safely tolerated in combination with antiplatelet agents, as an increasingly aging population will be prone to arterial as well as venous thromboembolic disease. Cost considerations are also important, especially from a population perspective.

Newer agents should, in theory, fulfill the following requirements of an ideal anticoagulant: a rapid onset with predictable response characteristics, predictable pharmacokinetics, pharmacodynamics with low plasma protein binding, no required monitoring, a half-life that provides both safety and ease of use (particularly during temporary withdrawal), lack of food or drug interactions, an excellent safety profile (particularly with respect to immune-mediated thrombocytopenia, hepatotoxicity, and potential for thrombotic rebound phenomenon), and reversibility or availability of an antidote. In addition, oral agents with predictable intestinal absorption/bioavailability used in a simple, fixed-dose once or twice daily regimen and for which compliance can be monitored would be even more advantageous. At this time, drugs at the most advanced stage of development with respect to VTE management include the parenteral indirect FXa inhibitor idraparinux and biotinylated idraparinux, the oral DTI dabigatran, and the oral selective direct FXa inhibitors rivaroxaban and apixaban. Whether there are inherent advantages in blocking initial thrombin formation via the prothrombinase complex early in the coagulation system or blocking thrombin directly and preventing feedback amplification is still a matter of debate, as is the notion of whether there is any clinically meaningful effect of small molecule DTIs that target both clot-bound and free thrombin. Long-term clinical data with respect to efficacy of anti-Xa inhibitors will be available shortly, while long-term data is currently available on the efficacy of direct thrombin inhibition. The lessons from ximelagatran reveal the importance of long-term safety data in different patient populations. Ximelagatran had shown significant potential as a possible replacement to warfarin therapy, but was withdrawn because of potential liver toxicity. Its contrast, dabigatran appears to have a better safety profile and has recently entered a phase III randomized clinical trial in AF. Oral direct FXa inhibitors (rivaroxaban, apixaban, and others) may prove to be more potent and safe. Selective inhibitors of specific coagulation factors involved in the initiation and propagation of the coagulation cascade (FIXa, FVIIa, circulating TF) are at an early stage of development. Additional new agents in clinical development include nNAPc2, protein C derivatives, and soluble thrombomodulin.
References


C hronic myeloid leukemia (CML) is a rare disease. Although its incidence is low, its prevalence is increasing. CML is the first malignant disease for which a direct genetic link has been found and, as a result, there has been much recent progress in understanding the biology of the disease.1-4 The cytogenetic hallmark of CML is the Philadelphia chromosome (Ph), created by a reciprocal translocation between chromosomes 9 and 22 [t(9;22)(q34;q11)].5 The conjugation of the breakpoint cluster region gene on chromosome 22 and the Abelson kinase gene on chromosome 9 creates the chimeric BCR-ABL oncogene, which codes for the deregulated tyrosine kinase, bcr-abl.6 Bcr-abl displays transforming activity owing to its constitutive kinase activity, which results in multiple signal transduction pathways, including the Ras/Raf/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase, STAT5/Janus kinase, and Myc, leading to uncontrolled cell proliferation and reduced apoptosis, and resulting in the malignant expansion of pluripotent stem cells in bone marrow.7,8 The bcr-abl kinase itself enhances genomic instability leading to accumulation of secondary genetic errors, which may be responsible for resistance to drugs, and transformation of the typically indolent chronic phase (CP), to advanced phases (accelerated phase [AP] and blast phase [BP]).6 One of the major differences between the CP and BC phases of CML is their differential responsiveness to anti-leukemia treatment. Whereas most CP patients respond well to the current front-line treatment and responses are usually durable, the majority of BC patients respond, but then relapse fairly rapidly. Historically, CML was treated with busulfan or hydroxyurea, and was associated with a poor prognosis.9 These agents controlled the hematologic manifestations of the disease, but did not delay disease progression. Treatment with interferon-alfa (IFN-alfa) produced complete cytogenetic responses (CCyR) in 5% to 25% of patients with CML in CP, and improved survival compared with previous treatments.10 With IFN-alfa, a survival benefit was seen compared to what was achieved with chemotherapy. This survival
benefit was dependent on the achievement of cytogenetic responses: patients achieving a CCyR have a 10-year survival rate of 78%, falling to 39% in patients with a partial cytogenetic response (PCyR) and 25% in those without any cytogenetic response.19 Combining IFN-alfa with cytarabine produced additional benefits.11 Allogeneic stem cell transplantation (SCT) may be curative in CML. However, compatible donors are not available for the majority of patients, and even when a fully matched donor is available, transplant-related morbidity and mortality can limit this approach.

The tyrosine kinase inhibitor imatinib mesylate (Gleevec or Glivec, Novartis Pharmaceuticals, East Hanover, NJ) was developed to specifically inhibit the bcr-abl fusion protein12 and it has become the golden standard frontline therapy for all CML patients in early CP, on the basis of the impressive response rates and good tolerability shown in numerous clinical trials. This conclusion is mainly based on the results of the International Randomized Study of Interferon and STI571 (IRIS) trial. In fact, in the IRIS trial arm with patients receiving imatinib 400 daily, the best observed rates for major CyR and CCyR at 7 years were 89% and 82%, respectively.13 The estimated event-free Survival (EFS) at 8 years was 81% and freedom from progression to AP/BC was 92%; estimated overall survival (OS) was 85%. It was 93% when only CML-related deaths and those prior to SCT were considered. The annual rates of progression to AP/BC in years 4 to 8 after initiation of therapy were 0.9%, 0.5%, 0%, 0%, and 0.4%, respectively.14 Only 15 (3%) patients who achieved CCyR progressed to AP/BC, all but one within 2 years of achieving CCyR.

**Definition of optimal and suboptimal response**

The European Leukemia Net (ELN) has published internationally accepted clinical practice recommendations, including monitoring practice, and formal definitions of optimal responses, warning signs, suboptimal responses, and treatment failure, using hematologic, cytogenetic, and molecular criteria.15 Commonly used time-based landmarks of optimal and suboptimal responses, and treatment failure, are shown in Table 1. The evaluation of the response is based on blood counts and differential (hematologic response, HR), on the examination of marrow cell metaphases (cytogenetic response) and on a quantitative assessment of BCR-ABL transcripts level (molecular response). An optimal response means that there is no indication that a change of therapy may improve a survival that is currently projected as close to 100% after 6 to 7 years.16 An optimal response to imatinib is defined by complete HR and at least minimal CyR (Ph+< 95%) at 3 months, at least partial PCyR (Ph+<35%) at 6 months, CCyR at 12 months and major molecular response (MMR=BCR-ABL/ABL<0.1%) at 18 months.16 The value of the ELN recommendations in predicting the prognosis of patients in early CP CML has been demonstrated. In fact, after 8 years, achieving landmark cytogenetic responses (minor CyR at 3 months, partial CyR (PCyR) at 6 and 12 months, CCyR at 18 months) were prognostic indicators of stable CCyR and lack of events, whereas suboptimal responders had a poorer prognosis.14 Patients with minor to PCyR at 3 months and those with PCyR at 6 and 12 months were more likely to achieve a stable CCyR than have an event. Among patients with less than CCyR at 18 months, the probability of an event was comparable to the probability of achieving stable CCyR. The IRIS study also confirmed that the achievement of CCyR and MMR at 12 months predicted for a low risk of events or progression in the long term and no patients with MMR at 12 months progressed to AP/BP, emphasizing the value of achieving a molecular response early in the treatment course. Thus failure is defined by incomplete HR at 3 months, no CyR (Ph+> 95%) at 6 months, less than PCyR (Ph+> 35%) at 12 months, less than CCyR at 18 months and loss of a complete HR or a complete CyR; treatment failure indicates that treatment at the current schedule is no longer appropriate for a patient and a change in therapy is indicated.15 In any other situation, the response is defined as suboptimal:16 suboptimal responses indicate that although patients may continue to receive a benefit from imatinib treatment with the present schedule, the chances of an optimal outcome are reduced, so that the patient may be eligible for alternative approaches.16 Suboptimal responders defined at 6 and 12 months had a significantly poorer PFS and lower probability of CCyR. Suboptimal responders by the 12-month criteria also had significantly worse survival. The 18-month criteria failed to identify patients with worse OS or PFS.17 Following initial therapy with imatinib, patients should be routinely assessed for response, and therapy should be adjusted to maximize the chance of response for patients with lack of response or suboptimal response. The US National Comprehensive Cancer Network (NCCN) offers another widely recognized set of recommendations, but does not include time-based molecular response landmark.

**Molecular Response Monitoring**

Vigilant monitoring aids prompt detection of imatinib failure, and ensures that patients receive the most suit-
Table 1. Time-based landmarks of optimal and suboptimal responses, and treatment failure.\textsuperscript{16}

<table>
<thead>
<tr>
<th>Time</th>
<th>Optimal Response</th>
<th>Suboptimal Response</th>
<th>Failure</th>
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<tbody>
<tr>
<td>3 months</td>
<td>CHR, and at least minor CyR</td>
<td>No CyR</td>
<td>Less than CHR</td>
</tr>
<tr>
<td></td>
<td>At least PCyR</td>
<td>Less than PCyR</td>
<td>No CyR</td>
</tr>
<tr>
<td>6 months</td>
<td>CCyR</td>
<td>PCyR</td>
<td>Less than PCyR</td>
</tr>
<tr>
<td></td>
<td>MMR</td>
<td>Less than MMR</td>
<td>Less than CCyR</td>
</tr>
<tr>
<td>12 months</td>
<td></td>
<td>Loss of MMR</td>
<td>Loss of CHR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mutations still sensitive to imatinib</td>
<td>Additional chromosomal abnormalities on the Ph+clone</td>
</tr>
<tr>
<td>18 months</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any Time</td>
<td>Stable or improving MMR</td>
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Eligible therapy as early in the disease course as possible. ELN guidelines recommend measuring BCR-ABL transcript levels using RT-PCR every three months following treatment start.\textsuperscript{16} There is clinical evidence to support the use of the MMR as a treatment goal. In particular, BCR-ABL transcript levels at the time of CCyR have been shown to predict the duration of cytogenetic remission. In one study, patients with stable CCyR had significantly lower BCR-ABL transcript levels at the time of achieving CCyR compared with those suffering cytogenetic relapse.\textsuperscript{18} Furthermore, achieving a MMR at 12 or 18 months has also been shown to be associated with longer cytogenetic remission than in patients who do not achieve this goal.\textsuperscript{17} Achieving a MMR may also affect survival outcomes. In the IRIS trial, no patient who had both a CCyR and MMR at 12 months was shown to progress to advanced disease over five years.\textsuperscript{19} The MMR rates increased over time, and in patients who achieved an MMR at any time point, progression was rare. Achievement of a CCyR correlated well with a molecular 1% from 6 months onwards. At 12 months, patients with a molecular >1% or without CCyR, fared more poorly than those with a molecular of 1% or those in CCyR. At 18 months patients with a molecular 1% had excellent long term outcomes, with the best outcomes seen in those with a molecular 0.1%. The comparison at 18 months was the only one that was statistically significant.\textsuperscript{20} However, in another study, the achievement of MMR at 12 or 18 months had no significant effect on 5-year PFS or OS in patients with early CP CML.\textsuperscript{21} Failure to achieve a MMR within 18 months from start of treatment, or loss of MMR at any time, is regarded as a suboptimal response (Table 1).\textsuperscript{16} There is current controversy regarding the significance of a complete molecular response (CMR). Firstly, the rate of such responses, defined by the absence of detectable BCR-ABL transcripts, is inevitably dependent on the sensitivity of the molecular assay used. Furthermore, quiescent stem cells may lodge in marrow niches and be relatively occult, and in contrast to the bulk of the CD34+ cells, leukemia stem cells may be transcriptionally silent and require a genomic PCR for detection. On the other hand, some transplanted patients never relapsed as well as about 50% of patients who discontinued TKIs.\textsuperscript{21,22} Studies are ongoing to assess the depth of molecular response for patients treated with more potent inhibitors. Continued molecular monitoring throughout treatment is recommended, even if a sustained CCyR has been achieved. It is now becoming apparent that the dynamics of BCR-ABL transcript levels vary among patients with CCyR, and that this may affect response. In fact, transcript levels in patients who have achieved CCyR may progressively decline, plateau, or rise.\textsuperscript{23} A significant increase in transcript levels warrants closer monitoring. The prognostic value of loss of MMR is not clear, but is considered a suboptimal response and more frequent monitoring is needed, including cytogenetic analysis and mutational analysis.\textsuperscript{16,17} Rising levels of BCR-ABL transcripts (not outright loss of MMR) have been described as indicating a loss of response, with more rapid doubling times associated with relapse into accelerated or blast crisis.\textsuperscript{24} A later study also demonstrated that a half-log increase in BCR-ABL transcripts is significantly predictive of shortened cytogenetic remission.\textsuperscript{25} Monitoring BCR-ABL transcripts may therefore aid early identification of loss of CCyR in such patients. However, such rises are not always associated with treatment failure. In another study, increasing levels of subscripts predicted a loss of cytogenetic response only in patients who did not achieve a MMR.\textsuperscript{26} In the case of suboptimal
response, there are no published data to suggest that switching treatment in response to rising BCR-ABL levels in CCyR patients improves long-term outcome. Enrollment in a clinical trial examining early detection and treatment is recommended. Few studies have examined the effect of dose escalation in suboptimal molecular responders, showing the achievement of MMR by 5% to 38% of patients with high-dose imatinib, but the small size of the samples precludes firm conclusions being drawn. Furthermore, dose escalation to ≥600 mg/day is associated with lower patient compliance, most likely due to associated toxicity. Second-generation TKIs may provide a better benefit-risk assessment than imatinib in cases of suboptimal response but data are still not available. The association between rising BCR-ABL transcript levels and the emergence of imatinib-resistant mutations underlines the requirement for molecular monitoring. Mutation analyses are recommended by the ELN following treatment failure, a suboptimal response, or if BCR-ABL transcript levels are observed to rise. An increasing BCR-ABL value should also prompt an inquiry into compliance.

Resistance and progression
It is well recognized that with continued imatinib treatment a significant number of patients show resistance to imatinib. The incidence of primary (i.e., patients who never respond to imatinib) and secondary (i.e., patients who become resistant after an initial response) resistance increases with more advanced phases of CML. In a 4.5-year follow up of patients with CP, AP or myeloid BP-CML (total n=300), primary resistance occurred in 3%, 9% and 51% of patients, respectively. Secondary resistance (i.e., a hematological recurrence) was noted in 22, 32 and 41% of patients, respectively. Patients classified as failure according to the ELN recommendations had a significantly lower OS, PFS, and probability of achieving CCyR than the patients classified as responders. For example, based on the assessment at 12 months, the 5-year survival was 87.1% versus 95.1% (P=.02), PFS 76.9% versus 90% (P=.002), and CCyR rate 26.7% versus 94.1% (P<.001). Patients classified as failure at any time point who did eventually achieve CCyR (after 18 months) had a significantly higher probability of losing their CCyR. For example, at 12 months, the cumulative incidence of loss of CCyR for these patients was significantly higher than that of responders (51% vs 10.3%, P=.001). Various mechanisms, bcr-abl dependent or independent, may contribute to imatinib resistance, including increased expression of bcr-abl kinase through gene amplification, decreased intracellular imatinib concentrations caused by drug efflux proteins, imatinib binding by plasma proteins, and clonal evolution. Activation of members of the Src-kinase family or of other pathways downstream of bcr-abl (like the PI3K-AKT pathway) has been reported and it is increasingly becoming evident that multiple mechanisms and events can be involved in the development of imatinib resistant subclones. All types of resistance can be ascribed to the high degree of genomic instability characterizing the Ph-positive clone. So far the molecular mechanisms leading to this instability are only in part understood and, although it is proved that bcr-abl activation is able to induce some degree of genomic instability, at least in some cases, a stem cell disease causing genomic instability and pre-existing to the acquisition of the Ph-chromosome cannot be excluded. Most of the mechanisms of resistance, however, involve bcr-abl kinase domain mutations, with resulting impairment of the ability of imatinib to bind the ATP binding pocket of the bcr-abl tyrosine kinase domain. Up to now, more than 100 different mutants of bcr-abl have been described, but some of them show a higher frequency than others. Most of the clinically relevant mutations develop at just a few residues in the in the P-loop (G250E, Y253F/H, and E255K/V), contact site (T315I), and catalytic domain (M351T and F359Y). Another example of imatinib failure is intolerance, which is the discontinuation of treatment necessitated by drug toxicity. Intolerance to imatinib is a significant clinical issue, with 29% of patients requiring dose interruption, and 26% of these patients discontinuing therapy. Intolerance is more frequently observed in patients in advanced stages of CML who have experienced a long duration of therapy.

Imatinib dose escalation is typically the first option for CML-CP patients with cytogenetic resistance or relapse to 400 mg/day imatinib. Support for imatinib dose escalation is provided in a retrospective analysis of imatinib patients dose escalated in the IRIS trial. A dose increase to 600-800 mg/day was reported for 106 of 551 (19%) of patients enrolled in the study. Clinical responses were achieved within 12 months for 21 of 48 (44%) patients dose escalated per ELN recommendations with 6 of 11 (55%) achieving a major cytogenetic response (MCyR) within 12 months of dose escalation and with 2 of 10 (20%) patients with failure at 18 months attaining CCyR 12 months after dose increase. For the entire cohort of 106 patients who had imatinib dose escalation, the estimated PFS
was 89% and the OS was 84% at 36 months after escalation of imatinib dose. In another study, 84 patients with CML-CP were dose escalated to imatinib 600-800 mg/day following hematologic (n=21), or cytogenetic failure (resistance: n=30; relapse: n=33) to imatinib standard dose.38 Among patients that met the criteria for cytogenetic failure, 75% responded to imatinib dose escalation. For patients dose escalated due to loss of hematologic response, 48% achieved a CHR and 14% attained a CyR after imatinib dose increase.

Strategies to cope with resistance have led to the development of second-generation bcr-abl inhibitors. The largest experience with these second-generation TKIs has been gained with dasatinib and nilotinib.39-41 Nilotinib and dasatinib have a higher affinity for bcr-abl, as compared with imatinib, and inhibit the majority of clinically relevant imatinib-resistant mutant subclones; they have similar potencies in inhibiting bcr-abl, but significantly differ in their target specificity. Dasatinib inhibits a large number of tyrosine kinases, including the Src kinases and several receptor tyrosine kinases, whereas nilotinib, like imatinib, inhibits mainly Abl, c-KIT and PDGFR. Both these two agents have been used mostly in the context of patients who are either resistant to or intolerant of imatinib, and have included patients in all stages of the disease, as well as patients with Ph-positive acute lymphoblastic leukemia. Both agents have resulted in hematologic and cytogenetic responses in a large proportion of patients, particularly those treated still in the CP.42 Dasatinib was approved by EMEA and FDA for the second-line treatment of all phases of disease on the basis of its efficacy and safety profiles shown in a series of phase II trials in patients who failed or were intolerant to first-line imatinib therapy.43-45 Recent 24-month follow-up data demonstrated a 2-year MCyR rate of 62% and a CCyR rate of 53%.46 The PFS at 2 years was 80% and the OS rate was 94%. Responses to dasatinib in patients with imatinib-resistant CP CML enrolled in phase II studies of dasatinib have been assessed by baseline mutational status.28,47 CCyR rates were similar among patients with unmutated bcr-abl and those harboring mutations, with the exception of the T315I and the F317L mutations. Importantly, responses to dasatinib did not appear to be diminished among patients harboring the P-loop mutations. Dasatinib was approved following an open-label phase II study in patients with CML who failed or who were intolerant to imatinib therapy.48 Follow-up data confirm the effectiveness of this compound particularly in CP and AP CML (Table 2).50-52 No CCyR were observed in patients harboring T315I, L248V, Y253H, or E255K/V and F359C/V mutations most frequently associated with progression.53,54 Unfortunately, neither nilotinib or dasatinib are affective against the T315I mutation. A number of new TKIs are in development, including bosutinib (SKI-606, Wyeth Pharmaceuticals, NJ, USA), a dual BCR-ABL and SFK inhibitor currently in phase II and III trials. Bosutinib is 30-fold more potent than imatinib against BCR-ABL and inhibits Src kinases, but not c-KIT and PDGFR.56 In a phase II trial, CCyR rates after a median 8 months of treatment in patients who had received imatinib (but not dasatinib or nilotinib) were 29% in imatinib-resistant patients and 50% in imatinib-intolerant patients.57 INNO-406 is also a dual BCR-ABL/SFK inhibitor, with activity against PDGFR and c-Kit.58 Moreover AP24534 is an orally available multiple TKI that potently inhibits the enzymatic activity of BCR-ABL-T315I, the native enzyme and all other tested variants. Not all effective therapies for CML are kinase inhibitors. Omacetaxine is a first-in-class cetaxine with clinical activity against Ph+ CML and a mechanism of action independent of tyrosine kinase inhibition.59

Bone marrow transplant

According to the ELN recommendations, an allogeneic stem cell transplant (allo-SCT) is suggested for

<table>
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<tr>
<th>Table 2. Phase II trials in patients who failed or were intolerant to first-line imatinib therapy.43-45,50-52</th>
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<tr>
<td><strong>Cytogenetic Response (%)</strong></td>
</tr>
<tr>
<td><strong>Major</strong></td>
</tr>
<tr>
<td>CML Chronic</td>
</tr>
<tr>
<td>CML Accelerated</td>
</tr>
<tr>
<td>CML Blastic</td>
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<tr>
<td></td>
</tr>
<tr>
<td>D=dasatinib; N=nilotinib; Ly=Lymphoblastic; My=Myeloblastic</td>
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patients in AP or BP or with the T315I mutation and for patients who experience failure on second-line TKIs.16 Allo-SCT is also a significant option in the patients who have a suboptimal response to dasatinib or nilotinib second line (Table 3). Treatment recommendations are different for patients who are referred in AP or BP. These patients should preferentially receive an allo-SCT, if eligible, after a pretreatment with imatinib 600 or 800 mg if TKI naive, or with second-generation TKIs if imatinib resistant. In fact, prior imatinib therapy is not exclusive to the efficacy of ASCT, and indeed the two approaches may be favorably combined.60-62 The German CML Study Group (Study IV) considered allo-SCT in three instances: early allo-SCT (in case of low EBMT score, high risk patients, or patient request); allo-SCT after imatinib failure or intolerance in first CP, and allo-SCT in second CP or accelerated phase or blast crisis. Overall survival rate at two years for the first group was 87.8%, for the second group was 93.8%, and for the last group 49.5%. By EBMT score, survival rates were 100% for risk score 0-2, 82.2% for risk score 3-4, and 43.5% for risk score 5.62

### First-line therapy optimization

According to the IRIS and other studies,14,19,63 a notable proportion of patients either do not respond adequately or do not achieve a durable response to imatinib. At 8 years, 45% of imatinib-treated patients had discontinued the IRIS study, including 14% for resistance and 5% for intolerance.14 To improve responses to imatinib, imatinib 400 mg was compared with imatinib 800 mg in the TOPS study, but the higher 12-month MMR and faster response with imatinib 800 mg did not confer a long-term survival.64 Randomized phase III trials are ongoing to compare imatinib monotherapy with combination treatment.65,66 In the French SPIRIT trial, patients were randomized 1:1:1:1 to receive imatinib 400 mg/day, imatinib 600 mg/day, imatinib 400 mg/day plus cytarabine, or imatinib 400 mg/day plus pegylated IFN. Twelve-month CCyR rates were 55%, 62%, 63%, 65%, respectively (differences not statistically significant);67,68 were observed, whereas at 18 months MMR was 41%, 52%, 53%, and 62% (P=.0001), and CMR was 4%, 7%, 5% and 15%, respectively (P=.0013), with a potential advantage for the imatinib plus IFN arm. However, further follow-up is needed to establish whether these early differences will confer a long-term survival advantage. Overall, 45% of the patients discontinued IFN during the first 12 months, with higher rates of MMR, optimal molecular response and undetectable minimal residual disease in patients who were treated with IFN for more than 12 months. A similar study in more than 1300 patients is being performed by the German CML Study Group (Study IV), with patients randomized to imatinib 400 mg/day or 800 mg/day alone, or imatinib 400 mg/d in combination with IFN.69 In the imatinib 400 mg, imatinib 800 mg, and imatinib 400 mg plus IFN arms, 12-month CCyR rates were 52%, 65%, and 51%, respectively, and 12-month MMR rates were 30%, 54% and 35%, respectively. After 5 years of follow-up, no difference was reported between arms in PFS or OS, as seen in the TOPS study.69 Following the success of nilotinib and dasatinib in the second-line setting, both drugs have been assessed in phase 2 studies with newly diagnosed CML patients. Sixty-two newly-diagnosed CP CML patients were treated with dasatinib 50 mg twice daily (BID) or 100 mg once daily (QD) at the MD Anderson Cancer Center, with a median follow-up of 24 months. Most patients achieved a rapid CCyR (94% at 6 months), with a cumulative CCyR ratio of 98%. After 12 and 18 months, a MMR was achieved by 71% and 79% of patients.70 Two phase II studies demonstrated substantial activity of nilotinib 400 mg BID in newly diagnosed CP CML patients. Among 61 patients treated at the MD Anderson Cancer Center with nilotinib with a median follow-up 17 months (range 1-43) MCyR was achieved by 96% of patients at 6 months, with a cumulative CCyR of 98%, and after 12 and 18 months, 81% and 79% had achieved a MMR.71 Seventy-three newly-diagnosed CP-CML patients were treated with nilotinib in Italy, with a CCyR of 78% at 3 months, 96% at 6 and 12 months and MMR rates were 52%, 66%, and 85%, respectively.72,73 The toxicity profile with dasatinib and nilotinib front line was favourable. In order to perform a full comparison of imatinib and next-generation TKIs, randomized phase III trials have been initiated. In CA180-056 (DASISION), an international trial, patients are randomized to receive either dasatinib 100 mg QD or imatinib 400 mg QD. CAMN107A2303 (ENESTnd) is an international phase III trial with 3 arms: 846 CP CML patients were randomized to receive nilotinib

### Table 3. Suboptimal response to dasatinib or nilotinib second line.16

<table>
<thead>
<tr>
<th>Suboptimal response</th>
<th>3 months</th>
<th>6 months</th>
<th>12 months</th>
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<tbody>
<tr>
<td>minor CyR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCyR</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>less than MMR</td>
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CML
CML

300 mg BID vs nilotinib 400 mg BID vs imatinib 400 mg/day.74 Preliminary data from ENEStnd have shown that nilotinib (300 mg or 400 mg BID) was superior to imatinib, with a significantly higher 12-month CCR (80% [P<.0001] vs 78% [P=.0005] vs 65%), MMR (44% [P<.0001] vs 43% [P<.0001] vs 22%) and, most importantly, a significantly lower rate of progressions (0.7% vs 0.4% vs 3.9%). Bosutinib has also been proposed as first line in a phase III trial of bosutinib vs imatinib.

Conclusions

CML is an important model for understanding oncologic diseases. It is the first malignant disease for which a direct genetic link has been found and there has been much recent progress in understanding the pathophysiology and finding a cure. Targeted agents have significantly improved the prognosis in CML. Imatinib represented a significant turning-point in the natural history of the disease and in patient survival. However, a considerable proportion of patients (up to 45%) discontinue imatinib due to resistance or intolerance. The achievement of CCR at 12 months predicted for a low risk of events or progression in the long term and the achievement a MMR represents a ‘safe haven’ for CML patients. In fact, no patients with MMR at 12 months progressed to AP/BC. Targeted therapies with TKIs in CML need molecular evaluations throughout the whole time of therapy to maximize the chance of response for patients with lack of response or suboptimal response. The recent availability of highly potent TKIs has further improved the outcome of many patients. The advent of novel agents, with the possibility of combining drugs with

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Profile for the GIMEMA CML Working Party. ASH Annual Meeting Abstracts. 2009;114:2205-.

Significance of sCD117 as a Tumor Marker in Acute Myeloid Leukemia

Huda AlSayed,* Faisal Rawas,** Amal AlSuraihi,*** Fahad AlShareef,**** Lila Bashawri,* Tarek Owaidah**

A
cute leukemias account for approximately 10% of all human cancers.1 Acute leukemia is classified into two categories based on the lineage of the blast cells: acute lymphoblastic leukemia (ALL) and acute myeloblastic leukemia (AML).

The diagnosis of AML is enhanced by identification of characteristic cluster of designation (CD) antigens expressed on the surface and/or in the cytoplasm of acute leukemia cells that are committed to the myeloid lineage regardless of the subtype. The proteolytic cleavage of this cell surface receptor results in soluble CD117.

MATERIALS AND METHODS: We have investigated the diagnostic and prognostic role of sCD117 in Saudi patients with AML. Detailed immunophenotyping of the bone marrow blast cells of 20 patients was performed by flowcytometry, and the serum c-kit level was measured using the ELISA assay.

RESULTS: CD117 surface expression showed no significant correlation with sCD117, but there was a significant elevation of sCD117 pre-chemotherapy and a significant decrease post-chemotherapy.

CONCLUSION: sCD117 was had good diagnostic value for early AML cases and a prognostic value post-chemotherapy. This suggests the usefulness of sCD117 as a tumor marker in AML patients.

KEYWORDS: Immunophenotyping, sCD117, AML, tumor marker.
sCD117 in AML

AML patients who were positive for CD117 on immunophenotyping. Peripheral blood (PB) or bone marrow (BM) samples from each patient were collected in sodium heparin or EDTA tubes. CBC was performed on Cell Dyne 4000 (Abbott). A sample of whole blood was collected from each patient in a serum separator tube (SST), which was allowed to clot for 30 minutes before centrifugation for 15 minutes at 1000g. Serum was then separated and kept in a deep freezer (-80°C) for subsequently measuring the sCD117 by ELISA. A second sample was collected from all patients at day 28 post-induction chemotherapy and at 3 months or more during follow up.

A total of 50 normal Saudi donors of different age groups and sex were used as controls for the ELISA method.

Immunophenotyping

The diagnosis of acute leukemia was confirmed using detailed immunophenotyping including 32 monoclonal antibodies (obtained from Becton Dickinson, San Jose, CA, USA) performed on each bone marrow sample at KFSHRC, Riyadh using FACSCalibur instrument (Becton Dickinson, San Jose, CA, USA). The monoclonal antibodies included: CD2, CD3, CD4, CD7, CD8, CD9, CD10, CD19, CD20, CD22, CD25, CD38, CD79a, kappa and lambda light chains, terminal deoxynucleotidyl transferase enzyme (TdT); (T cell and B cell lymphoid markers), CD13, CD33, CD117 and myeloperoxidase enzyme (MPO); (myeloid cell markers), CD11b, CD11c, CD14, CD15, CD64, CD65 (monocytic markers), others include CD34, HLA-DR, CD58, CD66 and NG2, CD45 been used for gating. Three- and four-color staining methods were used through the following combinations of fluorochrome-conjugated monoclonal antibodies: fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridin chlorophyll protein (PerCP) and allophycocyanin (APC) directed against surface antigens.

An aliquot from the BM aspirate was washed three times using phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA) and 0.1% sodium azide (NaN3). After the last wash the aspirate was filtered and the count adjusted to 0.5-1×10⁹/L of which 50 µL was placed in each tube and incubated with the appropriate combination of monoclonal antibodies for 15-20 minutes in the dark at room temperature. After the incubation period, the red cells were lysed using lysing kit (Intra-Prep Permeabilization Reagent Kit from Beckman Coulter Company) according to the recommendations of the manufacturer.

Data Acquisition and Analysis of Flowcytometry

Flowcytometry was performed on a FACS Calibur (Becton Dickinson) equipped with a 15,10±5% mW Argon laser. Non-specific antibody binding was monitored using an FITC and/or PE conjugated mouse IgG1 isotype control (obtained from Becton Dickinson). The forward scatter (FSC) and the side scatter (SSC) parameters were adjusted so that cells with high FSC and high SSC could be observed. The cell acquisition rate was adjusted to approximately 500-1000 events/second. Small particles up to 7 µm were excluded by FSC gate. A fluorescence threshold was set at 100 so that the background fluorescence was not greater than 0.5%. Data acquisition and analysis were performed using Cell Quest Pro software (Becton Dickinson) following daily calibration of the instrument using Calibrite Beads (Becton Dickinson) and a two-level control for CD3/CD8/CD45/CD4 and CD3/CD16+56/CD45/CD19 to ensure the consistency in the instrument function. Data analysis was performed by Cell Quest Pro software using CD45 versus side scatter as a gating strategy to gate on the blast cells. Gating is critical to isolate the abnormal cells because the leukemic phenotype should be determined on as pure a population as possible. The frequency of positive cells was determined after channel-by-channel subtraction of the background (isotype control). The criteria for scoring the positivity of blast cells for a given marker were defined as follows: positive if more than 20% of the cells had expression of the marker, and negative if less than 20%.

Measurement of sCD117 by ELISA

The frozen serum of the 20 patients as well as healthy control samples were assayed for sCD117 by quantitative ELISA kit (Solid Phase Sandwich Enzyme Linked Immunosorbent Assay) using Quantikine Human SCF sR/c-kit Immunoassay (obtained from R&D Systems, Inc. Company, Minneapolis, USA). In this assay a monoclonal antibody specific for CD117 has been pre-coated onto a microplate. Standards, controls, and serum samples, 100 µL each, were pipetted into the wells and coated by the immobilized antibody during the 2-hour incubation period at room temperature for
binding sCD117, if any. After washing off any unbound substances, an enzyme-linked monoclonal antibody specific for sCD117 was added and incubated for another 2 hours at room temperature. Following a wash to remove any unbound antibody–enzyme reagent, a substrate solution was added and color developed within 30 minutes incubation in proportion to the amount of sCD117 bound. The color development was stopped by stopping solution and the intensity of the color was measured; the optical density of each well was determined within 30 minutes using a microplate reader set to 450 nm using ETI Max 3000 ELISA reader (from DiaSorin company, Italy).

According to the manufacturer, the sensitivity of this method was evaluated and the minimum detectable level of sCD117 ranged from 0.057 ng/mL. As to the specificity of this assay method, it showed no significant cross-reactivity or interference. The mean was used as a cut-off value as there was no other method to compare the sensitivity and accuracy with the ELISA method used in the study.

**Statistical Analysis**

Collected data was revised and entered into the statistical software (SPSS version 10). Data are described by percentage, mean, range and by graphs. Data was analyzed using student t-test, paired t-test, one-way ANOVA and simple correlation.

**Results**

Table 1, depicts the characteristics of the 20 patients studied: 16 AML cases and 4 biphenotypic acute leukemia (BAL) cases. There were 13 adults (>14 years) with ages ranging from 18 to 53 years and 7 children with ages ranging from 2 to 10 years with an overall median age of 21 years; 9 of the patients were females and 11 were males. The most frequent clinical presentation was lymphadenopathy in 9 (45%) patients followed by hepatomegaly in 5 (25%) patients, while 6 (30%) of the patients presented with bleeding symptoms. More than 50% of the cases had fever at presentation. The WBC count at presentation ranged from 0.84 to 96.8×10⁹/L with a mean of 23×10⁹/L. The blast count at presentation ranged from 0.84 to 96.8×10⁹/L with a mean of 69%. The most common FAB classification of AML was M2 in 5 (25%), while the other cases were as follows: M3 (n=3); M1 (n=3); M4 (n=2), M5 (n=1), M0 (n=1). The BAL cases were B/myeloid in 2 patients and T/myeloid in the other 2 patients. Four cases (20%) had a normal karyotype, 4 (20%) cases had various chromosomal deletions and 4 (20%) had a complex karyotype. All patients, except 2 (10%), had achieved complete remission by day 28 post-chemotherapy.

**Surface markers expression**

As shown in Table 2, all cases were positive for CD117 surface antigen. The myeloid antigens CD33, CD13, CD15 and MPO were expressed in 19 (95%), 17 (85%), 9 (45%) and 11 (55%) respectively. CD34 was expressed in 14/20 (70%) of cases. B-cell markers, CD19 was positive in 8/20 cases (40%), CD22 and CD79a were positive in 2 cases (10%). T-cell markers, CD2 and CD3 were positive in 2/20 cases (10%).

**The expression of surface CD117 and soluble CD117**

The mean fluorescence intensity (MFI) of surface CD117 at diagnosis was 434.5 MFI ranging from 51-1193 MFI; 15 cases (75%) had MFI of <500 (dim expression), while 5 cases (25%) had MFI of >500 (moderate to bright expression). (Table 3).

The serum level of sCD117 in 50 normal subjects was of 7.2 to 17.5 ng/mL with a mean value of 12 ng/mL in the patient group. The concentration of sCD117 at diagnosis ranged from 3.3 to 50 ng/mL with a mean of 20.6 ng/mL. Five cases (25%) showed a concentration of sCD117 <12 ng/mL, while 15 cases (75%) showed concentration of sCD117 more than 12 ng/mL at time of diagnosis (Table 4). No significant correlation was detected between MFI of surface CD117 and sCD117 before chemotherapy (P=.054). All the 15 cases with sCD117 level in their serum at presentation more than the cut off (>12) were studied for sCD117 on day 28 post-chemotherapy. All these cases showed decreased sCD117 concentration in their serum, with sCD117 concentration was ranging from 1.8 to 18.9 ng/mL (P=.0001). (Table 3).

Eighteen patients (90%) showed complete remission on day 28 defined by BM blasts <5%. All these patients showed a decrease in the level of sCD117 concentration compared to the level at diagnosis. The MFI of CD117 for the two patients with no remission and another two with suspected residual leukemia was performed at day 28. In comparison with the changes that had been seen with sCD117, there was no clear relationship between the level at presentation and at end of induction.

**Discussion**

Management of acute leukemia requires risk stratification to tailor the chemotherapy according the disease severity. Most of the risks stratifying factors
<table>
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<tr>
<th>Serial #</th>
<th>Acute Leukemia subtypes</th>
<th>Sex</th>
<th>Age</th>
<th>Signs/Symptoms</th>
<th>WBC (x10^9/L)</th>
<th>BLAST %</th>
<th>Karyotyping</th>
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Table 2. Flowcytometry immunophenotyping results Showing Antigen expression of different AML subtypes:

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</table>
are based on molecular and genetic changes. The diagnosis of acute leukemia is enhanced by “immunophenotyping” that is performed by flow cytometry which can be used as a tool to monitor the response to treatment, however, this technique is demanding. It requires a skillful technologist to detect the minimal amount of residual disease and has to be done on a fresh sample.  

CD117 is considered to play a crucial role in hematopoiesis. It was first identified in a subgroup of AML using monoclonal antibody against AML blast cells. CD117 monoclonal antibody is claimed to be especially useful in the immunophenotyping of acute myeloid leukemia with specificity and sensitivity of CD117 for AML as 100% and 69%, respectively. Although CD117 has a high specificity, it is not as sensitive as CD13 or CD33 for detecting myeloid leukemic processes. The prognostic value of CD117 has been suggested in adult AML, but was not of value in pediatric AML. 

CD117 can be proteolytically shed from the cell surface to produce soluble c-kit. Soluble CD117 levels have been determined in a number of clinical conditions. Among the hematopoietic neoplasms examined, acute myeloid leukemia subtypes M1 and M2 were associated with a modest increase in sCD117 levels.

### Table 3. The mean fluorescence intensity (MFI) of surface CD117 and concentration of sCD117 and during follow-up.

<table>
<thead>
<tr>
<th>Serial #</th>
<th>MFI of CD117 At diagnosis</th>
<th>Concentration Of CD117 In Serum At diagnosis</th>
<th>Remission Status</th>
<th>MFI of CD117 At 28 days post-chemotherapy</th>
<th>Concentration of CD117 In Serum At 28 days post-chemotherapy</th>
<th>Concentration of CD117 In Serum At 3 months or more post-chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>86</td>
<td>10.4</td>
<td>Remission</td>
<td>223</td>
<td>2.9</td>
<td>NA**</td>
</tr>
<tr>
<td>2</td>
<td>818</td>
<td>16.4</td>
<td>Remission</td>
<td>NA*</td>
<td>6.8</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>51</td>
<td>9.3</td>
<td>Remission</td>
<td>NA*</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>316</td>
<td>42.6</td>
<td>Remission</td>
<td>NA*</td>
<td>18.9</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>162</td>
<td>10.2</td>
<td>Remission</td>
<td>NA*</td>
<td>1.7</td>
<td>NA**</td>
</tr>
<tr>
<td>6</td>
<td>289</td>
<td>3.3</td>
<td>Remission</td>
<td>NA*</td>
<td>2.8</td>
<td>NA**</td>
</tr>
<tr>
<td>7</td>
<td>57</td>
<td>15</td>
<td>Remission</td>
<td>55</td>
<td>4.2</td>
<td>12.8</td>
</tr>
<tr>
<td>8</td>
<td>266</td>
<td>17.5</td>
<td>Remission</td>
<td>385</td>
<td>6.6</td>
<td>NA**</td>
</tr>
<tr>
<td>9</td>
<td>974</td>
<td>13.6</td>
<td>Remission</td>
<td>NA*</td>
<td>7.4</td>
<td>10.8</td>
</tr>
<tr>
<td>10</td>
<td>496</td>
<td>18.3</td>
<td>No Remission</td>
<td>405</td>
<td>7.8</td>
<td>8.7</td>
</tr>
<tr>
<td>11</td>
<td>122</td>
<td>9.8</td>
<td>Remission</td>
<td>NA*</td>
<td>5.7</td>
<td>11.3</td>
</tr>
<tr>
<td>12</td>
<td>413</td>
<td>18.9</td>
<td>Remission</td>
<td>NA*</td>
<td>12.5</td>
<td>NA**</td>
</tr>
<tr>
<td>13</td>
<td>419</td>
<td>14.7</td>
<td>Remission</td>
<td>NA*</td>
<td>8.5</td>
<td>4</td>
</tr>
<tr>
<td>14</td>
<td>416</td>
<td>29.3</td>
<td>Remission</td>
<td>NA*</td>
<td>11.5</td>
<td>16.5</td>
</tr>
<tr>
<td>15</td>
<td>276</td>
<td>29.5</td>
<td>No Remission</td>
<td>NA*</td>
<td>6.4</td>
<td>11.6</td>
</tr>
<tr>
<td>16</td>
<td>1193</td>
<td>&gt;50</td>
<td>Remission</td>
<td>NA*</td>
<td>1.8</td>
<td>7.2</td>
</tr>
<tr>
<td>17</td>
<td>436</td>
<td>37.4</td>
<td>Remission</td>
<td>NA*</td>
<td>7.8</td>
<td>6.5</td>
</tr>
<tr>
<td>18</td>
<td>455</td>
<td>22.3</td>
<td>Remission</td>
<td>138</td>
<td>12.8</td>
<td>13.6</td>
</tr>
<tr>
<td>19</td>
<td>595</td>
<td>27.3</td>
<td>Remission</td>
<td>NA*</td>
<td>14.7</td>
<td>16.7</td>
</tr>
<tr>
<td>20</td>
<td>850</td>
<td>16.7</td>
<td>Remission</td>
<td>NA*</td>
<td>7.4</td>
<td>8.5</td>
</tr>
<tr>
<td>MEAN</td>
<td>434.5</td>
<td>20.6</td>
<td></td>
<td>263</td>
<td>7.5</td>
<td>10.9</td>
</tr>
<tr>
<td>RANGE</td>
<td>51 to 1,193</td>
<td>3.3 to &gt;50</td>
<td></td>
<td>55 to 405</td>
<td>1.8 to 18.9</td>
<td>4 to 16.7</td>
</tr>
</tbody>
</table>

NA*: not done because it is not indicated (no residual blasts). NA**: not done (no sample collected).
CD117 expression does not seem to be a reliable predictor of FAB AML subtype. In the present study, the highest frequency of CD117 expression was found in the AML M2 category, which is similar to previously reported studies and may indicate over expression in more immature myeloid leukemia.

The level of sCD117 in normal controls varies, although in a previous study it was as high as 719 ng/mL. In our study the normal range was 7.2 to 17.5 ng/mL, which was in agreement with another study reported from Egypt with a closer range 4.05-5.45 ng/mL, which suggests some correlation with ethnicity. In this study sCD117 was detected in all 20 cases, but using a cut off value of 12 mg/mL only 15 subjects showed an elevated level at diagnosis, which had dropped in all patients at day 28 of chemotherapy.

Despite the high specificity of CD117 in acute myeloid leukemia, the intensity of surface CD117 expression was dim in 75% of the cases in our study. The correlation between cellular and soluble forms of CD117 was not significant ($P=.054$). The mean concentration of sCD117 before chemotherapy was 20.6 ng/mL while its mean concentration after chemotherapy was 7.5 ng/mL, and the difference was statistically significant ($P<.0001$).

No significant correlation was detected between the mean fluorescence intensity of surface CD117 and sCD117 before chemotherapy. This finding is the same as that reported by Kamel et al.

There was no significant difference in the level of sCD117 found among the different FAB subtypes of AML whether before or after chemotherapy. CD117 expression does not seem to be a reliable predictor of FAB AML subtype. This finding is compatible with the study of Kamel et al. In contrast, Rajima, et.al. reported an increased level of sCD117 in M1 and M2 cases.

In conclusion, although the study group is small, we could show that surface expression of CD117 in comparison to the soluble CD117 has no significant correlation. Yet sCD117 could be considered a good indicator for response to therapy as its level in the serum dropped significantly post-chemotherapy in most cases, but it could not detect the presence of residual leukemia nor can it be used as a predictor of relapse. (Further studies have to be done to evaluate the significance of sCD117 as in monitoring AML patients on chemotherapy marker for remission).
References


Serum CA-125 correlates with staging, prognosis and survival of Non-Hodgkin Lymphoma (NHL)

Tarek Ashour,* Mohamad Qari **

BACKGROUND: The clinical importance of the serological markers in NHL is based on their role in staging, prognostic assessment and in monitoring NHL patients pre- or post-treatment.

OBJECTIVE: To assess the correlation of CA-125 with the clinical and pathologic features of NHL. To evaluate its role in the staging, follow up of patients, monitoring of treatment and whether it has independent predictive value on survival of patients.

PATIENTS AND METHODS: Serum levels of CA-125 were determined in 50 patients with NHL. Serum samples were obtained from all patients at diagnosis, during chemotherapy and at remission. Thirty age- and sex-matched healthy individuals were included as a control.

RESULTS: The serum levels of CA-125 were significantly increased when compared to the control group \((P<.05)\) with 48% patients having levels above the median. The high values were significantly associated with stage III-VI disease. The event-free survival (EFS) was significantly highest in the group with low sCA-125 (76.9%) versus 52.5% in the group with the high levels. The EFS was even better when both sCA-125 and LDH were low.

CONCLUSION: sCA-125 might be employed to aid in diagnosis, staging and prognostic assessment of NHL. It can be used not only in monitoring the response to primary therapy, but also in planning treatment duration. It has independent influence on the survival of patients as proved by the Cox proportional hazard test. Combination of both sCA-125 and sLDH could improve the prognostication of patients.

KEYWORDS: Non Hodgkin’s lymphoma, Prognosis, serum CA-125, lactate dehydrogenase, survival, tumor marker, staging.

Non-Hodgkin lymphomas (NHLs) are heterogeneous tumors with different patterns of clinical behavior and response to chemotherapy. The treatment strategy is based on the independent prognostic factors of the International Prognostic Index (IPI) namely B symptoms (fever, night sweats, weight loss), performance status, age, serum lactate dehydrogenase (LDH) level, serum b2 microglobulin, tumor bulk, and number of nodal and extranodal sites of disease. The IPI is a widely accepted prognostic tool. However, some patients do not respond well to the standard therapy and thus more potent treatments are used. The information derived from this novel prognostic factors combined with the previously known ones, should be added to the existing basic system, to establish treatment strategies.

The CA125 antigen is a glycoprotein expressed by epithelial ovarian cancer. CA125 serum levels are used to monitor response to therapy and for follow-up of patients with ovarian cancer. Serum levels may be elevated in many other malignant and nonmalignant diseases, including lymphoma.

The aim of this work was to assess the serum levels of CA125 in correlation with the clinical and pathologic features of NHL. Also, we evaluated its role...
Subjects and Methods
This prospective controlled study was carried out at the Hematology/Oncology Unit, Ain Shams University Hospital in Cairo, Egypt. All of the patients who were newly diagnosed were informed on the nature of the study and consented to participation. These patients were enrolled from 2006-2008. Patients with any coexisting disease like tuberculosis or HIV infection were excluded from the study.

Recategorization of the patients according to the levels of sCA125 using the median as a cut off value was done. Also, the patients were divided into 4 groups by combination of sCA125 with sLDH (very low, low, intermediate and high risk group). Very low risk=Low sCA125+low sLDH, low risk=low sCA125+high sLDH, intermediate risk=high sCA125+low sLDH and high risk group=high sCA125 and high sLDH.

Group II: Control group: Thirty age- and sex-matched apparently healthy individuals with a mean age of 30±10.4 years and a median age of 25 years were chosen as a control from individuals free from known diseases.

At diagnosis all the patients were subjected to the following:
1. Full clinical history and physical examination.
2. Plain chest x-ray, chest and abdominal computed tomography and abdominal ultrasonography.
3. Bone marrow aspiration and examination.
4. Lymph node and trephine biopsy and histological classification. All these investigations (1-4) were performed as a staging examination according to the Ann Arbor staging classification (8).
5. Routine investigations included a complete blood count and differential blood count on Coulter S plus Cell Counter (Coultronics, FI, USA). Total protein, albumin, uric acid and LDH on a Synchron CX7 (Beckman-Coulter, Nyon, Switzerland).
6. Immunophenotyping: Cell surface markers were evaluated by flow cytometry (FCM) on a Coulter EPICS XL (Coulter electronics, Healeh FI, USA). The immunophenotyping was performed on whole blood, lysis of red blood cells was done by using IQ lyse (IQ products, Zernikepark, Groningen, The Netherland). The panels of FITC/PE-labeled monoclonal antibodies used are: CD5/CD19,CD10/CD20,CD23/CD19,CD103/CD22,CD25/CD19,Kappa/CD19,CD19/Lambda, CD4/CD8,CD3/CD56
A sample was considered positive when >20% of cells showed the marker. Monoclonal antibodies were purchased from Coulter.
7. Cytogenetic analysis: bone marrow heparinized samples were taken aseptically and processed immediately following the direct preparation technique (9).
8. Serum CA125 assay: it was determined by enzyme immunoassay (ELISA) supplied by Roche Diagnostics Products Ltd (Welwyn Garden City, Hertfordshire, USA) (10). The values were expressed in µ/mL.

N.B.: The routine laboratory investigations and assay of CA125 were done for control subjects too.

Statistical analysis
Statistical analysis was performed using an IBM personal computer with statistical program SPSS version 8 for Windows. Differences between groups were evaluated by the Mann-Whitney U test and the Wilcoxon ranked-sign test. Qualitative data are presented and the comparison between the two groups was done using the chi-square test. Also, the multiple regression analysis and Spearman correlation were done. Survival analysis was done using the Kaplan-Meir method. A P value less than .05 was considered statistically significant. Multivariate analysis of the prognosis was performed using Cox proportional hazards regression model.

Results
A total of 80 subjects were included and divided into 2 groups. Group I included 50 patients (29 males and 21 females) with a male-to-female ratio of 1.3:1. Their ages ranged from 23 to 70 years with a mean (SD) age of 46.7±10.6 years. These 50 patients included 26 with indolent type and 24 with aggressive lymphoma according to the revised European American classification of lymphoid neoplasm (REAL) scheme (7).

The indolent lymphoma included 14 with chronic lymphocytic leukemia (CLL), 2 with hairy cell leukemia (HCL), 2 with splenic lymphoma, 4 with mucosa-associated lymphoid tissue (MALT) and 4 with follicular lymphoma grade 1. The aggressive lymphoma group included 9 with diffuse large cell lymphoma (DLCL), 4 with follicular large cell lymphoma grade III (FLCL), 4 with mantle cell lymphoma (MCL) and 7 with prolymphocytic leukemia (PLL). Recategorization of the patients according to the levels of sCA125 using the median as a cut off value was done. Also, the
patients were divided into 4 groups by combination of sCA125 with sLDH (very low, low, intermediate and high risk group). Very low risk=Low sCA125+low sLDH, low risk=low sCA125+high sLDH, intermediate risk=high sCA125+low sLDH and high risk group=high sCA125 and high sLDH.

Group II: Control group: Thirty age- and sex-matched apparently healthy individuals with a mean age of 30 ±10.4 years and a median age of 25 years were chosen as a control from individuals free from known diseases. laboratory findings of all patients versus control group are summarized in Table (1). Immunophenotyping was carried out in 37 cases only, the remaining of the 50 patients were diagnosed by histopathology only. Cytogenetic analysis was done in 10 cases only. The analyzed cases revealed normal karyotype in 5 cases (50%), numerical aberration in 3 cases (30%) and structural aberration in 2 cases (20%). When serum CA125 was compared to the control group in general, it showed a significant increase ($P<.05$) (Table 1).

<table>
<thead>
<tr>
<th>Parameters studied</th>
<th>Control group</th>
<th>Patients Groups</th>
<th>$P$ and sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±SD</td>
<td>Median</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>T. protein (g/dL)</td>
<td>6.8± 0.6</td>
<td>6.5</td>
<td>7.2±1.6</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>4.1±0.6</td>
<td>4</td>
<td>2.9±0.5</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>3.6±1.0</td>
<td>4</td>
<td>5.8±3.0</td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>346±62</td>
<td>390</td>
<td>827±86</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>13.1±1.4</td>
<td>13</td>
<td>7.7±2.8</td>
</tr>
<tr>
<td>TLCx10^9/L</td>
<td>6.2±1.3</td>
<td>6</td>
<td>44.3±26.9</td>
</tr>
<tr>
<td>Plateletsx10^9/L</td>
<td>216.6±54</td>
<td>200</td>
<td>83.9±79.1</td>
</tr>
<tr>
<td>sCA125 (U/mL)</td>
<td>13±8</td>
<td>12</td>
<td>110.3±153.3</td>
</tr>
</tbody>
</table>

The frequency of cases with elevated sCA125 in NHL histological subtypes are illustrated in Table 2. In the indolent group the lymphoma of the MALT type showed the highest frequency of elevation (100%), followed by the chronic lymphocytic leukemia group (57%), and follicular lymphoma grade I (25%). While patients with splenic lymphoma and HCL showed no elevation of sCA125 levels. In the aggressive group the follicular large cell lymphoma had the highest elevation (100%), followed by diffuse large cell lymphoma (44%) and by prolymphocytic leukemia (29%) and lastly by MCL (25%).

<table>
<thead>
<tr>
<th>Parameters studied</th>
<th>Control group</th>
<th>Patients Groups</th>
<th>$P$ and sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>Median</td>
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<tr>
<td>Albumin (g/dL)</td>
<td>4.1±0.6</td>
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</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>3.6±1.0</td>
<td>4</td>
<td>5.8±3.0</td>
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</tr>
<tr>
<td>TLCx10^9/L</td>
<td>6.2±1.3</td>
<td>6</td>
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<tr>
<td>Plateletsx10^9/L</td>
<td>216.6±54</td>
<td>200</td>
<td>83.9±79.1</td>
</tr>
<tr>
<td>sCA125 (U/mL)</td>
<td>13±8</td>
<td>12</td>
<td>110.3±153.3</td>
</tr>
</tbody>
</table>

The elevated sCA125 was found to be associated with certain clinical features at presentation. Twenty-four (48%) patients had serum CA125 above the median. These high values were significantly associated with stage III-IV ($P<.05$), aggressive histology ($P<.05$), abdominal involvement ($P<.05$), high LDH ($P<.01$), high tumor burden ($P<.05$), presence of effusion ($P<.01$) and high IPI score ($P<.01$) (Table 3).

Correlations between sCA125 and other parameters using multiple regression analysis are demonstrated in Table 4. sCA125 showed a highly significant positive correlation with CD19, uric acid, IPI and effusion ($P<.01$) and a significant positive correlation with GIT infiltration ($P<.05$). It also showed a highly significant negative correlation with CD5 ($P<.01$).

In the group of patients found to have a high level of sCA125, 13 patients (54.2%) showed complete re-
mission, 2 patients (8.3%) were resistant to therapy and 9 patients (37.5%) died. While in the group with a low level, 17 patients (65.4%) showed complete remission, 3 patients (11.5%) were resistant to therapy and 6 patients (23%) died. Using the Cox F test to compare the survival time in patients with higher and lower values than the median, the event-free survival were consecutively 52.5% versus 76.9% ($P<.05$) (Table 5).

In the group of patients with values higher than the median of sCA125, all those who achieved complete remission (13/24) had normalization of sCA125 by the end of the treatment, while none of the patients failing first line of therapy (2/5) had a return of sCA125 to normal levels. On the other hand, in the group of patients with values lower than the median levels of sCA125 (17/26) at presentation, all responders maintained normal values at final restaging (Table 6). Of the nonresponder patients (3/5) showed elevated values at final restaging.

To detect which of the variables studied is associated with an influence on the survival of the patients (Table 7), the Cox proportional hazard test was performed. It showed that sCA125, serum uric acid ($P<.01$), bone marrow infiltration, IPI score, lymphocyte count, sLDH and age of the patient ($P<.05$) had an independent influence on survival.

The role of combined sCA125 and sLDH as an accurate prognostic factor was investigated; thus, patients were divided into 4 risk groups based on this combination (very low, low, intermediate and high-risk group). The very low risk group=low sCA125+low sLDH; (EFS 100%). The low risk group=low sCA125+high sLDH; (EFS 85%), intermediate group=high sCA125+low sLDH; (EFS 66.7%). High risk group=high sCA125+high sLDH; (EFS 50%) (Figure 1).

**Discussion**

The clinical importance of the serological markers in NHL is based on their role in staging, prognostic assessment and in monitoring malignancy progression following therapy. In this study, sCA125 was studied beside sLDH which is an old serological marker.

In the present study, patients with NHL had significantly elevated levels of sCA125 when compared to the control group. This is in agreement with many previous reports. The frequency of elevated sCA125 in this study revealed increased levels of sCA125 in both the indolent and aggressive form of lymphoma. Patients were classified into two groups above and below median by this parameter. It was found that 48% of patients had elevated levels of sCA125 above the
median value. This is in agreement with previous studies.\textsuperscript{13-15}

An analysis of the relationship between pretreatment levels of sCA125 and the clinical and pathological features of NHL showed that a statistically significant association between high sCA125 levels and disease stage III-IV, with aggressive clinical presentation such as effusion, abdominal involvement, aggressive histology, high IPI score, high LDH and high tumor burden. The significant association with tumor burden is confirmed in our work by the significant positive correlation between sCA125 and each of CD19, serum uric acid and high IPI score and also by the significant negative correlation with CD5, which are all a close reflection of tumor load and tumor growth. No patients with disease stage I-II or with low tumor burden had an abnormal level of sCA125. This is in agreement with many authors.\textsuperscript{14-18} sCA125 is a glycoprotein produced by mesothelial cells, not released by lymphoma cells.\textsuperscript{14,19} For that reason, the increased sCA125 may represent the response of pleuropericardial or peritoneal mesothelial cells to the direct involvement by the tumor.\textsuperscript{13,20} On the other hand, Pui and Ludwig\textsuperscript{21} proposed that the lymphokines released by the tumor might represent the stimulus for sCA125 production. Thus, sCA125 could reflect the patient’s response to the invasiveness potential of the tumor acting as a measure of its infiltrative activity. It may also be an index of the clinically undetected extranodal extension of the tumor; therefore, it is a useful marker that may improve staging and be a promising complementary parameter to other tumor markers in the initial staging of NHL.\textsuperscript{13}

In our study, the EFS of patients with lower than the median sCA125 was significantly better than EFS in those with higher levels (76.9% versus 52.5%). Also,

### Table 4. Correlation between serum CA125 levels (µ/mL) and all the parameters studied using multiple regression analysis in NHL.

<table>
<thead>
<tr>
<th>Dependent variables</th>
<th>Beta</th>
<th>F</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>sCA125</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD19</td>
<td>0.91</td>
<td>6.1</td>
<td>&lt;.01 HS</td>
</tr>
<tr>
<td>CD5</td>
<td>-0.9</td>
<td></td>
<td>&lt;.01 HS</td>
</tr>
<tr>
<td>Uric acid</td>
<td>0.46</td>
<td></td>
<td>&lt;.01 HS</td>
</tr>
<tr>
<td>IPI score</td>
<td>0.4</td>
<td></td>
<td>&lt;.01 HS</td>
</tr>
<tr>
<td>Effusion</td>
<td>0.32</td>
<td></td>
<td>&lt;.01 HS</td>
</tr>
<tr>
<td>GIT infiltration</td>
<td>0.3</td>
<td></td>
<td>&lt;.01 S</td>
</tr>
</tbody>
</table>

### Table 5. Response to treatment and comparison of survival between patients with higher and lower than the median of sCA1.

<table>
<thead>
<tr>
<th></th>
<th>Remission (30)</th>
<th>Resist to treatment (5)</th>
<th>Died (15)</th>
<th>Event-free survival (EFS)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Total no. (50)</td>
<td>30</td>
<td>60</td>
<td>5</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>sCA125</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;Median (24)</td>
<td>13</td>
<td>54.2</td>
<td>2</td>
<td>8.3</td>
<td>9</td>
</tr>
<tr>
<td>&lt;Median (26)</td>
<td>17</td>
<td>65.4</td>
<td>3</td>
<td>11.5</td>
<td>6</td>
</tr>
</tbody>
</table>

EFS: event-free survival

### Table 6. Comparison of sCA125 levels at diagnosis and at remission with the control group.

<table>
<thead>
<tr>
<th></th>
<th>At diagnosis</th>
<th>At remission</th>
<th>Control group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt;Median 13/24</td>
<td>230.6±138.4</td>
<td>14.8±5.6</td>
<td>13±8.8</td>
</tr>
<tr>
<td></td>
<td>&lt;Median 17/26</td>
<td>11.6±5.0</td>
<td>11.3±5.1</td>
<td></td>
</tr>
</tbody>
</table>

P1: between at diagnosis and at remission, P2: between remission and control group.
this applies to the complete remission, which was significantly better when the sCA125 was lower (65.4%) versus when the sCA125 is higher (54.2%).

It is clear thus that the elevated levels of sCA125 influence complete remission duration and survival negatively. Patients with normal levels exhibited longer remission and survival than those with elevated or borderline levels. Several investigators have addressed the prognostic significance of sCA125, and all reported that serum CA125 levels at diagnosis had a strong correlation with event-free and overall survival where patients with increased levels had worsened survival.14,15,22

Among the patients presenting with abnormal sCA125, all complete responders had normalization of the values by the end of the treatment. In contrast, the nonresponding patients maintained sCA125 above the normal limit throughout therapy. On the other hand, for those with values less than the median at diagnosis, all responders switched back to normal values, while the nonresponders showed elevated values at final restaging. This indicates that there is a close relationship between this marker and disease activity and points to its usefulness, not only in predicting the response to primary therapy, but also in determining the outcome of treatment. It also can help to adjust the optimal treatment duration following the disappearance of sCA125 from the patient’s serum. These results are confirmatory to those reported by previous studies.13,23

To detect if sCA125 has an independent influence on the survival of patients, the Cox proportional hazards test was performed. It revealed that sCA125, IPI score, uric acid, TLC and sLDH had an independent predictive value on survival. In contrast to our results, Bonnet et al15 reported that sCA125 does not enhance the performance of standard prognostic markers in the management of patients with NHL or HD.

In our work, in order to attempt to improve the prognostication we have examined the value of combining the sCA125 and sLDH. It was found that in the group of patients who had the best EFS, (100%, very low risk group) both parameters were low, followed by a low risk group with an EFS of 85%; the sCA125 was low while the sLDH was high. This was followed by the intermediate risk group with an EFS of 66% where the sCA125 was high and the sLDH was low. The worst risk group was associated with a rise in both parameters and EFS of 50%. It was obvious that when the two markers were low the EFS was good. When one marker was elevated the EFS was variable, while the EFS was poorest when both markers were elevated.

In only one study was the combination of sCA125 with sLDH used and it was reported that the high risk group had a shorter survival time and the combination of both markers were found to be an important prognostic factor in low grade lymphoma.11

In conclusion, sCA125 might be employed for diagnosis, staging and prognostic assessment of different grades of NHL. It can be used not only in monitoring the response to primary therapy, but also in determining the number of chemotherapy cycles that should be given to patients to achieve cure and also the optimal treatment duration. The combination of both sCA125 and sLDH could improve the prognosis and assessment of the outcome of treatment.
References

The hemoglobin disorders are one of the most common single gene disorders encountered in the region and in the Kingdom of Saudi Arabia. The hemoglobinopathy genes occur with a variable frequency in different regions of the Kingdom. Genetic screening is an important tool to control and prevent genetic disorders. The aim of this study was to estimate the frequency of hemoglobin disorders among the Saudi population at Jeddah, in western Saudi Arabia. A total of 7,584 candidates were screened, divided into two groups. Group I: The general population, 6,750 unrelated Saudi adult volunteers of both sexes (5,050 males, 1,700 females). Group II: Cord blood samples of 834 Saudi neonate (422 males, 412 females). Our results showed the prevalence of sickle cell trait was 5.4%, α-thalassemia trait 6.69%, Hb E trait 0.85% and 1 (0.12%) of 834 neonates screened had sickle cell anemia. The prevalence of alpha-thalassemia in this study was 40%. The outcome of this study indicated that the Saudi population in this area is at risk for hemoglobin disorders. Screening programs are essential and should be implemented in the prevention program as a routine practice.

**KEYWORDS:** Prevalence of Sickle Cell Disease, Thalassemia Disorders, Hemoglobinopathies, Diagnosis Hb variants, Western Saudi Arabia.

In the thalassemias, excess of the normally synthesized globin chain results in ineffective erythropoiesis, phenotypically most apparent in homozygotes or compound heterozygotes for thalassemia mutations.

In this paper our aim was to estimate the prevalence of hemoglobin disorders and compare two methods of screening for hemoglobin variants by using standard hemoglobin electrophoresis and high performance liquid chromatography among the Saudi population from the Jeddah area, western Saudi Arabia.

**Methods**
A total of 7,584 healthy candidates were screened, to estimate the frequency of abnormal hemoglobin in this area, divided into two groups. Group 1: representing the general population, consisted of a total of...
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6,750 unrelated Saudi adult volunteers of both sexes (5,050 males, 1,700 females) There ages ranged from 20-45 years old, with a mean age of 28 years. Blood samples were collected from blood donors, students, and health care workers. Group II were 834 consecutive cord blood samples prospectively analyzed from Saudi neonates of both sexes (422 male, 412 females). The study was conducted at the Hematology Laboratory and Hematology Research Lab at King Abdulaziz University (KAU) in Jeddah. Five mL of venous blood samples were collected in ethylene diamine tetra-acetic acid (EDTA) and processed within 2 hour after the collection to obtain hematological analysis and blood films. Routine hematology was carried out using a Coulter LH 750 analyzer (Coulter Electronics, USA) with blood smears by Wright stain, H inclusions bodies using brilliant cresyl blue stain (BCB) to detect alpha thalassemia disorders. Data interpretation including family history were examined by an expert hematologist. Hemoglobin studies were analyzed by both twin-tier hemoglobin electrophoresis (alkaline/acid) Hydrasys Sebia and by cation exchange high performance liquid chromatographies (HPLC) using a variant TM. Using Hemoglobin Testing system with the β-Thalassemia Short Program, we followed the analytical protocol developed by the manufacturer's instruction and the Genetic Disease Laboratory.9-15 Hemoglobin A2 (HbA2) was estimated by microcolumn chromatography. All specimens from cord blood were screened by the β-thalassemia variant program (Bio-Rad Laboratories, USA). The β-thalassemia program was therefore able to provide a reliable quantitative Hb Bart’s and to detect alpha thalassemia genotype in cord blood. To detect Hb Bart’s, required a slightly longer run time. Cord blood of normal infants has <1% Hb Bart’s, but that of infants with β-thalassemia II has levels of 1-3% Hb Bart’s and β-thalassemia I had levels of >3-10% Hb Bart’s. Hemoglobin H disease has 15-30% of Hb Bart’s. After one year of age Hb Bart’s cannot be detected except for Hb H disease with 1-5%. We followed the analytical protocol developed by the manufacturer’s instruction and the Genetic Disease Laboratory.9,16-18

Results of hemoglobin studies were used to classify the individuals as normal (HbAA), sickle cell trait/carrier (HbAS), and sickle cell anemia (HbSS). A sickle cell trait was diagnosed if the proportion of the sickle cell hemoglobin (HbS) was 35% to 45% and the sickling test was positive. If the HbS was less than 35% of the individual with HbAS was supposed to be investigated for co-existing β-thalassemia.20-22 Subjects were considered to have thalassemia trait if a blood indices compatible with the thalassemia picture (high RBC count, low MCV <79fl, low MCH <27 pg with mild alteration of the erythrocyte morphology), and the individual was declared to have β-thalassemia trait if he had a thalassemic blood picture and hemoglobin A2 (HbA2) level >3.5%. An individual was diagnosed to have β-thalassemia trait if he had a blood indices compatible with thalassemia trait and the presence and detection with H inclusion bodies. The diagnosis of hemoglobin H (Hb H disease) was made on the basis of the presence of Hb H, Hb Bart’s on the electrophoresis/HPLC, supplemented by the demonstration of Hb H inclusion in the red blood cell.

**Table 1.** Hemoglobin studies of 6750 Saudi Adult in Jeddah, Western of Saudi Arabia.

<table>
<thead>
<tr>
<th>Screening Result</th>
<th>Total number of adult screened and percentage</th>
<th>6750</th>
<th>100.00%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Screen (Hb AA)</td>
<td>5996</td>
<td>88.8%</td>
<td></td>
</tr>
<tr>
<td>Abnormal Result</td>
<td>754</td>
<td>11.2%</td>
<td></td>
</tr>
<tr>
<td>Sickle Cell Trait (Hb AS)</td>
<td>365</td>
<td>5.4%</td>
<td></td>
</tr>
<tr>
<td>β-Thalassemia Trait (Hb AA2)</td>
<td>316</td>
<td>4.6%</td>
<td></td>
</tr>
<tr>
<td>Hb E Trait (Hb A E)</td>
<td>57</td>
<td>0.85%</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin H disease</td>
<td>13</td>
<td>0.19%</td>
<td></td>
</tr>
<tr>
<td>Hb C Trait (Hb A C)</td>
<td>2</td>
<td>0.03%</td>
<td></td>
</tr>
<tr>
<td>Heterozygous HPFH</td>
<td>1</td>
<td>0.015%</td>
<td></td>
</tr>
</tbody>
</table>

HβH=Hereditary persistence of fetal hemoglobin

**Results**

The initial screen of 6,750 healthy candidates showed that about 11.2% of the general populations in this area were heterozygous for one of the abnormal Hb genes: 365 (5.4%) had sickle cell trait with Hb S quantity (range 23.4-45%) and 109 (30%) of this population also had (β-thalassemia trait. 316 (4.69%) had β-thalassemia trait, with HbA2 (range 3.6 - 7.6%). 57 (0.85%) had Hb E trait, with (range of HbA2/E 19.2 -31%). The HbA2 quantity in β-thalassemia trait did not exceed 8.0% in our population while the HbA2 quantity in HbE traits ( 19%). 0.19% diagnosed as Hb H disease, and 2 (0.03%) had Hb C trait. One (0.015%) was a symptomatic student was diagnosed as heterozygous for hereditary persistence of fetal hemoglobin (HPFH) and confirmed by Kleihauer-Betke Acid elution test. The screening result of he-
moglobin studies of 6750 candidates is shown in Table 1.

Neonatal screening showed an additional finding of the high prevalence of $\beta$-thalassemia gene 39.57% with a predominance of $\beta$-thalassemia II 35.86%. One neonate was diagnosed as having sickle cell anemia out of 834 screened (0.12%) (Table 2).

**Discussion**

Screening programs for congenital diseases especially for detection of hemoglobin disorders have been available in the Kingdom since early 1980. This present study tested the possibility of deploying modern high performance liquid chromatography (HPLC) as a successful approach from the efficiency and cost effectiveness point of view in the detection and screening of the general population—including neonates and adults who are carriers or patients for genetic hemoglobin disorders. The results show that standard electrophoresis and HPLC are comparable. HPLC is efficient and cost effective in detecting hemoglobin disorders. HPLC, coupled with the development of an automated and rapid method, achieves excellent sensitivity and specificity, while adding the very important quantitative element to the analysis. It also has proven to be clinically accurate, and in line with other studies it confirms the occurrence of various genetic hemoglobin disorders at varying rates.

One of the limitations of most screening methods including HPLC in the diagnosis of thalassemia major and persistent fetal hemoglobin, is that there is often confusion between sickle cell anemia and sickle cell $\beta$-thalassemia. A similar screening limitation is true of other compound disorders such as E/$\beta$-thalassemia. HPLC is good for neonatal screening, which requires consistent demonstration of the presence or absence of small quantities of HbA, which can be detected at 0.5%. The common variants, Hbs, Hbc, Hbe and Hbd, were also detected consistently at the level of 1%.14-16

In our communities, with the cultural preference for consanguinity, gene variants are spread among families and tribes. Hence, an affected child is a marker for a group at a high genetic risk. In the Kingdom the percentage of consanguinity varies from 37 percent to 50 percent, which increases the incidence of these disorders.5,24

The results of the present study indicated that the use of HPLC is efficient and cost-effective in detecting subjects who are carriers or patients of genetic hemoglobin disorders at various rates. Our finding confirms the previous studies in the area with the additional characterization of the various Hb disorders.1,5,22

**Summary of the finding**

The prevalence of $\beta$-thalassemia among the population in the Jeddah area was high (40%), with alpha thalassemia II the highest at 35.86% (Table 2). Sickle cell trait was 5.4% with 30% co-existence with $\alpha$-thalassemia trait, followed by $\beta$-thalassemia trait (4.69%), Hb E trait (0.85%), and others non common variants.

<table>
<thead>
<tr>
<th>Newborn Pattern</th>
<th>Screening Result</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of neonates screened and percentage</td>
<td>834 (100.00%)</td>
<td>422 (50.6%)</td>
<td>412 (49.4%)</td>
</tr>
<tr>
<td>Normal Screen (Hb FA)</td>
<td>441 (52.88%)</td>
<td>196 (23.5%)</td>
<td>245 (29.38)</td>
</tr>
<tr>
<td>Abnormal Result</td>
<td>393 (47.12%)</td>
<td>193 (23.14%)</td>
<td>200 (23.98%)</td>
</tr>
<tr>
<td>Total $\alpha$-thalassemia (Hb AF Bart’s)</td>
<td>330 (39.57%)</td>
<td>143 (17.15%)</td>
<td>187 (22.42%)</td>
</tr>
<tr>
<td>$\alpha$-thalassemia II (a a/ a-)</td>
<td>299 (35.86%)</td>
<td>133 (15.95%)</td>
<td>166 (20.73%)</td>
</tr>
<tr>
<td>$\alpha$-thalassemia I (a/-a)</td>
<td>30 (3.60%)</td>
<td>13 (1.56%)</td>
<td>17 (2.04%)</td>
</tr>
<tr>
<td>Hb H Disease (-/-a)</td>
<td>1 (0.12%)</td>
<td>0 (0.00%)</td>
<td>1 (0.12%)</td>
</tr>
<tr>
<td>Sickle Cell Trait (Hb FAS)</td>
<td>37 (4.44%)</td>
<td>18 (2.16%)</td>
<td>19 (2.28%)</td>
</tr>
<tr>
<td>Sickle Cell Anemia (Hb F5)</td>
<td>1 (0.12%)</td>
<td>1 (0.12%)</td>
<td>0 (0.00%)</td>
</tr>
<tr>
<td>Hb E Trait (Hb F A E)</td>
<td>16 (1.92%)</td>
<td>7 (0.84%)</td>
<td>9 (1.08%)</td>
</tr>
<tr>
<td>Hb C Trait (Hb F A C)</td>
<td>1 (0.12%)</td>
<td>1 (0.12%)</td>
<td>0 (0.00%)</td>
</tr>
<tr>
<td>$\beta$-Thalassemia Trait (Hb F A A2)</td>
<td>8 (0.96%)</td>
<td>5 (0.6%)</td>
<td>3 (0.36%)</td>
</tr>
</tbody>
</table>
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e.g. heterozygous Hb C and heterozygous HPFH. An accurate prevalence of sickle cell anemia in this area requires a larger and multi-center study. Compared with the results of previous studies in this area, the prevalence of sickle cell carrier seems to the same but the frequency of β-thalassemia carrier is substantially higher with the additional presence of hemoglobin E carrier. Genetic screening is an important tool to control, minimize, and prevent genetic disorders.25

Acknowledgments

The author would like to thanks King Abdulaziz University for partial grant support for neonatal Screening in this study, and would like to acknowledge Dr. Magdi Anwar, for reviewing all the hematological studies (blood films, hemoglobin electrophoresis and BCB methods) and thanks for Ms. Mofida Tablawi and Mrs. Oljat Sheite Omer for their technical effort in hemoglobin studies and Mrs. Junmanah Jarullah in data entry.

References

Safe transfusion therapy is a basic requirement in advanced medical care. Although blood transfusion saves lives and reduces morbidity in many clinical situations, it is associated with certain risks. An adverse transfusion reaction is any unfavorable event occurring in a patient during or after receiving a blood transfusion. In spite of enormous progress in blood safety over time, leading to a decline in the risk of the infectious hazard of blood, non-infectious adverse events still remain the most common complication associated with transfusion. About 0.5% to 3% of all transfusion results in some adverse events, but most are minor without any significant consequence. Most transfusions are safe and without complications. However, mild reactions occur frequently; severe and even fatal reactions occur rarely. The most common reactions are allergic reactions (hypersensitivity) which occur in about 1 to 2 percent of transfusions. The acute reaction of blood transfusion consist of acute hemolytic reaction (AHTR), transfusion-related acute lung injury (TRALI), febrile non-hemolytic transfusion reaction (FNHTR), urticaria reaction and anaphylaxis. FNHTR and urticaria reaction are the most frequent non-life-threatening acute transfusion reactions. AHTR is the most frequent severe reaction and the leading cause of death associated with transfu-
TRALI and transfusion associated graft-versus-host disease (TA-GVHD) are the most fatal transfusion reactions. A significant proportion of adverse events may occur as a result of errors in the preparation, ordering or administration of blood products, causing many complications in blood recipients. Despite careful typing and cross-matching of blood, mismatches can still cause the transfused red blood cell to be destroyed shortly after the transfusion (hemolytic reaction). These reactions can have multiple causes; some are related to transfusion practice at the hospital, others to the clinical condition of the recipient or they may be due to interaction among the biological products being transfused and the recipient characteristics.

In one study, it was shown that the number of errors rose from 1238 to 2052 from 1993 to 1999. It is particularly alarming that errors involving patient samples had a 4-fold increase from 1993 to 1999. These errors caused a lot of morbidity and mortality associated with hospital-based errors. The research has focused on infectious complications of blood so far, but there are very real non-infectious hazards of transfusion.

A previous study has shown that in reports of adverse occurrences in the United Kingdom mistransfusion accounted for over 50% of hazards of transfusion and non-infectious hazards accounted for over 95% of adverse events. France and Canada suggested that innovation to address non-infectious hazards should be given high priority. Any adverse reaction to the transfusion of blood or blood products should be reported immediately. Rapid distinction of non-infection hazard is essential because of the possible life threatening effect of these events. Hospitals should report these adverse events to blood banks. Blood banks must evaluate the rate, type and the cause of non-infection hazards. A blood bank physician should be consulted for evaluation of the appropriate blood component for future transfusions.

Improvement in transfusion reaction reporting can make us aware of the frequency of transfusion reactions and assist in planning for the reduction of transfusion risks. This research aimed to collect data on adverse reactions of transfusion to estimate the magnitude of the risks and measure them in relation to epidemiological characteristics.

Patients and Methods
This was a cross-sectional study conducted in Shiraz from 23 September 2008 to 23 September 2009. This surveillance targeted 33 hospitals (all hospitals) in Shiraz. Collaboration between hospitals and blood transfusion organizations was necessary. The manager of blood banks and the head nurses of all Shiraz hospitals were invited and educated about the definition of transfusion reactions and how to distinguish, manage and report them. Then, a questionnaire was distributed among them. It consisted of demographic characteristics, the type of blood product, the time of occurrence of transfusion reaction, the patient symptoms and signs, the type of reaction, the modalities performed for managing the patient, and the severity of these reactions. These hospitals transfused 100% of blood products in Shiraz. Then, the numbers of units of blood transfused was recorded for estimating the risk of adverse events when transfusion reaction occurred. The hospitals filled out the form and initiated the investigation of the reactions. The forms were submitted to the blood bank. We entered and analyzed the data by SPSS software. Descriptive analysis of the data included the number and proportion of each category of adverse events, their distribution by age, sex, severity, and their relationship to transfusion.

Results
Of 86849 blood units were transfused, 100 adverse reactions were reported (0.01%). Sixty-four percent of the reactions occurred in women. The average age of all patients was 22±12.05 years. A history of previous transfusion was reported for 90.9%. The re-

<table>
<thead>
<tr>
<th>Adverse reactions</th>
<th>Relationship to Transfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Probable</td>
</tr>
<tr>
<td>Major allergic</td>
<td>5 (10.9%)</td>
</tr>
<tr>
<td>ABO incompatibility</td>
<td>0</td>
</tr>
<tr>
<td>Febrile non hemolytic transfusion reaction</td>
<td>0</td>
</tr>
<tr>
<td>Bacterial contamination</td>
<td>0</td>
</tr>
<tr>
<td>Anaphylaxis</td>
<td>1 (25%)</td>
</tr>
</tbody>
</table>
The relationship of adverse reaction to blood transfusion is shown in Table 1. The most frequent symptoms of patients were urticaria (59.6%), dyspnea (23.1%), chills (32.3%), fever (21.2%), nausea (11.1%), tachycardia (11.1%) and hypotension (5.1%). The most frequent reaction was major allergic reaction and the next, febrile non-hemolytic transfusion reaction (Table 2). The mean time of occurrence of transfusion reaction was 93±64 minutes. The largest proportion of adverse reaction (43%) occurred in patients aged less than 19 years. The diagnosis of the patients who developed reactions were 90.6% thalassemia, 2.1% leukemia, and 7.3% were in patients that received blood during operations. The severity of the outcome is shown in Table 3. The type of blood product that caused a transfusion reaction was 93.2% packed RBC, 5.7% platelets and 1.1% fresh frozen plasma.

**Discussion**

This study was conducted to evaluate the reporting system of transfusion reactions, and evaluate the efficacy of this system. Any adverse reaction to the transfusion of blood or blood products should be reported immediately. Rapid distinction of non-infection hazards of blood is essential because of the possible life-threatening effects of these events. Hospitals should report these adverse events to blood banks. Blood banks must evaluate the rate, type and cause of non-infection hazards. A blood bank physician should be consulted regarding the evaluation of the appropriate blood component for future transfusions.\(^{11,12}\) The current incidence of non-infectious complications of blood is unknown. Improvement in the transfusion reaction reporting can make us aware of the frequency of transfusion reactions and assist us in planning for the reduction of transfusion risks.

The risks of blood transfusion should always compare to the benefits of blood transfusion to treat critical clinical diseases. Nevertheless, much effort has been made to provide a zero-risk blood supply to eliminate both infectious and noninfectious complications of transfusion. With the improvements in reducing the transfusion of infectious diseases, the non-infectious complications still cause serious morbidities or mortalities following transfusion. Patients should be accurately informed about the risk of blood transfusion and the possible consequences of refusing transfusion and how those risks compared with those for other common medical therapies.

The rate of transfusion reactions in this study was

---

**Table 2. Diagnosis of adverse transfusion reactions reported to Shiraz blood bank.**

<table>
<thead>
<tr>
<th>Adverse reactions</th>
<th>Valid percent</th>
<th>Proportion</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major allergic</td>
<td>77%</td>
<td>47%</td>
<td>47</td>
</tr>
<tr>
<td>ABO incompatibility</td>
<td>1.6%</td>
<td>1%</td>
<td>1</td>
</tr>
<tr>
<td>Viral infection</td>
<td>0%</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>Bacterial contamination</td>
<td>1.6%</td>
<td>1%</td>
<td>1</td>
</tr>
<tr>
<td>Febrile hemolytic transfusion reaction</td>
<td>13.1%</td>
<td>8%</td>
<td>8</td>
</tr>
<tr>
<td>Anaphylaxis</td>
<td>6.6%</td>
<td>9%</td>
<td>4</td>
</tr>
<tr>
<td>Not mention the type</td>
<td>0%</td>
<td>39%</td>
<td>39</td>
</tr>
</tbody>
</table>

**Table 3. Adverse transfusion reaction by severity of outcome.**

<table>
<thead>
<tr>
<th>Adverse reactions</th>
<th>Total</th>
<th>Not Determined</th>
<th>Minor/No Sequel</th>
<th>Life-threatening</th>
</tr>
</thead>
<tbody>
<tr>
<td>Febrile hemolytic transfusion</td>
<td>7 (100%)</td>
<td>0</td>
<td>5 (71.4%)</td>
<td>2 (28.6%)</td>
</tr>
<tr>
<td>ABO incompatibility</td>
<td>1 (100%)</td>
<td>0</td>
<td>0</td>
<td>1 (100%)</td>
</tr>
<tr>
<td>Major allergic</td>
<td>42 (100%)</td>
<td>1 (2.4%)</td>
<td>39 (92.9%)</td>
<td>2 (4.8%)</td>
</tr>
<tr>
<td>Bacterial contamination</td>
<td>1 (100%)</td>
<td>0</td>
<td>1 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>Anaphylaxis</td>
<td>4 (100%)</td>
<td>0</td>
<td>3 (75%)</td>
<td>1 (25%)</td>
</tr>
</tbody>
</table>
0.001% that was lower than the prevalence rate of this reaction in other settings. Other studies showed that about 0.5% to 3% of all transfusion results in some adverse events. In another study in France the rate of transfusion reaction was 4.02 per 1000 for platelet transfusion, 1.71 per 1000 for RBC transfusion and 0.34 per 1000 for FFP transfusion. The lower incidence of reported transfusion reaction in compare to other studies showed the deficiency in reporting system. Probably because most of the transfusion reactions were not distinguished or reported well and mild reactions are possibly not reported. Perhaps, the only reactions being reported to Shiraz blood transfusion organization (BTO) were the severe ones.

Most of the reactions reported were in thalassemic patients, probably due to greater prevalence of adverse reactions among these patients as a result of repeated blood transfusions and better supervision given to them. In this study, the most frequent reaction was allergic reaction that was similar to a previous study. Because there is no specific sign or symptom for distinguishing the type of reactions; therefore, the type of reaction may not distinguished well. In this study, we had only one report of a bacterial complication of blood due to the prevalence of this reaction in other settings. It seems that in our setting, no attention was paid to its signs and symptoms and they are not regarded as a cause of fever and sepsis in patients.

We had only one death report in the transfusion reactions, which may be due to lack of consideration of this problem as the possible cause of death and it might be due to the fact that delayed transfusion reaction did not report because of transfusion history in patients and not assume it as a leading cause of death.

These data should not be considered as national figures and must be interpreted cautiously. These figures only showed the defect in reporting system but only represented a substantial underestimate of incidence of important failures in transfusion process. This data are inadequate to determine the true incidence of transfusion reactions. Due to lower reported reaction, we could not do any statistical analysis on data. This is a new surveillance system and the cooperation of Shiraz hospitals may have been low. There was too little information transferred to Shiraz BTO to interpret the diagnoses and to be assumed as valid.

It is not possible to evaluate the extent of under-reporting since a clinical condition must be recognized first at the primary level as a transfusion reaction within a hospital and it needs education and some of the reactions were possibly missed.

However, these data probably do not reflect real transfusion reaction rates, but it shows the importance of implementation of a surveillance system and should certainly be included for future endeavors in order to enable the estimation of risks of adverse reactions.

This study has limitations in the capacity to capture transmission of viral infections because of the current inability to link with public health data. Such a link is necessary, as these infections are often not recognized until weeks or months later when the patient is no longer in hospital. Estimating this risk needs a longitudinal study.

There is, currently, an initiative to develop and pilot a system for error surveillance that would eventually be incorporated into blood bank so that the national surveillance system would be more comprehensive observes the patient during transfusion. It seems that implantation of hemovigilance network to improve knowledge of blood transfusion-related mortality and morbidity is essential. That needs education regarding the observation, diagnosing, distinguishing, managing and reporting these events. Finally, the system for reporting serious hazard of transfusion should expand in the future to include surveillance of major errors in transfusion medicine.

Understanding the real transfusion reaction rate can help blood banks to plan preventive measures and consider appropriate blood products according to patient clinical status. It is important to share information and develop standards in transfusion medicine in Iran.

Conclusion
The rate of transfusion reaction was lower than that of other studies, showing the deficiency in distinguishing and reporting of these events. It seems that implantation of hemovigilance system to improve knowledge of transfusion-related mortality and morbidity and measure the effect of new procedures on it, is essential.

Acknowledgment
We would like to appreciate from Asad Asadi and Mehrishalife for nice help in data collection and the head nurses and managers of Shiraz hospital blood banks for reporting the reactions. In addition, we would like to thank Maryam Shirmohammadi for entering data, Fariba Farhadi for typing the manuscript. I really appreciate Dr. Mehrdad Vosough for data analysis and Dr. Nazin Shokrpoor for editing the manuscript.
NON-INFECTIONOUS HEMOVIGILANCE

References

In his classic 1953 paper, Medawar pointed out that survival of the genetically disparate (allogeneic) mammalian conceptus contradicts the laws of tissue transplantation. Mammalian reproduction poses an immunological paradox because fetal alloantigens encoded by genes inherited from the father should provoke responses by maternal T cells leading to fetal loss. Medawar proposed the concept of the “fetal allograft” to explain the immune relationship between mother and fetus. In this model, immunological interaction between mother and fetus is suppressed, either through lack of fetal-antigen presentation to maternal lymphocyte or maternal lymphocyte functional suppression. In a further advance, Wegmann et al(2) suggested that even if fetal antigen were presented, maternal immune responses would be biased to the less damaging, antibody-mediated T-helper 2 (Th2) - type(2)pathway.

Recurrent spontaneous abortion (RSA) is defined as a sequence of three or more consecutive spontaneous abortions. RSA is a heterogeneous condition, which may have many possible causes; more than one contributory factor is suggested to underlie the recurrent pregnancy losses. The major causes are attributed to genetic, endocrinological, or immunological basis. In the immunological causes, it may be autoimmunity or alloimmunity. In autoimmune disease, a woman may develop antiphospholipid antibodies, antithyroid antibodies, antinuclear antibodies, and antineutrophilic cytoplasmic antibodies. Alloimmune cases of RSA show involvement of human leukocyte antigen (HLA) and
blood group antigens. The literature review reveals association of HLA alleles with RSA in various populations. Strong association of HLA DR alleles have been shown with RSA.3,8

The Hypothesis of HLA sharing and Recurrent Abortion
The MHC is a region of highly polymorphic genes whose products are expressed on the surfaces of a variety cells. Benacraff and McDevitt and their colleagues9 showed that immune responsiveness is an autosomal gene that share with HLA antigens between husband and wife. The early observations of association of HLA DR alleles have been shown with RSA.3-8

HLA sharing and recurrent abortion
Early investigators reported compatibility or sharing of histocompatibility antigens (HLA) in some couples with unexplained recurrent abortion at the HLA-A and HLA-B loci. The early observations of an increased sharing of HLA antigens between husband and wife were both confirmed and extended by studies conducted by McIntyre and Fauu,10,11 and by Beer et al.12 They included in their studies the HLA-A, -B, -C and DR loci. The postulated mechanism is that histoincompatibility between husband and wife permits sufficient maternal-fetal disparity in inherited HLA loci to induce maternal blocking antibodies, which prevent the rejection of the foreign trophoblastic material. The concept behind the immunologic mechanism as explained by Beer and coworkers postulates that the sharing of HLA antigens is simply the detectable marker for the segment of chromosome 6 which carries recessive MHC-linked genes responsible for chronic spontaneous abortions.

The protective function of HLA-G
At least three additional class I genes, HLA-E, HLA-F and HLA-G (known as non-classical Class I genes) have been identified which are highly homologous to classical HLA genes. HLA-E and HLA-F are expressed in many fetal and adult tissues. By contrast, HLA-G is only expressed on fetal placental tissues at the materno-fetal interface where classical HLA-class I and II antigens are absent. This restricted expression suggests that HLA-G products may play an important role in the immune tolerance of the semi-allogenic fetus by the mother.13 HLA-G is strongly expressed on trophoblasts from the first trimester and this suggests that HLA-G is strongly involved in materno-fetal immune tolerance.14

Interaction between NK cells and HLA-G
Many investigators have demonstrated that NK activity can be regulated by HLA class I antigens.15 Some HLA-C and HLA-B alleles present on target cells act as protective molecules against NK lysis and constitute the specific ligands for P58 and bp 43, respectively and HLA-B51, B58 and B27 are specific ligands for NKB1. With regard to the materno-fetal barrier, it was demonstrated that HLA-G is capable of inhibiting NK activity of decidual large granular leukocytes against the trophoblast from the first trimester of gestation. Human trophoblast tissue lacks classical HLA-A, -B, -C molecules, except for limited HLA-C expression during the first trimester. These class I molecules are ligands for killer-cell inhibitory receptors (KIRs) and their absence would normally triggers cytolytic NK-cell response. Since this does not happen it indicates the presence of a separated trophoblast KIR ligand. It has been shown that HLA-CW6 and HLA-CW7 are protective against lysis by NK cells only with the relevant KIR specificity, while HLA-G confer protection against all NK-cell lines. Therefore HLA-G inactivates NK-cell mediated lysis and its protective function for the trophoblast may be paramount in maternal tolerance of the fetus.

Many studies indicate that there is much sharing of HLA antigens in couples with RSA. In 1977, Komlos and his associates demonstrated the HLA allele-sharing hypothesis concerning RSA.16 Several studies have confirmed HLA antigen-sharing between spouses in RSA.17,18 Further meta-analysis of selected case-control studies suggested a slightly increased and significant risk of RSA among couples who shared at least one allele in the HLA DR locus.12 Other studies have also found non-sharing of HLA antigens.20,21 The fetus, in part, evades maternal immune rejection because the trophoblast does not express class I (HLA-A and HLA-B) or class II molecules. The expression of non-classical HLA-G on extravillous cytotrophoblast does not appear to stimulate cytotoxic T-lymphocyte (CTL)
activity\textsuperscript{22} and actively inhibits natural killer (NK) cells.\textsuperscript{23} Furthermore, Th1 responses are suppressed by placental products such as progesterone, prostaglandin E\textsubscript{2} and cytokines such as interleukin 4 (IL-4) and interleukin 10 (IL-10).\textsuperscript{24,25} Such modulation is apparent in maternal peripheral blood lymphocytes.\textsuperscript{26}

Unexplained RSAs have many etiologies. Many of them involve immune dysfunctions, which include the presence of cytotoxic antibodies, an absence of maternal blocking antibodies, sharing of HLA antigens, and disturbances of killer cell immunoglobulin-like receptor dysfunction. The roles of immunological and genetic characters have been implicated in RSA. Very little information has been obtained in Saudi Arabia. Therefore, the present study was conducted to evaluate the role of HLA in RSA.

Materials and Methods

Two hundred and twenty-five, consecutive couples, attending the Obstetric & Gynecology clinic at King Khalid University Hospital, with a history of unexplained fetal loss were selected for this case-control study. Couples with diagnosed anatomic, hormonal, chromosomal or infectious cause were excluded. Fifteen normal child-bearing couples were recruited as controls.

Twenty-five mL blood samples were obtained by venepuncture from each husband and wife. 20 mL blood was collected in ACD tubes for immunological studies and 5 mL in red top tubes for serological studies.

HLA typing, for both husband and wife, was performed using Lambda Monoclonal HLA class I and Class II tissue typing trays (one Lambda, Inc. 21001 Kittredge Street, Canoga Park, CA 91303-2801, USA). The trays are kept at –70°C until use.

1. T-lymphocytes and B-lymphocytes. Lymphocytes were isolated by magnetic heads.
2. Trays were thawed at room temperature (20-25°C) for 15 minutes.
3. To each well, 1 µL of a 2×10\textsuperscript{6}/mL suspension of either T or B lymphocytes to the class I tray or B-lymphocytes to the class II tray were added.
4. The micro droplets microplates were mixed using an electrostatic mixer.
5. The trays were incubated at room temperature (20-25°C) for 1 hour.
6. For fluorescence testing, 5 µL of fluoro quench Acridine Orange/Ethidium Bromide (FQAE-500) were added.
7. The trays were covered with Terasaki Insta Seal (T1s250) and left to stand at room temperature for 15 minutes to allow lymphocytes to settle.
8. The trays were then examined by a Lambda Scan Plus II Fluorescent Microscope.

Since the lymphocytes were incubated with a mixture of complement-binding monoclonal antibodies and complement. In a negative reaction the lymphocytes remain viable. If the lymphocytes have an antigen recognized by a monoclonal antibody, complement is bound and this leads to cell lysis. Under the microscope, lymphocytes will be dead in a positive reaction. The results are then analyzed by a computer program and results obtained in a print out for further analysis.

Results

HLA-antigen sharing, at one or more loci, was detected in 225 couples (75%) (Table 1). The distribution of sharing at one or more locus are presented in Table 2. A comparison of HLA antigen sharing in patients with a history of primary abortion versus patients with secondary abortion, is presented in Table 3. HLA-sharing at the A-locus was higher among the primary aborters patients (65.2%) as compared to secondary aborters (57.9%). The same trend was observed at the DR-locus (76.1%) for primary aborters versus (68.4%) for secondary aborters, but the difference between these prevalence rates was not statistically significant. HLA-sharing at the B-locus was greater in the secondary aborters (52.6) as compared to primary aborters (43.5%).

HLA-sharing in relation to intra-uterine fetal death (IUFD) appear in Table 4. The result show that the prevalence of HLA-sharing at the three loci was higher in patients with a history of IUFD’s than in patients without such history, but the differences between the prevalence rates were not statically significant. HLA-sharing at the DR-locus was higher in patients with history of IUFD’s occurred in couples having HLA-sharing at the DR locus.

Results comparing normal child-bearing women and couples with unexplained fetal loss are presented in Table 5. Although the degree of sharing was almost identical in the 2 groups (73.3%), the pattern of sharing was different. The following observations were noted:

1. None of the normal child-bearing couples shared all the four antigens. This was shown by two patients in the group with recurrent abortion.
2. HLA-DR3 and HLA-DR7 occurred with more
frequency in the group with unexplained fetal loss. HLA-DR3 did not appear in the group with normal child-bearing.

3. In the control group there was more sharing at the HLA-B locus as compared to the patient group.

4. These findings are in agreement with the report by Kishore et al, 1996 where they documented significant HLA-sharing in couples with recurrent spontaneous abortion at the HLA-A and HLA-DR loci compared to normal controls. Other workers could not detect significant differences between the 2 groups of patients. In our study group, although the prevalence rates were not statistically significant, however, we observed greater sharing at the HLA-A and HLA-DR locus for patients with primary abortion.

Analysis of HLA-CW6 and CW7, as forming a protective layer on the extra-villous cytotrophoblast, in the control and patient group showed interesting findings. The comparison appears in Table 6. More patients with unexplained fetal loss lack both antigens (41.5%) as compared to 33.3% in the control group. On the other hand, a very small number of the patients (5.7%) have both antigens present as compared to 33.3% in the normal-child bearing group.

In the light of the importance of certain HLA antigens acting as protective molecules against NK cell activity, we analyzed our HLA data for these antigens. The result is shown in Table 7. All protective antigens, HLA- B51, B58, B27 and -B7 appeared in very low frequencies in abortion cases.

Discussion
Our analysis revealed that 75% of couples with recurrent fetal loss shared HLA antigens. The most frequent HLA alleles shared were HLA- A2 (22.9%) followed by HLA-DR7 (16.9%). Many studies have associated recurrent fetal loss with the Dr. Locus. A study by Takamizawa et al 1987 associated recurrent abortion with locus HLA-DRW8. Another study associated recurrent abortion with HLA-DR5. An investigation by Christiansen, 1999 concluded that HLA-DR1, - DR5 and DR4 show association to recurrent miscarriage in Caesarian women. However, an important point to be noted is that the discrepancies in antigen and haplotype frequencies among the different reports could be explained by ethnic differences in HLA frequencies.

In this study, we also attempted to analyze further the pattern of HLA-antigen sharing in the different

### Table 1. The frequency of sharing HLA antigens in patients with unexplained recurrent abortion.

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>Patients with sharing</th>
<th>Patients with no sharing</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Couples) Number</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>225</td>
<td>75%</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>25%</td>
</tr>
</tbody>
</table>

### Table 2. Distribution of HLA sharing in couples with unexplained recurrent abortion.

<table>
<thead>
<tr>
<th>Degree of HLA-sharing</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sharing at one locus</td>
<td>86</td>
<td>38.2%</td>
</tr>
<tr>
<td>Sharing at two loci</td>
<td>67</td>
<td>29.8%</td>
</tr>
<tr>
<td>Sharing at three loci</td>
<td>42</td>
<td>18.7%</td>
</tr>
<tr>
<td>Sharing at four loci</td>
<td>30</td>
<td>13.3%</td>
</tr>
<tr>
<td>Total</td>
<td>225</td>
<td>100%</td>
</tr>
</tbody>
</table>

### Table 3a. Sharing of HLA antigens in Patients with unexplained recurrent fetal loss.

<table>
<thead>
<tr>
<th>Couple No.</th>
<th>Shared HLA Antigens</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HLA-A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HLA-B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HLA-C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HLA-DR</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>19 (29)</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>7.21 (50) W7</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>35 W4</td>
</tr>
<tr>
<td>4</td>
<td>19 (30)</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>68</td>
<td>8 W6</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>21 (49) W7</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>2</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 3b. Number of Antigens.

<table>
<thead>
<tr>
<th>Number of Antigens</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>One antigen</td>
<td>4</td>
</tr>
<tr>
<td>Two antigens</td>
<td>4</td>
</tr>
<tr>
<td>Three antigens</td>
<td>1</td>
</tr>
<tr>
<td>Four antigens</td>
<td>2</td>
</tr>
</tbody>
</table>
**Table 3b.** HLA-sharing in normal child-bearing couples.

<table>
<thead>
<tr>
<th>Couple No.</th>
<th>HLA-A</th>
<th>HLA-B</th>
<th>HLA-C</th>
<th>HLA-DR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>35</td>
<td>W6</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>W52 (5)</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>W63 (15)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>7</td>
<td>-</td>
<td>W11 (5)</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>-</td>
<td>W7</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>W57 (17), 7</td>
<td>-</td>
<td>W15 (2)</td>
</tr>
<tr>
<td>11</td>
<td>31 (W19)</td>
<td>W51 (5)</td>
<td>-</td>
<td>W13 (W6)</td>
</tr>
</tbody>
</table>

**Table 4.** Comparison of HLA-sharing between primary and secondary aborters.

<table>
<thead>
<tr>
<th>Locus of HLA-Sharing</th>
<th>Parity</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0+Fetal Losses (n=46)</td>
<td>&gt;1+Fetal Losses (n=57)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-A</td>
<td>30 (65.2%)</td>
<td>33 (57.9%)</td>
<td></td>
<td>.5791</td>
</tr>
<tr>
<td>HLA-B</td>
<td>20 (43.5%)</td>
<td>30 (52.6%)</td>
<td></td>
<td>.4680</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>35 (76.1%)</td>
<td>39 (68.4%)</td>
<td></td>
<td>.5224</td>
</tr>
</tbody>
</table>

**Table 5.** Loci of HLA-sharing in relation to history of IUFDs.

<table>
<thead>
<tr>
<th>Locus of HLA-Sharing</th>
<th>IUFDs</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes (n=11)</td>
<td>No (n = 88)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-A</td>
<td>8 (72.7%)</td>
<td>55 (62.5%)</td>
<td></td>
<td>.3791</td>
</tr>
<tr>
<td>HLA-B</td>
<td>7 (63.6%)</td>
<td>41 (46.6%)</td>
<td></td>
<td>.4553</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>9 (81.8%)</td>
<td>61 (69.3%)</td>
<td></td>
<td>.3179</td>
</tr>
</tbody>
</table>

**Table 6.** Comparison of HLA-CW6 and HLA-CW7 in patients with recurrent fetal loss and normal child-bearing women.

<table>
<thead>
<tr>
<th>(Couples)</th>
<th>Patients with recurrent fetal loss (n=176)</th>
<th>Normal child-bearing women (n=15)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
<td>Number</td>
</tr>
<tr>
<td>Both antigens present</td>
<td>10</td>
<td>5.7%</td>
<td>5</td>
</tr>
<tr>
<td>Both antigens absent</td>
<td>73</td>
<td>41.5%</td>
<td>5</td>
</tr>
<tr>
<td>HLA-CW6 only</td>
<td>25</td>
<td>14.2%</td>
<td>3</td>
</tr>
<tr>
<td>HLA-CW7 only</td>
<td>68</td>
<td>38.6%</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 7. Frequency of protective HLA antigens in patients with RFL

<table>
<thead>
<tr>
<th>HLA Antigens</th>
<th>Number</th>
<th>Percentage</th>
<th>Frequency in Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-B51</td>
<td>20</td>
<td>8.9</td>
<td>36.1</td>
</tr>
<tr>
<td>HLA-B58</td>
<td>2</td>
<td>0.8</td>
<td>11.2</td>
</tr>
<tr>
<td>HLA-B27</td>
<td>1</td>
<td>0.4</td>
<td>2.6</td>
</tr>
<tr>
<td>HLA-B7</td>
<td>6</td>
<td>2.7</td>
<td>11.4</td>
</tr>
</tbody>
</table>

Extensive studies have been performed in an attempt to associate HLA-antigen sharing with recurrent abortion. An interesting Danish study relates several specific haplotypes of the HLA loci with a susceptibility to recurrent pregnancy loss. It was unclear which exact loci were important, but they concluded that these alleles were less efficient at initiating a Th2-type response (beneficial) while still generating a Th1-type response (embryotoxic). This link remains intriguing but unproven.

Analysis of HLA-CW6 and CW7, as forming a protective layer on the extra-villous cytotrophoblast, in the control and patient group showed interesting findings. More patients with unexplained fetal loss lack both antigens (41.5%) as compared to 33.3% in the control group. On the other hand, a very small number of the patients (5.7%) have both antigens present as compared to 33.3% in the normal-child bearing group.

In the light of the importance of certain HLA antigens acting as protective molecules against NK cell activity, we analyzed our HLA data for these antigens. All protective antigens, HLA-B51, B58, B27 and B7 appeared in very low frequencies in abortion cases. Varla-Leftherioti et al. have demonstrated that NK activity can be regulated by HLA class I antigens: some HLA-C and HLA-B alleles present on target cells act as protective molecules against NK lysis, and constitute the specific ligand for NK-cell receptors. HLA-CW4 and HLA-B7 are the specific ligand for p58 and kp43 respectively. HLA-B51, HLA-B58 and HLA-B27 are specific legands for NKBI. The hypothesis is that the numerous uterine NK-like cells may act to control placentation. This provide a mechanism whereby maternal leukocytic recognition of fetal trophoblast may result in maternal cytokine production which would act locally in a paracrine manner important for trophoblast growth and differentiation. On the other hand, any basic MHC-non restricted cytolytic activity of NK-cells would be suppressed by their expression of the killer-cell inhibitory receptor (KIR) molecules. HLA antigens such as HLA-C and HLA-G would act as KIR ligands. Therefore, trophoblast would be susceptible to cytolysis by cells lacking both NK1 and NK2 KIRs. It has also been demonstrated that preconception natural-killer (NK) cell activity could be a predictor of miscarriage and that women with high preconception NK activity, had a significantly higher abortion rate in the next pregnancy.

A comparative analysis of HLA in RSA patients reported from the world revealed a differential association among our patients. HLA B17 has been reported to be associated in Italian RSA patients.

Various studies on HLA DR and DP loci have shown associations with RSA in Italian, Danish, and Japanese populations. Recently, HLA DRB1*1502 has been reported to be significantly associated in Japanese RSA patients. The reports on antigen sharing in couples with recurrent miscarriage led to several studies assessing the role of HLA antigens and their influence on the outcome of pregnancy. In fact, couples sharing at HLA A, HLA B, HLA C, HLA DR, and HLA DQ loci has been reported to be positively associated with the risk of RSA. Since 1977, increased HLA sharing among spouses has been associated with RSAs; later more specific HLA DR and/or DQ antigens were suggested. HLA sharing has also been reported in couples that fail to achieve pregnancy with multiple cycles of assisted reproductive techniques. Studies examining the association of HLA sharing with a risk of RSA have yielded inconsistent results, in terms of whether or not couple sharing is significantly related to the outcome and which particular HLA gene may be responsible. Meta-analysis of selected case-control studies suggested a significant risk of RSA among couples who shared at least one allele at the HLA-DR locus, but not at the other HLA loci. The discrepant results in the HLA and RSA studies...
were attributed to inconsistent definitions of recurrent miscarriage and of the control groups, the small number of samples, and the different tissue typing methods used to investigate the HLA loci.

References

case report

Acquired prothrombin inhibitor

Saud Abu-Harbesh, Hisham Yousef

Acquired inhibitors of coagulation are antibodies that either inhibit the activity or increase the clearance of a clotting factor. A common clinical manifestation in an affected patient is a hemorrhagic diathesis. Antiphospholipid antibodies represent a different problem as these antibodies prolong certain clotting assays but result in thrombosis rather than bleeding. We describe a patient with an initial presentation of deep venous thrombosis, found to be secondary to antiphospholipid syndrome (APS), which is well known to be a hypercoagulable state. However, her clinical presentation changed to a bleeding disorder, as she developed hematuria; that was only manageable with immunosuppressive medications. Patients with APS should be warned about probable bleeding. Antiprothrombin antibodies should be looked for if the patient continues to bleed in spite of stopping anticoagulant therapy. Immunosuppressive medications are successful in the correction of prolonged coagulation assays and in treating the bleeding clinically.

A
toantibodies can be directed against a variety of clotting factors, with variable clinical manifestations. Prothrombin antibodies are most often detected in patients with antiphospholipid antibodies.1-3 The prothrombin antibodies, which can cause significant clinical bleeding, usually bind a non active portion of the molecule, resulting in accelerated clearance of prothrombin.2 Laboratory testing is most consistent with a factor deficiency rather than an inhibitor, since the functional activity of prothrombin is not impaired in a clotting assay when additional prothrombin is added. The possible presence of prothrombin antibodies should be suspected if a patient with antiphospholipid antibodies develops bleeding rather than the expected thrombotic event.3-4 An autoantibody directed against prothrombin has been recognized in one patient who did not have a lupus anticoagulant.5 The autoantibody bound to a different epitope on the prothrombin molecule, but still led to depletion of prothrombin without affecting prothrombin function. Specific immunochemical measurement of the prothrombin concentration is required to establish the diagnosis.3-5

Case
The patient was a 19 years old female, from a small town about 200 km from the capital Riyadh. She presented to our hospital on 31/01/2005- with left lower limb pain and swelling. She was known to be completely healthy before, with no significant past medical or surgical problems and was receiving no medications. She denied any cardiac, respiratory, gastrointestinal, urinary or neurological symptoms. She had normal and regular menstrual cycles. She was single with no history of use of oral contraceptive pills.

On examination she was hemodynamically stable, and chest and cardiac examinations were normal, with no signs of pulmonary hypertension. Abdominal and neurological examinations were unremarkable. She was afebrile and oxygen saturation was 97% on room air. There was no malar rash, discoid skin rash, photosensitivity, mouth ulcers, serositis or arthritis. The left lower limb was swollen on examination up to mid thigh, with a 5 cm difference below the knee and a 4 cm difference above the knee when compared to the other limb. The swollen limb was red, hot and tender up to the mid thigh. Doppler ultrasound study of left lower limb confirmed the presence of thrombosis of the deep venous system involving the popliteal vein and reaching up to the femoral vein (deep venous system).

Laboratory Studies showed a CBC-WCC=6.5, hemoglobin=12.6, platelets=320,000, INR=1.2, APTT=59.6. A mixing study for APTT showed NO correction. The patient was treated with heparin followed by warfarin aiming for an INR 2-3. She was...
positive anticardiolipin antibodies IgG level=76 GPL/ml and high APTT=59.6 which didn't correct in mixing study suggesting lupus anticoagulant. She was diagnosed antiphospholipid syndrome (APS) by 2 lab. and 1 clinical criteria. A hypercoagulable state which was complicated by deep venous thrombosis of the left lower limb. She also had low protein C level. The ANA was positive (titer 1/360); but anti DNA was negative. Urine analysis was free of any abnormality. She was discharged 08/02/2005, on warfarin 1 mg PO OD, to be followed up in the anticoagulation clinic, aiming for an INR 2-3 Elastic stockings were prescribed.

Last clinic before the next admission was on 08/03/2005. INR was 2.3, and APTT was 64.8. The patient was clinically better with less swelling of the left lower limb with no more pain and less tenderness. The impression was therapeutic range INR, and the decision was to continue anticoagulation for total of 6-12 months, and to continue on elastic stocking with regular follow up.

However she presented to our hospital ER on 26/06/2005, with a 20-day history of hematuria. She sought medical advice in her local town hospital 20 days before when she developed the hematuria, and physicians there stopped warfarin, gave her medication and she was transfused, but she continued to have hematuria.

In our hospital her hemoglobin found to be 76 g/l (HB drop), platelet count 165,000. Inspite of no warfarin for 20 days the INR was 3.8 and APTT was 148. Hematuria did not show any response to vitamin K, and FFP transfusion showed only a slight and transient clinical effect then she continued to bleed again.

A mixing study for PT and APTT showed correction of the PT but no correction for APTT, which suggested either a high titre antiphospholipid antibodies or antiprothrombin antibodies. In this clinical setting of bleeding antiprothrombin antibodies was the most likely diagnosis. On 03/07/2005 factor II level was <3%. On 04/07/2005 INR was 3.6 and APTT was 139.1 Inferior vena cava filter was inserted.

On 04/07/2005, methylprednisolone 500 mg IV OD for 5 days, followed by PO prednisolone 60 mg PO OD, aiming to suppress the inhibitor.

On 10/07/2005, INR was 1.2 and APTT was 36.6, there was no more bleeding and she was discharged asymptomatic on a tapering dose of steroids.

ANA was positive since her first presentation on January 2005, but anti-DNA was negative at that time. On June 2005 both ANA and anti-DNA were positive. In June 2005 C3 and C4 were low. DAT was positive for both IgG and C3d. SLE was suspected and valuated by the rheumatology team as well.

Two years later, on October 2007 she developed proteinuria during clinic follow up, 24-hour urine proteins=3.7 g/l. There was enough criteria to diagnose systemic lupus erythematosus and lupus nephritis. She was given high dose pulse steroid therapy and monthly cyclophosphamide 500 mg IV-for 6 cycles-last cycle was on 08/03/2008.

Azathioprine (Imuran) was added as a steroid sparing agent. She was then followed in the clinic on 12/08/2009, Hb was 115 g/l, INR=1.1, APTT=33.9; she was on prednisolone 7.5 mg PO OD and azathioprine 100 mg PO OD. She was asymptomatic.

Discussion
This young patient was diagnosed with antiphospholipid syndrome based on the presence of one clinical criterion (vascular thrombosis), and two lab. criteria (moderately elevated anticardiolipin antibodies of the IgG type and the presence of lupus anticoagulant). Lupus anticoagulant was evidenced by the prolonged APTT that didn't correct upon adding an equal amount of normal plasma in the mixing study.

Antiphospholipid syndrome is considered to be a hypercoagulable state inspite of the prolonged APTT by the effect of the inhibitor lupus anticoagulant. There are several explanations for the hypercoagulable state of the antiphospholipid syndrome; these include platelet activation by the antibodies, endothelial damage by the antibodies, inhibition of the protein C pathway in endogenous anticoagulation, and decreased production of prostacyclin. These factors may work alone or together to produce the hypercoagulable state commonly found in the antiphospholipid syndrome.

As may be expected this patient developed deep venous thrombosis of the left lower limb, which reached proximally up to the femoral vein.

This patient had a prolonged APTT by the effect of the lupus anticoagulant, but the INR was normal.

Treatment of the DVT started with LMWH and warfarin aiming for INR 2-3, when INR was above 2 for two days, LMWH was discontinued and the patient was continued on warfarin. LMWH was a good choice in this setting when compared to unfractionated heparin, because following of the anticoagulant effect of the later by APTT was expected to be difficult as the APTT was already prolonged by the effect of the lupus anticoagulant. Warfarin anticoagulant effect monitoring using the INR was easy as the patient had normal INR before anticoagulation.

When the patient was followed up in the clinic, she
was found to have a therapeutic INR and the patient was improving clinically with decrease in the pain and swelling in the left lower limb.

Then she developed hematuria which led her to seek medical advice in her local town hospital 20 days before coming to our hospital ER. Warfarin was stopped, but the patient still continued to bleed. Twenty days later she presented to our hospital with hematuria, INR = 3.8 and APTT =148.

With the mixing study the INR showed correction but APTT showed no correction. This pattern in mixing studies can be seen in two clinical situations:

1. High titre of antiphospholipid antibodies. Because phospholipids are essential for both the intrinsic and extrinsic pathways of the coagulation cascade; it theoretically expected to have prolongation of both PT and APTT when phospholipids are attacked by the antiphospholipid antibodies. But because the reagent used to measure the PT contains higher amount of phospholipids when compared to the reagent used to measure the APTT, this renders the PT more liable to be corrected when even more amount of phospholipids in the normal plasma of the mixing study is added during the test. However the APTT will fail to be corrected as the reagent used for its measurement contains less phospholipids.

2. In case of antiprothrombin antibodies in the setting of antiphospholipid syndrome. Prothrombin being in the final common pathway of the coagulation cascade, a deficiency will lead to prolongation of both PT and APTT. APTT will not be corrected by a mixing study due to the presence of lupus anticoagulant. However, PT will be corrected as the antiprothrombin antibodies behave like factor deficiency rather than an inhibitor.

In this clinical setting of bleeding, the presence of antiprothrombin antibodies was the most likely diagnosis. The patient had a normal platelet count all the time. Even when patients with antiphospholipid syndrome have thrombocytopenia they rarely bleed as the younger newly released platelets are thought to be more hemostatically effective. The diagnosis was confirmed by finding a low factor II level.

Our aim was then to eradicate the inhibitor, but we considered that if this happened successfully, the DVT of the left lower limb may worsen and be complicated by pulmonary embolism, so an inferior vena cava filter was inserted. The patient responded perfectly to the treatment with high dose methylprednisolone followed by oral prednisolone 1 mg/kg/day. INR returned back to normal with no more bleeding.

More criteria developed with time from January to June/2005 that added weight to the diagnosis of SLE; anti DNA became positive, C3 and C4 were found to be low, also direct antiglobulin test turned to be positive but without evidence of hemolysis. However, the patient continued to be asymptomatic until October/2007 when she developed significant proteinuria and lupus nephritis that was managed with high dose steroids and cyclophosphamide with good response.

One report monitored 70 patients with apparent primary antiphospholipid syndrome over a 5-year period, and none developed SLE. In another observational study, 3 of 80 patients with primary antiphospholipid syndrome developed overt SLE during follow up (mean of 6.5 years).

Patients with SLE and APS have an increased incidence of thrombosis; this association was illustrated in a review of 21 studies of 1400 patients with SLE (39% of whom had antiphospholipid antibodies). Twenty-four percent of all patients with SLE had thromboses. The thromboses occurred in 42% of those antiphospholipid antibodies, 51% with lupus anticoagulant and 31% with anticardiolipin antibodies.

**Conclusion**

Patients with APS should be warned about probable bleeding. Antiprothrombin antibodies should be looked for if a patient with APS continues to bleed in spite of stopping the anticoagulant therapy. Immunosuppressive medications aiming to eradicate the antibodies are successful in correction of prolonged coagulation assays and in treating the bleeding clinically.

Patients with primary APS may develop SLE with time and regular follow up with a high level of suspicion is critical in diagnosing the natural progression of the disease process. Patients with SLE and APS have an increased incidence of thrombosis.
References

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening disorder that is characterized by microangiopathy and hemolytic anemia. It has been linked to a severe deficiency in ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 motif, member 13) activity that targets a cleavage sequence in von Willebrand factor (VWF). VWF normally circulates as multimers of 500-20,000 kDa. Ultra-large VWF (ULVWF) multimers (>20,000 kDa) have been detected in endothelial cells, but are not usually present in plasma. However, they are found in plasma from some patients with TTP.

Congenital or acquired deficiency of ADAMTS13 is associated with intravascular platelet aggregation manifested clinically as TTP.

Since the identification of ADAMTS13 and its role in TTP, several novel ADAMTS13 activity, antigen and autoantibody assays have been developed. These assays make indirect measurements and involve the incubation of patient plasma samples with a source of VWF and then measuring the amount of residual.

Most of these assays generally take 2-3 days and are technically difficult to perform. We are reporting the validation of a new automated rapid test for measuring of ADAMTS13 activity and Inhibitor in the plasma.

Materials and Methods

Samples from 14 patients who presented with classical clinical picture of TTP were collected prior to initiation of plasma exchange during a period of 5 years from 2003-2008. An aliquot of each sample was frozen while one aliquot was sent to a reference laboratory for measurement of ADAMTS13 activity and inhibitors based on a previously reported methodology. All samples were tested after removal of patient identification.

All the materials were bought locally except the VWF concentrate Wilfactin® from Laboratoire Fraincas du Fractionnement et des Biotechnologies LFB) with a final concentration of 100 u/ml, Pefabloc from (Merck®, Germany) and BC von Willebrand reagent from (Siemens).
The assay protocol was set up in the instrument BCS (Siemens). For each batch of samples a calibration curve is plotted using the Normal Pooled Plasma (NPP) with concentrations of 6.25%, 12.5%, 25%, 50% and 100%. Heat inactivated NPP (ADAM-TS13 depleted) was used for dilution. Both controls (Normal and Pathological) from Siemens were run in each batch. ADAMTS13 activity is determined by measuring the decrease in VW Ristocetin co-factor activity (VWF:RCo) of VWF substrate. After dilution of plasma with low ionic tris buffer and activation of the protease with barium chloride, vWF concentrate is denatured in the presence of urea prior to digestion. Digestion occurs during overnight incubation of the denatured substrate with diluted test plasma at 37°C. Subsequently, the residual VWF:RCo of the samples is assessed using the BC von Willebrand Reagent (Siemens) and the ADAM-TS13 activity in the test sample is calculated and the results were expressed in % activity.

For detecting ADAMTS13 inhibitors, the sample was depleted from endogenous ADAMTS13 activity by incubating test sample for 30 min at 56º and then centrifuge for 15 min at 15000g to separate the supernatant. A test sample is considered positive for ADAMTS13 inhibitors if it reduces the ADAMTS13 activity of NPP to less than 75% of the activity in the reference mixture.

We tested 24 normal subjects (13 male and 11 females) for ADAMTS13 activity and identified a reference range (51%-149%).

### Results

The test results from both methods were entered into an Excel spreadsheet and analyzed. The result from both the reference lab and the new validated method was reported as normal or reduced. In addition we are reporting percentage of activity with the new method. For comparison reason, the sample was considered normal if there were more than 51% activity. Out of the 14 cases studied by both methods, 10 (71%) were reported as normal and 4 (29%) were reported as reduced with various degrees of activity, (Table 1). Good agreement (100%) was observed in samples with decreased and normal activity of ADAMTS13 levels. All samples with reduced activ-

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### Table 1. Results of ADAMTS13 by both methods.

### Table 2. Comparison between the reference method and new method for ADAMTS13 Antibodies.
ity for ADAMTS13 were tested for inhibitors except one due to insufficient quantity. We had detected inhibitors in all the tested 3 samples by both methods (Table 2).

Discussion
Rapid recognition of the disease along with timely treatment has greatly improved the clinical outcome by inducing remission in more than 80% of patients. The specificity of low ADAMTS13 levels for the diagnosis of TTP, HUS, or other thrombotic microangiopathies is still debated. Although the current recommendation does not mandate having the result of ADAMTS13 to initiate management, yet the value of this test is in addition to establishment of the differential diagnostic criteria for the microangiopathic haemolytic anaemias. It is useful in the daily clinical management of TTP and as a follow up marker for detecting relapse. There are many methods that have been developed for testing ADAMTS13 levels and inhibitor. Although most of the methods displayed a linear relationship when studied in comparison to a reference method, yet the degree of agreement between them was variable. Tripodi A al showed in a large international collaborative study involving 11 different methods that the best concordance between observed and expected levels was achieved by two methods. One is our newly validated method using VWF ristocetin cofactor with $r^2$ value of 0.98.

In our study we confirmed the good concordance between this method and another reference method based on SDS-agarose in measuring the cleavage of ultralarge VWF by the protease.

In conclusion, we are reporting validity and sensitivity of an automated and rapid method for testing ADAMTS13 activity with ability to detect inhibitors.

References
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- FEIBA therapy has been used successfully for more than 30 years

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- Physicians in more than 50 countries rely on FEIBA therapy
- FEIBA therapy has been used successfully for more than 30 years

Help your patients by making FEIBA therapy an integral part of your inhibitor management strategy.

References:
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Abul Qasim Khalaf ibn al-Abbas al-Zahrawi (Abulcasis)

Mohamad Qari

Abul Qasim Khalaf ibn al-Abbas al-Zahrawi (known in the west as Abulcasis) was born in 936 C.E. in Zahra in the neighbourhood of Cordova. He became one of the most renowned surgeons of the Muslim era and was physician to King Al-Hakam-II of Spain. After a long medical career, rich with significant original contribution, he died in 1013 C.E.

Abulcasis wrote the first description on hemophilia, an inherited genetic disease, in his Al-Tasrif, in which he wrote of an Andalusian family whose males died of bleeding after minor injuries.

He is best known for his early and original breakthroughs in surgery as well as for his famous Medical Ecyclopaedia called Al-Tasrif, which is composed of thirty volumes covering different aspects of medical science. The more important part of this series comprises three books on surgery, which describe in detail various aspects of surgical treatment as based on the operations performed by him, including cauterization, removal of stone from the bladder, dissection of animals, midwifery, stypics, and surgery of eye, ear and throat. He perfected several delicate operations, including removal of the dead foetus and amputation.

Al-Tasrif was first translated by Gherard of Cremona into Latin in the Middle Ages. It was followed by several other editors in Europe. The book contains numerous diagrams and illustrations of surgical instruments, in use or developed by him, and comprised a part of the medical curriculum in European countries for many centuries. Contrary to the view that the Muslims fought shy of surgery, Al-Zahrawi’s Al-Tasrif provided a monumental collection for this branch of applied science.

Al-Zahrawi was the inventor of several surgical instruments. A special medical instrument called a cauter, used for the cauterization of arteries, was first described by Abu al-Qasim in his Kitab al-Tasrif. Abu al-Qasim also introduced the use of ligature for the blood control of arteries in lieu of cauterization. He was the first surgeon to make use of cotton (which itself is derived from the Arabic word qutn) as a medical dressing for controlling hemorrhage.

Al-Zahrawi became an eminent surgeon. He was appointed as the Court-Physician of King Abdel-
Rahman III. He spent a productive life in practicing medicine, especially in surgery and medical writings. He died at the age of 83. He wrote mainly four books. One of them is “Al-Tastif Liman Ajiz’an Al-Ta’lif” which is the best medieval surgical encyclopedia. It was used in Europe until the 17th century. He stressed the importance of basic sciences: “... Before practicing, one should be familiar with the science of anatomy and the functions of organs so that he will understand them, recognize their shape, understand their connections, and know their borders. Also he should know the bones, nerves, and muscles, their numbers, their origin and insertions, the arteries and the veins, their start and end. These anatomical and physiological bases are important, and as said by Hippocrates: ‘These are many physicians by title and a few by practice.’... If one does not comprehend the anatomy and physiology, he may commit a mistake that can kill the patient. I have seen someone, who pretended to be a surgeon, incised an aneurysm in the neck of a woman, mistaking it for an abscess. The woman bled to death.”

Heller stated that Al-Zahrawi described the ligation of arteries long before Ambrose Pare (Khairallah 1942). Al-Zahrawi also used cautery to control bleeding. He used wax and alcohol to stop bleeding from the skull during cranial surgery. Sprengel said that Al-Zahrawi was the first to teach the lithotomy position for vaginal operations (Khairallah 1942). Al-Zahrawi also described the tracheotomy operation and performed it as an emergency on one of his servants. He was the first to write on orthodontia. He showed evidence of great experience from details of clinical picture and surgical procedures e.g. his description of varicose veins stripping, even after ten centuries, is almost like modern surgery (Al-Okbi 1971): “... Have the leg shaved if it is much hairy. The patient gets a bath and his leg is kept in hot water until it becomes red and the veins dilate; or he exercises vigorously. Incise the skin opposite the varicose vein longitudinally either at the ankle or at the knee. Keep the skin opened by hooks. Expose, dissect, and separate the vein. Introduce a spatula underneath it. When the vein is elevated above the skin level, hang it with a blunt rounded hook. Repeat the procedure about three fingers from the previous site and hang the vein with another hook as previously done. Repeat the procedure at as many sites along the varicose vein as necessary. At the ankle, ligate and strip it by pulling it from the incision just above. When it reaches there, repeat at the higher incision until all of it is stripped. Ligate the vein and then excise it. If difficulty is encountered in pulling it, ligate its terminal part with a string and pass it under the spatula and dissect it further. Pull gently and avoid its tearing because if it does, it becomes difficult to strip all of it and can cause harm to the patient. When you have stripped it all, put alcohol spanges at the sites of the skin incisions and take care of the incisions until they heal. If the varicose vein is tortuous, you have to incise the skin more frequently, at each change of direction. Dissect it and hang it with the hooks and strip it as previously described. Do not tear the vein or injure it. If this happens, it becomes difficult to strip it. The hooks used should be blunt, eyeless, and rounded, otherwise it can injure the vein”.

During the time of Al-Zahrawi, surgery in the Islamic world became a respected specialty practiced by reputable physicians. On the contrary in Europe, surgery was belittled and practiced by barbers and butchers. In 1163 A.D., the Council of Tours declared the following resolution “Surgery is to be abandoned by the schools of medicine and by all decent physicians.”
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Haemophilia B

Thrombasthenia

For bleeding episodes and for invasive procedures/surgery administer 90 μg (range 80-120 μg) per kg b.w. every 2 hours (1.5-2.5 hours). At least three doses should be administered to secure effective haemostasis. For patients who are not refractory to platelets are first-line treatment. Contraindications: Known hypersensitivity to active substance, excipients, or to mouse, hamster or bovine protein.

Precautions: For severe bleeds NovoSeven should only be administered in hospital, especially in the treatment of patients with coagulation factor VIII or IX inhibitors or in close collaboration with a physician specialised in treatment of haemophilia. Ambulatory treatment should not exceed 24 hours. Possibility of thrombogenesis or induction of DIC in conditions in which tissue factor could be expected in circulating blood, e.g. advanced atherosclerotic disease, crush injury, sepsis, or DIC. Since NovoSeven may contain trace amounts of mouse, bovine and hamster proteins there is a remote possibility of the development of hypersensitivity. Monitor FVII deficient patients for thrombolytic time and FVII coagulant activity; suspect antibody formation if FVIIa activity falls below expected level or bleeding not controlled with recommended doses. Avoid simultaneous use of thrombolytic complex concentrates, activated or not. Use in pregnancy: Only administer to pregnant women if clearly necessary. Data known if administered in human milk; exercise caution when administering NovoSeven to nursing women. Side Effects: Adverse reactions are rare and non-serious reported during post-marketing period: Rash: < 1/10,000. < 1/1000. Lack of efficacy very rare: < 1/10,000. Coagulopathic disorders such as increased D-dimers and consumptive coagulopathy; myocardial infarction; nausea; fever; pain, especially at injection site; increase of ALT, AIP, LDH and alanine aminotransferase levels; cerebrovascular disorders including cerebral infarction and cerebral ischaemia; skin rashes; various thrombotic events; haemorrhage. Serious adverse reactions include: Arterial thrombotic events (such as myocardial infarction or ischaemia, cerebrovascular disorders and bowel infarction); various thrombotic events (such as thromboembolism, deep vein thrombosis and pulmonary embolism). In the vast majority of cases patients were predisposed to such events. No spontaneous reports of anaphylactic reactions, but patients with a history of allergic reactions should be carefully monitored. No reports of antibodies against FVII in haemophilia A. In patients with FVII deficiency patients developing antibodies against FVII after treatment with NovoSeven. These patients previously treated with human plasma and/or plasma-derived FVII. Monitor FVII deficient patients for FVII antibodies. One case angioedema occurred in patient with Glanzmann's thrombasthenia after administration of NovoSeven. Marketing Authorisation numbers: NovoSeven 60 KIU EU/15/060/001 / NovoSeven 120 KIU EU/15/060/002 / November 240 KIU EU/15/060/003. Legal Category: POM. Further information: Full prescribing information can be obtained from: Novo Nordisk Healthcare AG, Andreasstrasse 15, CH-4050 Zurich, Switzerland Tel: +41 43 222 4300. Fax: +41 43 222 4342.

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The society encourages all members and non-members to support the journal by submitting their research papers and clinical experiences for publication in the journal.

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