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Review

Monitoring of anticoagulant therapy in cancer patients with thrombosis and the usefulness of blood activation markers

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ABSTRACT

Thrombotic diseases caused by cancer progression have been reported as one of the major causes of cancer associated morbidity and mortality along with cancer invasiveness and infectious complications. Moreover, anticoagulant therapy with heparin and heparin-like drugs, or vitamin K antagonists, or the Direct Oral Anticoagulants, is seeing an extended application in cancer patients and offers prolonged life expectancy to oncology patients for whom blood activation and thrombotic events have a variable incidence, depending on cancer type. Laboratory tools are highly useful for identifying patients at thrombotic risk through the measurement of blood activation markers and selecting those appropriate for anticoagulant therapy. Among the pathological markers, DDimer or Extracellular Vesicles have the highest diagnostic value in these pathological conditions. Global assays are useful for dosage adjustment, such as assessing either an induced anticoagulant effect or the measurement of drug activity. Various assays are also developed such as platelet aggregometry techniques for evaluating drug induced- aggregates or methods allowing measurement of the drug activity to its targeted coagulation factors such as: heparin to thrombin or Factor Xa; DOACs to Thrombin or Factor Xa (Dabigatran to thrombin and DiXaIs, Rivaroxaban, Apixaban, and Edoxaban, to Factor Xa). Such explorative techniques help to find the right dosage adjustment to protect patients from developing thrombosis without exposing them bleeding. It also permits exploration of unexpected drug behavior in treated patients, to check the right adherence to therapy in long-term anticoagulant protocols, and prevention of bleeding in patients with impaired renal or hepatic function. Complementary use of blood activation markers brings additional information on the curative effects of the anticoagulant therapy, and allows identification of pro-thrombotic activity in the clinically silent state. These issues are concisely addressed below.

Contents

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1. Introduction

The Trousseau syndrome is known for 1 and a half century, and many years ago [1], occult cancer was diagnosed in patients with unprovoked thrombosis, and without genetic risk factors [2,3]. Malignant diseases are observed in a significant proportion of patients with thrombosis, in the absence of usual inducing causes such as anticoagulant protein deficiency or any identified epidemiological risk factors [4,5–7]. Today thrombosis is one of the major complications occurring in patients with malignant diseases, and it has an important impact on morbidity and mortality, being the second contributing cause of death in these patients. Thromboembolic diseases occur frequently in patients with cancer (from 20% to >50% in pancreatic cancer), with an incidence variable with cancer type and activity, in view of tumor evolution or chemotherapy treatment, and, conversely, malignant diseases are associated with about 20% of all thrombotic events. In addition, occurrence of unprovoked thrombosis is associated with the diagnosis of an occult cancer in a significant proportion of patients (10–15%), and the clinical complications or subsequent bleeding are more severe than in other patients with thrombosis [8]. Therefore, short-term and long-term anticoagulant therapy is widely used for treating these patients in the acute phase or for prevention, and this therapy is associated with an improved survival [9–14]. Vitamin K antagonists (warfarin, coumadin) were first used for treatment and prevention of thrombotic events in cancer patients, and are still the treatment applied by many sites [15,16]. Other anticoagulants are progressively preferred for their higher efficacy and the lower risk of associated bleeding. Mainly, low molecular weight heparin (LMWH) is now the most recommended therapy, even for the long-term prevention of recurrences [11,14,17–19]. In addition, Direct Oral Anti-Coagulants (DOACs) are seeing increasing applications but their efficacy and long-term protective effect is still insufficiently documented [12,20,21]. Unfractionated heparin (UFH) is more restricted to acute situations and in some surgeries, and this therapy is then switched to VKA or LMWH for long-term treatment [21].

Variations and limitations of anticoagulant therapy in cancer patients remain like those for other patients, although the associated clinical thrombotic or bleeding complications occur with a higher severity rate, and recurrence is frequent [8,15]. Treatment surveillance is very helpful for correct management and therapeutic adjustment of these patients, according to the risks present. Laboratory monitoring is requested for vitamin K antagonists (INR measured with prothrombin time/PT), and for dose adjustment for UFH (anti-thrombin or anti-FXa clotting or chromogenic assays). Drug measurement is better linked to specific clinical situations for the other types of anticoagulants. For LMWH preventive dosage regimen and DOACs (mainly Dabigatran and Edoxaban) the presence of an impaired kidney clearance can significantly increase the in-vivo half-life of the drug, and expose the patient to the risk of bleeding if not appropriately controlled [21–27]. Drug measurement in citrated or CTAD plasma [23] is then necessary for dose adjustment, as it is also useful in the presence of recurrence of thrombosis or bleeding, or in the presence of an unexpected clinical response. In addition, for DOACs, laboratory testing is useful in cases of extreme weight (<45 kg or >120 kg), or to check compliance with therapy, or when an urgent surgery is needed, and hemorrhagic risk due to anticoagulation must be excluded [21,26–28]. Surgery is then undertaken when the DOAC concentration is below the risk threshold concentration (<30–50 ng/ml). For treatment with UFH and long-term therapies with LMWH the Heparin Induced Thrombocytopenia risk must also be assessed, and a platelet count is recommended every two days at least during the 2 first weeks of treatment. It allows detection of most patients developing that immune complication due to generation of heparin dependent antibodies. These antibodies can be measured with immunoassays for their binding to heparin-platelet Factor 4 (PF4) complexes, or with functional assays detecting platelet activation in the presence of a low heparin concentration (0.1–1.0 IU/ml), which is abolished when heparin is present in excess (10–100 IU/ml). The combination of a clinical risk assessment along with a positive result of both an immunoassay and a functional assay is necessary to confirm the HIT diagnosis.

Hypercoagulability is frequently present in patients with malignancies and can be due to the tumor itself, or to the consequences of its treatment (especially when anti-angiogenic drugs and chemotherapy are used and combined) This is reflected by the presence of blood activation markers such as activated platelets, DDimer, P-Selectin, Micro-Particles (MPs) with pro-coagulant activity, Prothrombin fragment 1 + 2, and Tissue Factor (TF). This blood activation is associated with inflammatory markers such as Cross-Reactive Protein (CRP), and elevated coagulant Factor VIII (FVIII) concentrations, an acute phase reactant coagulation protein [29–31]. Other activation markers (reflecting blood cells or coagulation factor activation) are available and can be measured, but they remain mainly restricted to the research area. Elevated blood activation markers can alert to the possible existence of a tumor, and their concentration can decrease when there is a remission or a successful antithrombotic treatment [32,33]. They can then help in to follow-up of disease evolution, and in assessing the efficacy of anticoagulant therapy [34–37].

This article aims to review the various laboratory methods which can be of great usefulness for monitoring anticoagulant therapy in cancer patients when needed [37]. It also discusses the assays for blood activation markers which reflect the hypercoagulability stage of patients and their response to therapeutic management.

2. Blood activation and thrombosis in cancer patients

As early as in 1865 Trousseau already noted that unexpected or migratory thrombophlebitis could be a forewarning of occult visceral malignancy [1]. Since this time, many reports have highlighted the occurrence of thrombotic episodes in patients with cancer [38]. Nowadays, how tumor development and oncogenic events deregulate the hemostatic system, which can generate thromboembolism or cardiovascular complications in affected patients, but also bleeding episodes, is better understood [6,33,39]. Thrombotic complications in this clinical context involve a multifactorial phenomenon, that involves release of TF and PAI-1 by the malignant cells. This promotes coagulation and inhibits fibrinolysis, secretion of tumor derived cytokines, angiogenesis with tumor progression, inflammation, hypoxia, release of pro-coagulant extracellular vesicles (EVs), or direct contact of tumoral cells with blood [4,5,33]. The possible presence of an occult cancer in patients with unprovoked thrombosis must be suspected, and is now well documented [2,3]. An occult malignant disease is discovered in about 10% of patients with thrombosis, and is associated with the presence of elevated concentrations of blood activation markers in blood which can co-exist or precede the thromboembolic event. Venous thromboembolism and/or pulmonary embolism have been observed in these patients, and disease evolution tends to present a higher grade of severity than in non-cancer patients. Cardiovascular diseases, such as stroke or myocardial infarction have also been observed [5,7,10,15].

In patients with a diagnosed malignancy, thrombosis occurs in about 20% of cases, and frequency varies greatly according to the cancer type and evolution stage. It tends to be higher in breast, lung, prostatic cancers, and gastro-intestinal tumors, with the highest rate being observed in patients with pancreatic cancer, where the incidence can reach >50% in some studies. Thrombotic compli-

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cations are associated with an important morbidity and mortality, and they are estimated to be the second contributing cause of death in patients with cancer. Anticoagulation is therefore an important therapeutic strategy for better management of these patients [11,12–14,17,23]. However, despite this therapy, patients tend to develop recurrent clinical episodes of thrombosis or bleeding [8,15], and they require a specific follow-up. Causes of thrombosis are associated with the tumor progression itself, or can result from its treatment with chemotherapy or surgery [12,13,15,40–44]. The tumor can release pro-coagulant material into the blood circulation [33,44,45].

3. Anticoagulant therapies in malignancy

Various available anticoagulant therapies can be used in patients with cancer: vitamin K antagonists, heparin therapy, the recently introduced Direct Oral Anticoagulants (Direct thrombin inhibitors or direct factor Xa inhibitors), but also, in some cases, antiaggregant treatment or fibrinolytic drugs, on a case by case basis [13,14,43]. Anticoagulation has a beneficial effect on patients with malignancies by reducing the burden associated with recurrent thrombotic events, and specific guidelines are available for these therapeutic protocols [17,18,46,47]. Warfarin or dicumarol were the first anticoagulants used in patients with cancer and their benefit was documented early. UFH is used during the time of high-risk surgery, and can be initiated to treat the acute thrombotic episode but cannot be maintained for a long time although it has anti-inflammatory and anti-tumoral as well as anticoagulant activities [40]. Anticoagulation can then be continued with VKAs for a variable term, taking care to only withdraw heparin when the expected INR value is reached. However, there is now a greater tendency to use LMWH for a short or long term with successful results and a positive effect on the life expectancy of patients. Large cohort studies show that LMWH is more efficient for reducing the recurrence rate and bleeding events than VKAs [8,15]. DOACS are also increasingly used for that application [19,39,40], but are not yet fully recommended by guidelines. Anticoagulant treatment is needed in the acute phase of the thrombotic event until healing is complete. Nevertheless, its continuation is recommended during a defined time to avoid recurrence. LMWH therapy is frequently introduced for 3 months or longer, with positive feedback. There is now a strong trend to start preventive anticoagulation in cancer patients, by using LMWH and eventually DOACs. However, there is no strict recommendation to initiate a preventive anticoagulant therapy in all patients diagnosed with cancer and treatment is proposed on the basis of risk analysis for each patient. Recently, following an initial UFH treatment during the acute phase, VKAs and more frequently LMWH, is complemented by the possible use of DOACs. The incidence of recurrent thrombotic and bleeding episodes is reported to be lower in patients treated with LMWH or DOACs than in those treated with VKAs, with clinical efficacy remaining at least equivalent (non-inferiority).

4. Laboratory monitoring of anticoagulant therapies

Using the right drug dose for preventing or curing thrombotic episodes in cancer patients requires an accurate diagnosis and therapeutic protocol, and recommendations are described in guidelines. Laboratory assays are useful for achieving this objective, adjusting the right dosage for VKAs, UFH, or LMWH, and identifying any risk of unexpected patient response, this later approach also includes patients treated with DOACs. Alternatively, specific assays are available and used for those patients who receive another anticoagulant therapy on a case by case basis: aggregometry assays for antiaggregants and measurement of fibrinolytic proteins or evaluation of the fibrinolytic potential when fibrinolytic treatments are used. The various assays available and used for the different anticoagulant therapies are summarized below.

4.1. Vitamin K antagonists (dicumarol, warfarin)

Use of this anticoagulant for cancer patients is decreasing due to higher antithrombotic efficacy and lower recurrence of thrombosis and bleeding obtained with LMWH therapy; likeky, a similar pattern can be anticipated with DOACs (these later tending to also replace some indications for VKA therapy). However, VKAs still remain in use, although guidelines recommend their restriction and their replacement, first with the recommendation of LMWH therapy [17,18]. VKAs, by lowering vitamin K activity, generate hypo- or de-carboxylation of vitamin K dependent proteins (Factor II/prothrombin, Factor VII, Factor IX, Factor X, Protein C, Protein S, Protein Z), which are then present with decreased activity, and this lowers the global coagulolytic balance. Activities of anticoagulant proteins (Protein C and Protein S) are also reduced, but the global balance, when treatment is stabilized, is a lower procoagulant potency, and a lower thrombotic risk. If activities are decreased too much, a serious bleeding risk is present: VKA treatments produce among the highest rates of iatrogenic complications with their associated morbidity, and mortality. With this long-term therapy, the major difficulty is to reach the right dosage, as VKAs are difficult to adjust and individual specific, and have a strong interference with food (which can contain variable amounts of vitamin K). Close surveillance is required during therapy initiation and from time to time in the stabilized stage (this can take weeks to >3 months depending on the patient, treatment adherence and diet). However, and despite close follow-up, some patients cannot be stabilized (poor INR control, or INR > 5.0 more than twice in a year, while compliant) or develop side effects (such as alopecia), so VKA treatment cannot be maintained and needs replacement with another anticoagulant. The right dosage adjustment is monitored using the Prothrombin Time (PT) test, which now is mainly designed with recombinant human Tissue Factor (TF) and synthetic phospholipids. Results are reported as clotting time and % of normal range, and especially as the International Normalized Ratio (INR), which smooths differences between reagents and instruments used for performing the assay. This way of expression has introduced better comparability between centers, and variability of testing conditions with different reagents and instruments. PT reagents have a performance index (International Sensitivity Index/ISI) established by comparison with a WHO International Standard (provided by NIBSC, Potters Bar, UK), and uses the WHO reference method (tilt tube). This ISI value is specific for each reagent and each lot, and it allows establishing the INR value with the reagent used.

Another variability factor is the normal reference clotting time in testing laboratory (Mean Normal Prothrombin Time/MNPT), which needs to be defined in each laboratory or used through a commercial normal reference plasma, duly characterized for clotting time and % PT value. The INR calculation is then obtained by the ratio of clotting time of the tested patient's plasma on MNPT with the ISI exponent (Patient CT/MNPT^{ISI}). Although this approach has greatly improved variability between reagents, instruments and laboratories, many factors still induce some lack of homogeneity. Reagents do not always have the same sensitivity to calcium concentration, or to citrate concentration in the tested plasma, and this impacts the result comparability when insufficiently filled blood collection tubes are obtained from patients with deteriorated veins, and contain excess citrate, or in the presence of low or high hematocrits. Use of a PT reagent, using recombinant human TF, synthetic phospholipids, and with a low sensitivity to calcium concentration variations or to citrate concentration variations, in the tested specimen are preferred. When used, calibration with INR calibrators is

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also to be preferred, as it offers a constant matrix, a homogeneous fibrinogen content, and normal citrate concentrations (calibration curves obtained with serial dilutions of a normal citrated plasma pool are impacted if the successive dilutions do not have the same fibrinogen and citrate concentration). In practice, the right drug dosage adjustment for VKA treatments is obtained when the optimized reagent (as discussed here above) is used, and testing is done in the same laboratory, with the same instrument, although use of the INR allows a sufficiently safe evaluation when testing is done in different settings.

4.2. UFH and LMWH

UFH remains the antithrombotic therapy of choice in acute thrombotic events, as, in addition to its high anticoagulant efficacy, this drug also has anti-inflammatory and anti-tumoral activities [41] and this therapy is followed with VKAs, or LMWH, or DOACs treatment. There is a tendency in acute conditions to also start therapy with LMWH continued for long-term (>3 months or more), or switched by VKAs or DOACs, when the initial acute phase is under control. In some applications, DOACs now tend to become the first treatment choice for prophylaxis in cancer patients with associated high risk factors for thrombosis. Therapies with LMWH, and even with DOACs, are associated with a lower incidence of recurrence and bleeding episodes. Measuring LMWH regularly is useful at the onset of treatment, to achieve the right dose adjustment, and to exclude any impaired clearance due to organ dysfunction, mainly kidney [26,27]. This monitoring can be continued from time to time, and testing is immediately required in cases of an unexpected response to therapy or recurrence of thrombosis or bleeding. For UFH and LMWH the most popular laboratory methods in use are now the kinetic chromogenic anti-Factor Xa assays, fully automatable on all laboratory instruments [21,22].

Testing practice is now improved by the availability of liquid presentations, and methods can pre-calibrated and can be used at any time (7/7 and 24/24) in laboratories, without requiring skilled personnel. Including controls in the series is then sufficient for validating the measurements. We developed a kinetic anti-FXa assay based on the report by Lyon et al. [21], with an extended working range (0.05 to >1.50 IU/ml), and which has the same dose-response curves for UFH and all LMWHs, and is poorly sensitive to blood collection conditions and to the possible presence of Platelet Factor 4 (PF4) in the specimen. This homogeneous reactivity to the various UFH and LMWH presentations can be obtained thanks to the addition of a small amount of Dextran Sulphate in the reagent. This assay, Biophen™ Heparin LRT, is available in the liquid form and can be used on any of the laboratory instruments available. Fig. 1 reports the dose response curves for UFH and LMWH obtained with this reagent on the STA-R instrument. All the various LMWH brands generate the same dose response curve between the change in absorbance (measured at 405 nM and the concentration expressed in Anti-FXa International Units (IU)). This homogeneous reactivity has a very practical impact, as the heparin type used (UFH or any one of the LMWH available) is not always known for plasma samples from anticoagulated patients sent for testing. When used with dedicated plasma, calibrators and controls, the kinetics of the anti-FXa method can also be applied for measuring fondaparinux and sodium danaparoid. The method can be used on any coagulation automated platform available in laboratories, and a full correlation is obtained between the different instruments. As an example, Fig. 2 shows the correlation diagram obtained for the measurement of UFH or LMWH in plasma from treated patients, using the Biophen™ Heparin LRT reagent with STA-R (Diagnostica Stago) or CS-5100 (Sysmex). All concentrations measured are similar and within the assay variation range. The same results are obtained when ACL-Top (IL), BCS-XP (Siemens) or any other of the laboratory photometric coagulation instruments are used.

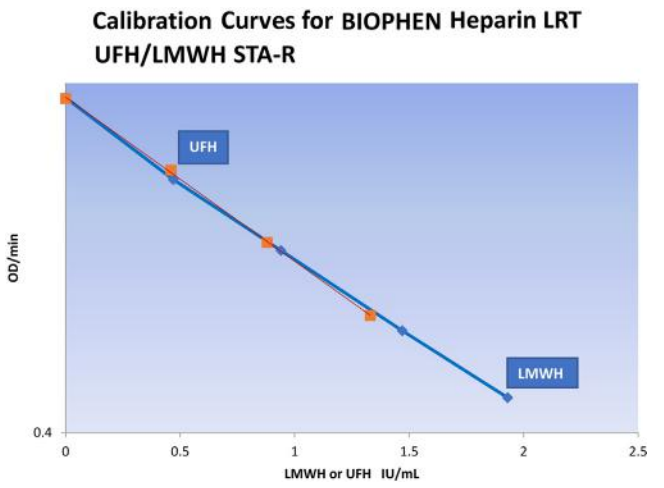

Fig. 1. Hybrid calibration curve for the measurement of both UFH and LMWH with Biophen™ heparin, in the same series, on the STA-R instrument: Unfractionated heparin (UFH) and any type of low molecular weight heparin (LMWH) are measured with the same accuracy and reliability, and without any bias due to the heparin type used.

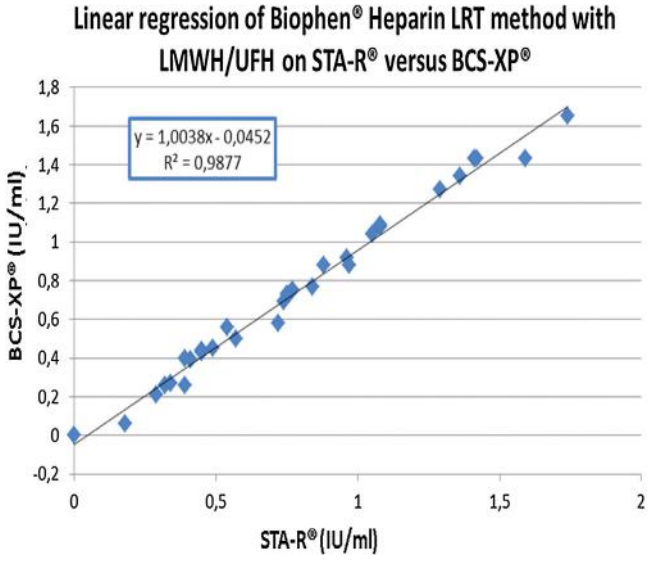

Fig. 2. Correlation diagram obtained for the measurement of unfractionated heparin (UFH) or low molecular weight heparin (LMWH) in various random plasmas from patients treated with the various heparin drugs, using the STA-R (Stago) or the BCS-XP (Siemens) instruments. Both assay systems give the same results for heparin content, with a strong correlation.

4.3. DOACs

Treatments with DOACs are claimed to not require any monitoring, as they offer a wide therapeutic window, have little interference with food or drugs, present large variations in kinetics and peak although standard drug dosages are used for most of the patients (with ethnic differences, dosages being slightly lower for Asians, who have a higher propensity to intra-cranial bleeding when anticoagulated). However, in some cases it is useful to measure their plasma concentration by using methods which evaluate their inhibitory activity to their targeted coagulation serine esterase: thrombin for DTI, i.e. Dabigatran, and FXa for DiXaIs, i.e. Rivaroxaban, Apixaban and Edoxaban [22,25–27].

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A summary follows of the major characteristics and performance of laboratory methods for measuring DOACs in plasma, when required. Dabigatran (DTI) is tested with a diluted thrombin time clotting assay or with a chromogenic anti-IIa assay, whilst Rivaroxaban, Apixaban and Edoxaban are measured with Anti-Factor Xa chromogenic assays, either with the kinetic method developed for LMWH, or with 2-stage assays better fitting the mode of action of DiXaIs [22,25–27]. Global assays, such as PT or APTT, are not recommended for evaluating DOACs concentration in plasma, as they are too variable and not accurate enough due to the many interferences possible. Dedicated calibrations are required for each drug, using drug specific calibrators obtained by adding the active drug to plasma which is then distributed and lyophilized in vials. For both DTI and DiXaIs, laboratory methods allow exploration from the low range (<30 ng/ml) to the high range (>500 ng/ml). Low range protocols can be used when a higher detection sensitivity is needed, and concentrations down to 5 ng/ml can be accurately measured. All these assays are automatable and reliable for measuring the drug anti-thrombin or anti-Factor Xa activity and they give comparable concentrations to those measured with LC:MS/MS (Liquid Chromatography, Mass Spectrometry), the usual reference method used for drug measurement during drug validation and initial clinical trials. As already indicated, there is usually no need to measure DOACs concentration in treated patients except in some circumstances which can be more frequently present in patients with malignancies. Among these indications the first one is to check compliance with the protocol (measured drug concentration at peak or at the trough must comply with the expected concentration ranges in plasma). Measurement of DOACS plasma concentration is also needed in the presence of recurrent thrombosis or in the presence of bleeding to verify if these complications can be related to unexpected kinetics of a drug. In the presence of bleeding, the possibility of overdose (which can be due to excessive intake) must be explored.

In cancer patients, organ failure can occur due to disease evolution or because of therapies. DOACs clearance is impacted in the presence of liver failure (mainly for rivaroxaban and apixaban), or of impaired kidney function (mainly for dabigatran and edoxaban). Drug dose must then be adjusted to the bleeding risk in treated patients or another therapeutic protocol can be requested. However, experience shows that the most frequent need to measure DOACs is when surgery is needed in an emergency and DOACs cannot be discontinued in enough time before surgery to avoid bleeding. Measurement of the plasma concentration is helpful to define the appropriate time when surgery can be undertaken safely. This approach is now greatly improved with the availability of antidotes for dabigatran or DiXaIs. Dabigatran is reversed by Praxbind®, a monoclonal antibody fragment (idarucizumab) with a high affinity for dabigatran and DiXaIs can be neutralized by Adexanet alfa, a recombinant human factor Xa with the active site mutated and inactive and with the phospholipid membrane binding domain deleted, or by ciraparantag (PER 977), a cationic small molecule which binds to all DOACs and neutralizes their activity [48]. Laboratory tools for the measurement of DOACs' concentration in plasma of treated patients help to render safer this novel anticoagulant treatment (which offers a better practicability and acceptance than LMWH as administration is oral), thanks to a better management of associated risk and a reduction of bleeding events compared to VKAs (an oral anticoagulant).

4.4. Antiaggregant treatment & fibrinolytic therapy

although not frequent, antiaggregant therapy can be used for some patients, and frequently involves aspirin or other antiaggregant drugs such as clopidogrel, and remains associated with other anticoagulant therapies. In some reports clopidogrel has been used, but it seems associated to an increased risk of bleeding complications [43,47]. The most recent guidelines, however, tend to prefer the use of LMWH for cancer patients as reported in the ASCO guideline update [11]. Laboratory monitoring can be performed with the current aggregometry tests. Concerning fibrinolytic therapy, it can be used on a case by case basis when rapid clot dissolution is needed and possible. No specific laboratory monitoring is required but, eventually, consumption of alpha 2- anti-plasmin (A2AP) or of plasminogen can be checked through the chromogenic measurement of activities for these factors. A global fibrinolytic capacity assay could be useful for the measurement of the body's potential to lyse clots. It can help to identify and evaluate the level of hyperfibrinolysis when present, or more frequently that of hypofibrinolysis, which could result from an increased PAI-1 concentration in patients with cancer, associated with hypercoagulability induced, more particularly, by TF and EVs.

5. Measurement and significance of blood activation markers

The presence of cancer, its treatments, its progression and its evolution have a strong impact on blood circulation and its coagulolytic balance. There are multiple factors which can favor blood clot formation in these patients: tumor infiltration into vessels, release of pro-coagulants by tumor cells (tumor EVs, TF), vessel obstruction, angiogenesis, surgery, medications and chemotherapy. All the components of the "Virchow's triad" can be affected: altered blood vessel wall; altered blood flow and venous stasis and an increase in blood coagulability. Patients with cancer have a 4 to 5 higher probability to develop thrombosis than the general population. The presence of pro-coagulant activity induces release of blood activation markers in the circulation. They can be derived directly from blood cell activation (mainly platelets and endothelial cells), or from tumor cells, leading to the presence of activated cells (which expose specific cell surface antigens) or to the generation of EVS or cell debris or release of cell specific proteins (as, for example, platelet Factor 4 or beta-thromboglobulin released by activated platelets). In addition, blood activation markers can be the result of the coagulation cascade activation and can effect the various steps of this process: thrombin generation, fibrinformation or fibrinolysis. Among these markers are activation peptides (prothrombin F1 + 2, fibrinopetide A, etc...), enzyme-inhibitor complexes (Thrombin-Antithrombin/TAT, Plasmin-A2AP/PAP, etc...), fibrin monomers, and fibrinogen or fibrin degradation products. DDimer, derived from cross-linked fibrin, is the most widely used marker as an indicator of thrombus formation (due to the dual presence of both thrombin and plasmin activities during fibrinformation) and its degradation (as a representative of cross-linked fibrin lysis). While EVs have a promising future as diagnostic parameters, they are still of limited laboratory use due to the lack of sufficiently friendly methods which are validated for clinical application and widely available. Methods used for measurement of EVs can be designed for identifying their cell origin. Evaluation of EV activity allows detection of their deleterious activities when present in the circulation through their pro-coagulant and pro-inflammatory effect. EVs are both consequences of disease and causes for their worsening. Measuring EVs in blood permits evaluation of blood cell activity and involvement in the thrombotic process. This investigation can be completed by the identification of activated cells thanks to the measurement of cell surface proteins, by using flow cytometry. Although this method is still mainly used for research applications, simplification of cytometers, measurement technology and cost reduction of instruments will make this technology available to point of care testing, or to intensive care units or emer-

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gency services. For plasma coagulation activation markers, most of them have very short half-lives (a few minutes), due to their rapid clearance. They are measurable only when "active blood activation" is present in the body at the time of sample collection. They are then mainly useful during the active stages of disease and they tend to decrease during the silent phase. DDimer, which is a mixture of fibrin degradation products exposing the "so-called" DDimer epitope (only present on fibrin derived products, but not on fibrinogen), is the only marker with a lasting presence in blood. This results from its prolonged and delayed generation (during fibrin-formation, then, clot lysis), and from its long half-life in the circulation, with an average of about 6 h from generation. Its plasma concentration is then fully representative of the disease state. DDimer concentration is also responsive to the efficacy of anticoagulant therapy, and its concentration decreases when the hypercoagulabity state is under therapeutic control with anticoagulant treatment.

Various laboratory tools can be used for the measurement of blood activation markers. Methods available for EVs can involve capture based assays or flow cytometry and other research methods are also available. Activated cells exposing specific surface proteins can be measured with flow cytometry. This technique is also useful for EVs, and can combine their detection by size and cell origin characteristics. However, the method is not sensitive enough for the small or very small EVs (below 250–400 nM, according to flow cytometer used and expertise of operator), which represent a vast population of EVs, with an important procoagulant surface originated from platelets. Conversely, capture based assays can measure these EVs. The various blood activation markers generated during the coagulation cascade and fibrinolysis activation are measured with immunoassays as they carry no specific activity. Most of them (F1 + 2, TAT, PAP, FPA, etc. . .) are present at low or very low concentrations and only highly sensitive immunoassays can be used. As their measurement remains a research application, they are mainly measured with two site ELISAs (designed with specific polyclonal or monoclonal antibodies) or competitive methods (in the case of FPA). But other high sensitivity immunoassays are possible such as the solid phase fluorescent assay, or chemiluminescent techniques and magnetic latex particles. DDimer is the major marker routinely tested for the diagnosis and follow-up of thromboembolic diseases. In clinical laboratories DDimer is measured in citrated plasma with latex automated assays, and the clinical cut-off value is 0.500 μg/ml for most of the assays which express DDimer as fibrinogen equivalent (i.e. the amount of fibrinogen clotted and lysed which produces the measured amount of DDimer). Automation is very helpful, as DDimer is now among the most tested coagulation markers. It is important to keep in mind the standardization method used for DDimer measurement as some assays express DDimer for its specific protein content, which represents about 50% of the lysed fibrin. The clinical cut-off value is then 250 ng/mL, and concentrations in patients are then about half of those measured when DDimer is expressed as fibrinogen equivalent. For all latex automated assays it is of the utmost importance to check that the clinical cut-off concentration can be accurately and reliably measured as it is at the low end of the assay range. To reach this objective, the analytical Lower Limit of Quantitation (LOQ) of the automated photometric latex assays used for DDimer must be <0.100 μg/mL, when DDimer is reported as fibrinogen equivalent. A "cassette Elisa" monotest, using a capture coated pin for catching the tested DDimer in assayed plasma is available (Vidas® DDimer from BioMérieux, France). It is pre-calibrated for each lot and is very useful when an individual patient must be tested within a short time frame out of a series. Other laboratory techniques are available or under development and offer a better sensitivity/specificity ratio in the low range to improve the assay usefulness. Historically, DDimer was used to rule out thrombosis when its concentration is below the clinical cut-off value. Some studies have reported the usefulness of DDimer for its positive predictive value for cardiovascular and thromboembolic risk or for monitoring the efficacy of anticoagulant therapy and helping to define its needed duration in each patient.

6. Control of thrombosis in cancer patients

There is an important interaction between cancer, angiogenesis, and thrombosis: tumor growth and its evolution contribute to blood cell and coagulation activation. Conversely hemostasis and fibrinolysis contribute to angiogenesis, cancer progression and favor metastasis. This context explains the higher incidence of thrombosis in patients with malignant diseases, as compared with the general population (odds ratio of about 5), and the association of occult cancer diagnosis in patients with unprovoked thrombo-embolic events. The high incidence of thrombotic and cardiovascular diseases in patients with cancer also explains the generation of activated cells in the circulation, the presence of the various blood activation factors, and the high prevalence of these indicators in this pathology. EVs have special implication in the pathological mechanism, as they originate from blood or tumoral cells and because they carry procoagulant activities, which can be disseminated in the circulation. They can contribute to disease evolution and complications. Thrombosis in cancer patients is characterized by the high incidence of recurrence, which is more frequent than in other clinical contexts, and by the association with bleeding episodes. In addition, in this pathology there is a tendency to develop multiple circulatory complications, partly due to neovascularization and to damaged vasculature, especially at the vicinity of the tumor. Anticoagulant treatment or transfusion are then useful for controlling the acute phase and managing clinical risk. UFH is used when vessel obstruction occurs as, in addition to its good anticoagulant efficiency, UFH also has important anti-inflammatory and anti-tumor activity which is of great benefit to cancer patients. Long-term antithrombotic therapy is now frequently used for prevention of recurrence. Protocols are adapted to the hemostatic risk in malignancy as documented by clinical studies and scientific societies publish recommendations. VKAs (dicumarol, warfarin) tend to be less often recommended than LMWH, because the recurrence rate for thrombosis and bleeding remains higher. Therapy durations can be variable according to patient risk and tumor type. Proposed protocols can range from a few months to >1year, or even lifelong, reduce the recurrence of thromboembolic events, and contribute to prolonged life expectancy by reducing the incidence of mortality associated with thrombotic complications. Recently, with the introduction of DOACs, new therapeutic strategies have become available, and can substitute for existing ones because of their ease of use and oral intake while providing a similar protective effect for treated patients with a lower associated incidence of bleeding.

Due to the multifactorial alterations in patients with malignancies, a personalized follow-up and specific drug dosage adjustment is often necessary. Laboratory assays contribute to the detection of any abnormal behavior or drug kinetics in anticoagulated patients, especially when the organs involved in drug clearance are affected and the drug can accumulate in the blood. As a complementary investigation, molecular markers of blood activation provide information on the existing pro-thrombotic activity in patients, and on the efficacy of therapy. DDimer, the most popular marker of thrombosis and in-vivo clot degradation, have been proposed as useful indicators for determining the duration of anticoagulant therapy in patients with thrombosis. Therapy discontinuation, when possible, is safe enough when the DDimer concentration is normalized, while treatment is recommended to be maintained in patients with elevated DDimer concentrations [30,34,35,37]. Many studies support the value of DDimer in that case. On the other hand, measurement

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of EVs and/or activated blood cells is very promising as a tool to detect hemostatic abnormalities early, before any clinical event occurs, and allows initiation of an antithrombotic treatment early in the disease course. This approach is still in the research field and not yet well enough documented for routine use in therapeutic protocols. No doubt this will be a strong contribution to the prevention and treatment of these complications.

7. Discussion and conclusions

Patients with malignant diseases can benefit from anticoagulant therapy which prevents and reduces the recurrence risk of thromboembolic or cardiovascular events and of subsequent bleeding episodes. It becomes an important adjuvant treatment in many patients with a high risk of thrombosis. The elevated recurrence rate of disease characterizes these patients, and thrombosis is frequently associated with or followed by bleeding events (especially during anticoagulant therapy). These complications are dependent on cancer type and stage of evolution [49,50]. Risk evaluation becomes possible thanks to the measurement of activated blood cells and plasma activation markers in the circulation and various recent or emergent technologies are available. Few methods offer the expected performances for individual testing in emergency situations or in intensive care units, at the time where the complication occurs, but new approaches are being introduced for addressing these needs. In many cases, the usual laboratory techniques, using automation on instruments, are convenient for following disease evolution and assessing the pathological procoagulant risk. Curative and preventive anticoagulant therapies are frequent in patients with cancer. Treatments can be initiated when a thromboembolic or a cardiovascular disease occurs for curative applications, and they can be maintained long-term in patients with a high risk. Anticoagulation can be initiated for preventive indications in patients with a high thrombotic risk cancer, and with individual evidence of coagulation activation, although there is no recommendation to systematically treat patients with malignancies. Molecular markers can allow a thrombosis risk profiling of these patients, and can help in selecting those who could benefit from this therapeutic preventive approach. UFH remains the major drug to use in the acute phase of the disease, and is followed using VKAs, but experience and guidelines tend to recommend the use of LMWH for long-term prevention. LMWH has been demonstrated to offer a higher efficacy than VKAs in treated patients with malignancies as it is associated with a significantly lower rate of thrombosis and recurrent bleeding episodes. More recently, DOACs have also been proposed for prevention but clinical experience is still less than with LMWH and fewer studies are available. In any case, therapeutic monitoring is useful and required in the presence of recurrence and unexpected behavior. Cancer patients have a coagulolytic balance with a higher propensity to be disturbed than in other patients so close surveillance helps to adjust to the most appropriate treatment protocol. Assays are mandatory for adjusting VKA therapy based on the right INR for UFH and remain of high usefulness for monitoring LMWH and controlling the risk associated with DOACs. Well standardized and reliable laboratory methods are available for these various applications: PT reagents with human TF and synthetic phospholipids for VKAs; highly performing Anti-FXa methods for UFH and LMWH (which permit measuring safely any heparin molecule when the hybrid calibration curve is used); anti-thrombin methods for DTI; and Anti-FXa methods for DiXaIs. In all cases, a drug specific calibrator is needed, except for UFH and LMWH which can be measured with a hybrid calibration curve. These assays can be completed with the measurement of markers that provide patient information by giving indications on the antithrombotic risk remaining during therapy. They allow adjusting, when possible, the treatment duration. DDimer has demonstrated a significant usefulness for this application. Laboratory tools are then highly useful companion diagnostics for antithrombotic treatments and are of special relevance in patients with malignant diseases, where an important interaction is present between tumor evolution and the circulatory system. In conclusion, diagnostic assays for activated blood cells and plasma activation markers are very useful for the early detection of a coagulolytic imbalance in cancer patients which exposes them to a higher risk of developing thrombotic diseases. Anticoagulant treatment is now becoming a necessary complementary therapy in many cases of malignancy where there is development or high risk of thromboembolic and cardiovascular disease, and drug monitoring is of special relevance in this clinical context to limit recurrence of both thrombosis and bleeding.

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