Study of the underlying mechanism of the hypercoagulable state in acute leukaemia and impact of specific treatments on the thrombotic risk

Dissertation présentée par
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En vue de l'obtention du grade de
Docteur en Sciences Biomédicales
Et Pharmaceutiques

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Acknowledgements

Mener à bien une recherche scientifique représente une quantité de travail importante qui ne peut pas être assumée par une seule personne en quatre ans. Ce travail est le fruit d'une collaboration étroite avec un grand nombre de personnes et de services. C'est aussi le produit de plusieurs investissements personnels.

Je voudrais d'abord reconnaître l'opportunité. L'opportunité d'être arrivé dans une telle équipe de chercheurs. Ce fut une très belle expérience d'avoir eu comme doctorant, lors de mon mémoire, François Mullier qui m'a fait confiance, m'a responsabilisé, m'a appris à faire de la recherche et m'a introduit auprès des personnes du CHU Dinant Godinne. Merci à lui pour m'avoir offert l'occasion de faire une thèse. Merci à lui pour son suivi lors de la réalisation de celle-ci et ses nombreuses corrections d'articles et autres travaux. Merci de m'avoir permis de collaborer avec le Professeur Alain Brisson, de l'Université de Bordeaux, et Philippe Poncelet, de chez Biocytex-Stago, pour l'étude des microparticules, mais aussi avec le Dr Nicolas Mardyla pour l'étude sur le L-PRF.

Pour mener à bien un tel travail, il faut aussi un brin de chance. Et j'ai eu la chance d'avoir eu d'excellents encadrants à commencer par mon promoteur le Professeur Christian Chatelain. Chercheur et clinicien passionné, enthousiaste, toujours curieux et qui sait transmettre sa passion pour la recherche et donner l'envie de persévérer dans les moments difficiles. Ce fut toujours un grand plaisir de discuter des résultats obtenus avec le Professeur Christian Chatelain et de voir l'enthousiasme suscité par de nouveaux résultats, un enthousiasme au combien communicatif.

J'ai aussi eu la chance d'avoir comme co-promoteur le Professeur Jean-Michel Dogné qui voit le meilleur chez les gens et qui pousse à le donner. Merci à lui pour tous ses conseils avisés sur le plan scientifique, technique et communicationnel, ses nombreuses corrections de manuscrits et de présentations. Merci à lui également pour sa passion pour la science, pour toutes ses connaissances en thrombose hémostase et pour ses qualités relationnelles.

J’ai également eu l’honneur d’être suivi par le Professeur Bernard Chatelain qui, grâce à ses connaissances scientifiques techniques et sa passion pour l’hématologie et l’hémostase, a apporté beaucoup à la réalisation de ce travail. Il m’a aussi permis avec le Professeur François Mullier de m’intéresser à la thrombose induite par l’héparine et à l’analyse morphologique des plaquettes en microscopie électronique.

Je souhaite remercier également l’équipe de la biobanque du CHU Dinant Godinne, le Dr Fabienne George, Mlle Laure Finet et le Professeur Carlos Graux, sans qui la récolte de plasma de patients n’aurait pas pu se réaliser. Cette équipe a toujours montré un grand dynamisme pour que cette recherche se poursuive dans les meilleures conditions pré-analytiques. Cette structure peut aussi s’appuyer sur la collaboration d’excellents médecins tels que le Professeur Chantal Doyen, le Dr Anne Sonet, le Dr Bérangère Devalet, le Dr Valérie Mathieux.

Merci aux membres actuels et anciens du Département de Pharmacie de l’UNamur, le Professeur Bernard Masereel, le Dr Lionel Pochet, le Professeur Raphaël Frederick, Mr Philipe Devel, Mr Jonathan Douxfils, le Dr Séverine Robert, le Dr Julie Laloy, Mlle Lutfie Alpan, Mlle Christelle Vancraeynest, Mlle Céline Bouvy, Mlle Valentine Minet et Mlle Julie Hardij, le Dr Emilie Varin et le Dr Stéphanie Rolin pour leur disponibilité, leur motivation, leur enthousiasme scientifique et pour les moments de complicité en dehors du travail. Un merci particulier à Mr Philippe Devel qui m’a appris la culture cellulaire, et au Dr Séverine Robert qui m’a initié à la technique de la génération de thrombine.

Merci aux membres du laboratoire d’hématologie du CHU Dinant-Godinne, Mr Nicolas Bailly, Mr Nicolas Neyman, Mr Alex Dromelet, Mr Sébastien Walbrecq, Mme Nicole Meunier et feu Mr Yvan Cornet pour leur disponibilité, le temps qu’ils m’ont consacré et pour les moments d’amitié.

Merci au Dr Renaud Vigneron, Mr David Degleize et Mr Corry Charlier de m’avoir accompagné dans l’apprentissage des techniques de microscopie électronique.

Merci aussi au Professeur Alain Brisson et son équipe pour m’avoir fait découvrir de la plus belle des manières la Cryo Microscopie Electronique.

Merci aux patients qui ont accepté, malgré l’annonce d’une telle maladie, de donner leur sang pour la recherche.

Je souhaiterais également remercier cette formidable association qu'est le TELEVIE et tous ses membres, ses bénévoles et donateurs. Merci à tous ces gens qui travaillent sans compter pour financer la recherche scientifique. Merci pour leur enthousiasme à soutenir la recherche. C'est grâce à eux que ce genre de travail peut être réalisé.

Je finirai les remerciements par mon entourage personnel sans qui tout cela n'aurait pas été possible. Ma compagne Hélène Wolak qui m'a toujours soutenu moralement, m'a donné confiance en moi, m'a encouragé, m'a aussi aidé dans différentes tâches dès qu'elle le pouvait. Merci aussi à ma famille, mes parents Claire et Robert qui m'ont permis de faire des études et qui m'ont aussi toujours soutenu et tiré vers le haut. Merci à mes frères, Ludovic et David, qui m'ont ouvert la voie, et particulièrement à ma sœur Céline, qui m'a beaucoup aidé pendant ces blocus interminables via ses explications, son soutien et ses cours.

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List of abbreviations

Ab:	Antibody
AL:	Acute Leukaemia
ALL:	Acute Lymphoblastic Leukaemia
AML:	Acute Myeloid Leukaemia
AnV:	Annexin V
APL:	Acute Promyeolocytic Leukaemia
ATE:	Arterial Thromboembolism
ATRA:	All *Trans* Retinoic Acid
BFU-E:	Blast Forming Units Erythrocyte
CFUs:	Colony-Forming Units
CLP:	Common Lymphoid Progenitor cell
CMP:	Common Myeloid Progenitor cell
DIC:	Disseminated Intravascular Coagulation
DNR:	Daunorubicin
ELISA:	Enzyme linked Immunosorbent Assay
ETP:	Endogen Thrombin Patential
EVs:	Extracellular Vesicles
F:	Coagulation serine protease
FAB:	French-American-British
FCM:	Flow Cytometry
Filt:	Filtrate
FITC:	Fluorescein IsoThioCyanate
GMP:	Granulocyte Monocyte Progenitor cell
Gs:	Granulocytes
LTHSC:	Long-Term Repopulating Hematopoietic Stem Cell
mAbs:	Monoclonal Antibodies
MDA:	MDA-MB-231 breast cancer cells
MEP:	Megakaryocyte-Erythroid Progenitor cell
MKs:	MegaKaryocytes
MPP:	MultiPotent Progenitor cell
MPs:	Microparticles
Ms:	Macrophage

MUC-1:	MUCin-1
MVs:	Microvesicles
NPP:	Normal Pooled Plasma
PBS:	Phosphate Buffer Saline
PCA:	ProCoagulant Activity
PE:	PhycoErythrin
PS:	PhosphotidylSerine
RBC:	Red Blood Cell
SD:	Standard Deviation
STHSC:	Short-Term Repopulating Hematopoietic Stem Cell
Sup:	Supernatant
TEM:	Transmission Electron Microscopy
TF:	Tissue Factor
TFPI:	Tissue Factor Pathway Inhibitor
TGA:	Thrombin Generation Assay
UC:	UltraCentrifugation
VTE:	Venous Thromboembolism

1. Introduction

1 Introduction

The production of new blood cells is ensured by pluripotent hematopoietic stem cells, which reside in specialized niches in the bone marrow (Wilson and Trumpp 2006). Haematopoietic stem cells have the ability to self-renew via asymmetrical cell division, in which a new stem cell and a hematopoietic progenitor cell are generated. This self-renewal ensures the number of stem cell (He, Nakada et al. 2009). While all haematopoietic stem cells divide via asymmetrical cell division, the frequency of these cell divisions varies. In this respect, the most primitive hematopoietic stem cells divide only rarely, whereas other stem cells divide more frequently (Zhang and Wang 2008). The formation of mature and functional blood cells by the stem cell occurs via several consecutive cell divisions and maturation stages (**Figure 1**). The initial asymmetrical cell division is followed by several symmetrical cell divisions, during which two identical daughter cells are generated. These newly formed cells are more differentiated than their parental cells and become increasingly committed to either the myeloid or the lymphoid lineage. In the myeloid lineage of haematopoiesis erythrocytes, platelets and some white blood cell types, such as monocytes and granulocytes are formed. In the lymphoid lineage, T cells, B cells and natural killer cells are generated. It may be obvious that this intricate process of haematopoiesis is highly regulated by complex signals from the microenvironment to ensure the proper generation of sufficient blood cells. Aberrations in cell division and differentiation can severely disturb normal haematopoiesis and even result in the onset of leukaemia.

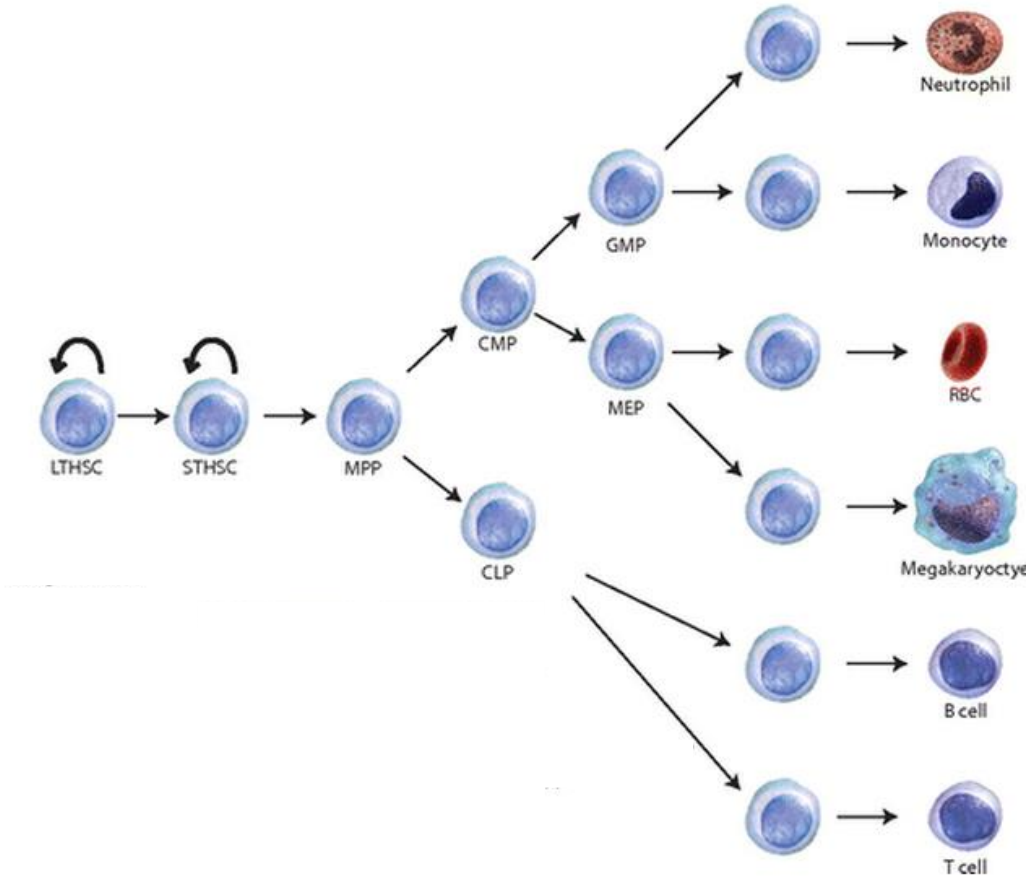

Figure 1: Overview of haematopoiesis. LTHSC, Long-term repopulating hematopoietic stem cell; STHSC, short-term repopulating hematopoietic stem cell; MPP, multipotent progenitor cell; CMP, common myeloid progenitor cell; MEP, megakaryocyte-erythroid progenitor cell; GMP, granulocyte monocyte progenitor cell; CLP, common lymphoid progenitor cell; RBC, red blood cell. Adapted from (Chute, Ross et al. 2010)

1.1 Leukaemia

In case of leukaemia, aberrations are often the result of inherited or acquired genetic alterations (Rowley 1998). Acute leukaemias are rare diseases, but have a disproportionally large effect on cancer survival statistics (Deschler and Lubbert 2006). The incidence is approximately 10 cases/100000/years. Even if leukaemias represent about 2% of all cancers, they constitute the leading cause of death due to cancer in children and persons aged less than 39 years (Greenlee, Hill-Harmon et al. 2001). The underlying mechanisms and factors leading to developing leukaemia in most patients are unknown. Several factors are associated with increased risk of developing the disease. The risk of developing haematological disorders such as leukaemia increases steadily with age (Hiddemann, Kern et al. 1999).

1.1.1 Definition of leukaemia

Leukaemia is a neoplastic disorder originated from a single haematopoietic cell. This change in its genetic making gives the capacity to proliferate independently of growth signals and to escape apoptosis. It leads to uncontrolled proliferation of hematopoietic cells in the bone marrow blood and other tissues.

A number of different leukaemias are classified according to the course of the disease and the predominant type of white blood cell involved (Walter, Othus et al. 2013).

1.1.2 Classification of leukaemia

The degree of maturity and aggressiveness of the leukemic cell population allows to qualify chronic and acute leukaemias. Leukaemias are also defined as either myelogenous or lymphocytic based on the cell type origin. These characteristics are used to designate almost all cases as one of four types—acute myelogenous, acute lymphocytic, chronic myelogenous, and chronic lymphocytic leukaemias.

Chronic leukaemias also occur more frequently in adults. These are characterized by a more gradual onset and a more protracted course. Chronic myelogenous leukaemia, which has a peak incidence among adults in their 40s, may remain quiescent for long periods before symptoms such as weight loss, low fever, and weakness develop. Before bone marrow transplantation and more recently the "Imib" drugs, the median survival was about 3 years. Chronic lymphocytic leukaemia occurs primarily in elderly people and may be inactive for months or years. The leukaemia itself is rarely the cause of death, but it renders the patient vulnerable to infection or haemorrhage.

The acute leukaemias (AL) affect immature cells by a blockage of differentiation during the haematopoiesis and a large increase in the numbers of immature blood cells primarily in the bone marrow. These leukaemic blasts can enter the circulation and spread throughout the body (Porcu, Cripe et al. 2000). The subsequent accumulation of cells at various stages of incomplete maturation reduces the production of healthy haemopoietic elements which cause cytopenias. The disease develops rapidly, with symptoms including anaemia, sever infections, haemostatic disorders (Colombo, Gallipoli et al. 2014), and sometimes swelling of the lymph nodes. Because acute leukaemia leads to haemostatic disorders such as haemorrhage, thrombosis or disseminated intravascular coagulation (DIC), we focused our research on acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL).

1.1.3 Acute myeloid Leukaemia

Acute myeloid leukaemias are characterized by an increase in the number of immature myeloid cells in the marrow. This results in anaemia (fatigue and dyspnea), neutropenia (infections) and thrombocytopenia (haemorrhage) (Ferrara and Schiffer 2013). Acute myeloid leukaemias are divided in eight subtypes M0 through M7, all are recognized following the French-American-British (FAB) classification (Walter, Othus et al. 2013). These subtypes are characterized by degrees of granulocytic differentiation (M1 – M3) and by the degrees of monocytic maturation (M4 and M5). The subtype M6 and M7

are characterized by the presence of erythroblasts and megakaryoblasts, respectively. The subtype M0 affects the myeloid precursor with minimal differentiation **(Figure 2)** (Venditti, Del Poeta et al. 1997).

AML can occur in people of all ages. It is the most common leukaemia in adults, and has the lowest survival rate among acute leukaemias. The prognosis is very poor for older patients. (Redaelli, Lee et al. 2003) (Greenlee, Hill-Harmon et al. 2001). AML is primarily a disease of later adulthood. 42.8% of leukaemia patient are at age >65 years. Age is one of the strongest independent predictors of prognosis in AML. The overall survival at 48 months of intensively treated patients with AML is 40 – 45 % for patient younger than 65 years and 15 – 20 % for patient older than 65 years (Ferrara and Schiffer 2013). Patient younger than 40 years have 75% to achieve a complete remission compared to 68% of patient between 40 and 65 years and 47% of patient older than 65 years (Mayer, Davis et al. 1994).

Patients previously treated by chemotherapy can develop AML, known as secondary AML or therapy related AML. Several drugs such as anthracyclines or epipodophyllotoxins, targeting topoisomerase II, can lead to the development of the disease with monocytic abnormalities such as translocations involving chromosome bands 11q23 or 21q22 (Godley and Larson 2008), and proliferation within months to 2 years of treatment (Pollyea, Kohrt et al. 2011). Alkylating agents such as melphalan or cyclophosphamide can induce disease with a dose-response relationship. This form of treatment related-myelodysplasic syndrome or treatment related AML typically occurs within 5–7 years after chemotherapy and/or radiotherapy have been given, and confers a poor prognosis (Curtis, Boice et al. 1992). The prognosis for AML related to therapy is generally unfavourable compared to *de novo* AML.

Ionizing radiation is another factor that influences the risk of developing AML. Studies on the Japanese A-bomb survivors revealed that leukaemia, primarily AML, exhibits the highest excess relative risk of all neoplasms (Preston, Kusumi et al. 1994). The same conclusions are drawn from a study on clean-up workers at the Chernobyl Nuclear Power Plant (Ivanov, Tsyb et

al. 1997). Although the risk associated with exposure to lower level radiation is not clear, studies have shown an increase in leukaemia following the use of radiotherapy. These AMLs induced by radiation were characterized by an unfavourable clinical course with resistance to conventional therapy, a shorter overall survival and a lower complete remission rate as compared to de novo case (Klymenko, Smida et al. 2011).

Inherited syndromes can also influence the risk of developing AML. Thus, children with Down syndrome have a incidence of leukaemia (myeloid and lymphoblastic) 10- to 20-fold higher than that in the general population (Fong and Brodeur 1987). Other inherited syndromes such as ataxia-telangiectasia, Blomm syndrome, Fanconi syndrome increase the risk of leukaemia (Swaen 1996).

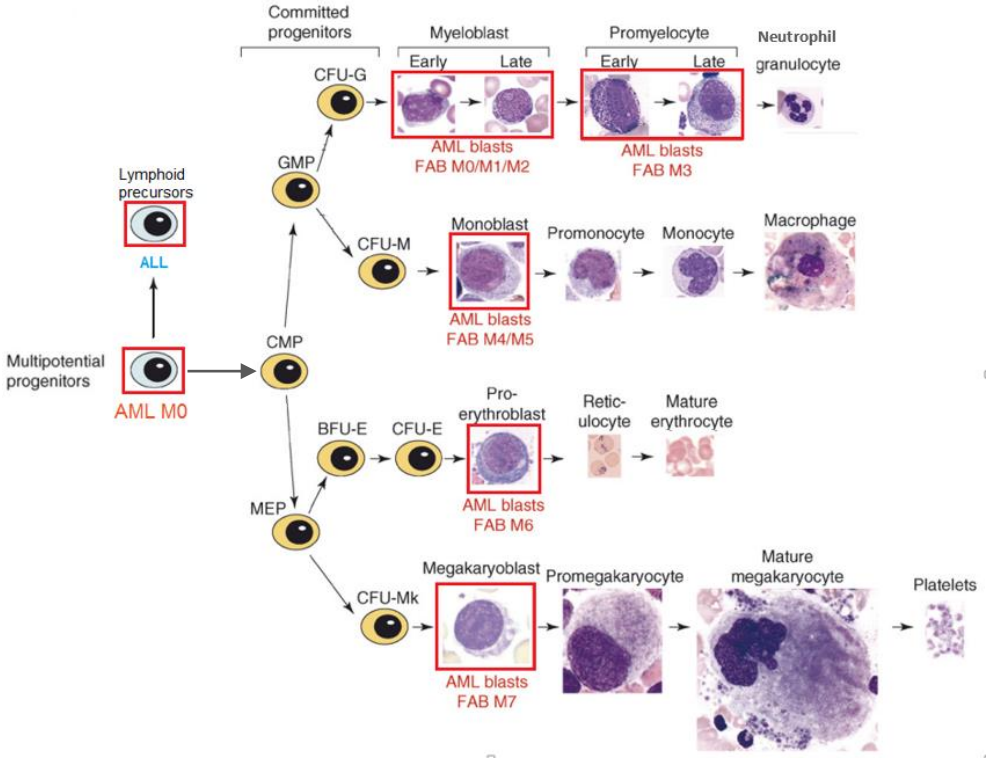

Figure 2: overview of haematopoiesis and progenitor cells maturation blockage affected by Acute Myeloid Leukaemia (AML) M0 to M7 according the FAB classification and Acute Lymphoid Leukaemia (ALL).Common Myeloid Progenitors (CMPs), Granulocyte-Macrophage Progenitors, Megakaryocyte-Erythroid Progenitors (MEPs), Colony-Forming Units (CFUs) for Granulocytes (CFU-Gs), macrophage (CFU-Ms) and megakaryocytes (CFU-MKs), Burst Forming Units Erythrocyte (BFU-E). Adapted from (Krause and Van Etten 2007)

1.1.4 Acute lymphoblastic leukaemia

Acute lymphocytic leukaemia affects lymphocyte precursor cells, or lymphoblasts that normally become one of three types of lymphocyte, B- and T- lymphocyte and natural killer. Leukemic lymphoblasts have an uncontrolled expansion, fail to mount a normal immune response and as AML cause a drop in production of normal bone marrow cells. The causes of ALL development are currently unknown. It appears that chromosomal alterations and mutations that are associated with the disease may be inherited from pregnancy or develop through infancy and childhood (Pendergrass 1985). These chromosomal aberrations can interact with some environmental exposures which can lead to some types of ALL (Pendergrass 1985). The precursors of T-cell and B-cell can give rise to ALL, however B-cell ALL represents about 88% of all cases. ALL represent less than 1% of adult cancers, and 25 % of all childhood cancers. It is more frequent in white than black and it affects more males than females (Abbasi, Maleha et al. 2013). ALL is also the most common form in children, 60% of cases occur in patients younger than 20 years (Stanulla and Schrappe 2009). ALL accounting for approximately 16% of all childhood leukaemia diagnoses and about 175-200 children are diagnosed with ALL each year in the Nordic countries (Norway, Denmark, Finland, Iceland and Sweden) (Gustafsson, Kreuger et al. 1998). The annual incidence rate in Europe and US is approximately 3.5 per 100,000 children younger than 15 years old. Before, this disease killed more than 90% of its victims within six months. With new drug therapies, more than 80% of children with ALL now achieve complete remission, with no evidence of malignant cells in the blood. With continued therapy, more than half remain free of disease for five years or longer. These patients are presumed to be cured.. Treatment related death for this disease is still 2-4% in childhood (Lund, Asberg et al. 2011, Rubnitz, Campbell et al. 2013). Infections, bleeding or thrombosis, tumour burden complications, and therapy induced organ toxicities are the most common causes of therapy related death.

1.1.5 Treatment of acute myeloid leukaemia

Conventional treatment for AML consists of two phases: the induction to achieve complete remission and consolidation to eliminate residual leukemic blasts after induction. Intensification with or without stem-cell transplantation may also be considered. The induction phase uses interlacing agents, such as daunorubicin or idarubicin, that wedge into the spaces between the nucleotides in the DNA (Kellogg, Scarsdale et al. 1998) (**Figure 3**) and cytarabin, a pyrimidine analogue that blocks the synthesis of DNA. The standard induction phase for adult consists of a combination of an anthracycline, usually daunorubicin (DNR), given for 3 days with continuous infusion of cytarabine for 7 days (3+7) (Hiddemann, Kern et al. 1999) (Ferrara and Schiffer 2013). The induction phase 3+7 induces a complete remission rate of 70% for patients younger than 60 years. The use of other anthracyclins such as idarubicin, hydrochloride and mitoxantrone, an anthracyclin-analog can improve the rate and the quality of complete remission (Arlin, Case et al. 1990). The addition of a third drug, usually etoposide, at high dose instead of cytarabine can also improve the complete remission rate. The induction 3+7 could be repeated if complete remission is not observed.

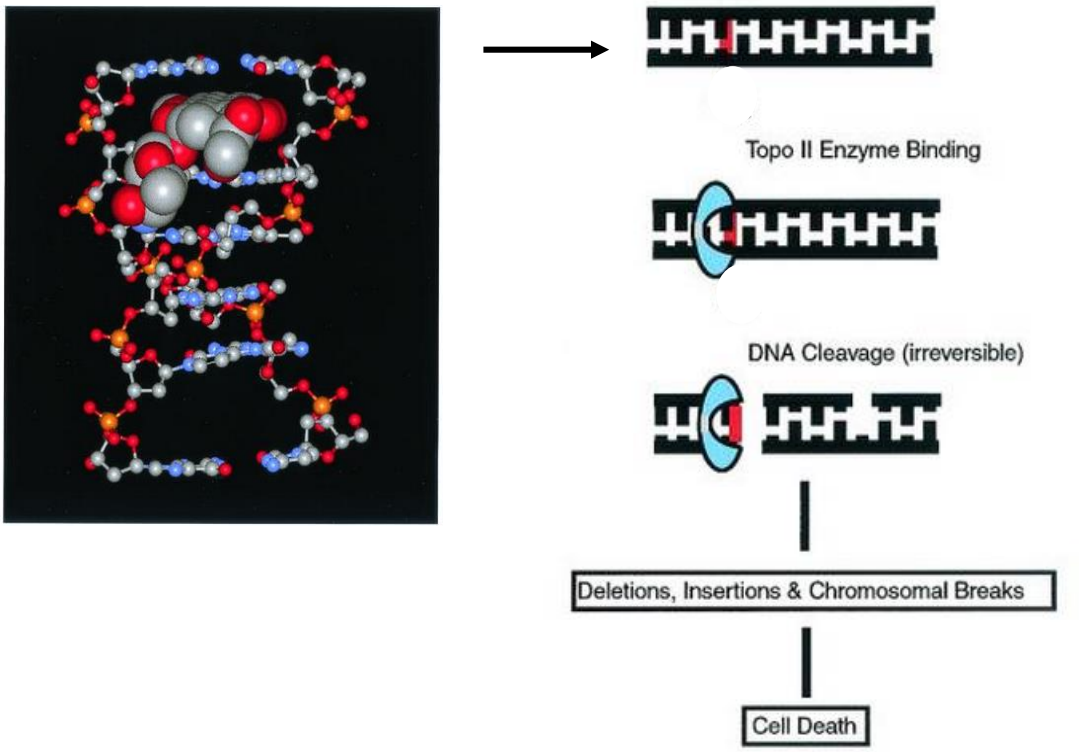

Figure 3: Crystal structure of anthracyclines-DNA complex and schematic involvement of drug in the dynamics of DNA cleavage by topoisomerase II. Adapted from (Qu, Wan et al. 2001)

The consolidation phase is essential to minimize further relapse after induction. A standard consolidation phase consists of several cycles of high dose or intermediate dose of cytarabin and/or complete radiation. For example, four cycles of high dose cytarabin treatment (3g/m2 every 12h over 3 days) give a survival advantage in patients up to 60 years old and decrease long term relapse. This survival advantage is more striking in patients with favourable cytogenetic (Arlin, Case et al. 1990, Wiernik, Banks et al. 1992).

With consolidation therapy at high dose, patients need hematopoietic stem cell transplantation either autologous or allogenic to repopulate the ablated bone marrow. Despite the complications of graft-versus-host disease, with a treatment mortality of 10-25% (Hiddemann, Kern et al. 1999), a number of studies suggest that long-term outcome is improved by allogeneic bone marrow transplantation during the first complete remission (Zittoun, Mandelli et al. 1995, Cassileth, Harrington et al. 1998). Moreover, the graft versus leukaemia effect could have an additional impact with high dose cytarabin. In older patients with untreated poor-prognosis leukaemia who can support high dose of cytarabin, a reduced treatment combined with allogenic stem cell transplantation sufficiently reduces the leukemic burden, resulting in a high complete remission through graft versus leukaemia effect (Bertz, Potthoff et al. 2003). For an increased rate of complete remission, induction, consolidation and stem-cell transplantation treatment are adapted for each patient. Major criterias such as the type of leukaemia, cytogenetic, chemo-resistance leukaemia, patient age and condition, the possibility of allogenic transplantation are used to define the appropriate treatment.

A distinct type of AL, acute promyeolocytic leukaemia (APL), was considered the most fatal type of acute leukaemia five decades ago and the treatment of APL was a nightmare for physicians due to lack of supportive care and cytotoxic-agent-related exacerbated coagulopathy. However, since 1970, APL benefit from all *trans* retinoic acid (ATRA) treatment. This vitamin A-derivate induces the differentiation of APL blasts and is able to induce complete remission. Experience has shown that the combination of ATRA and chemotherapy gives better survival in APL than chemotherapy alone (fewer relapses and higher complete remission) (Stein and Tallman

2014). Maintenance of ATRA treatment in combination with low-dose chemotherapy reduces the incidence of relapse. More than 90% of patients with newly diagnosed APL can achieve CR and about 75% can be cured by the combination of ATRA and chemotherapy (Zhou, Zhang et al. 2007, Sanz, Iacoboni et al. 2014).

1.1.6 Treatment of acute lymphoid leukaemia

Acute lymphoid leukaemia treatment usually stretches from 2 to 2.5 years. This includes three phases, remission induction, consolidation and maintenance (Pui, Robison et al. 2008). The induction therapy spans 4 to 6 weeks and is used to reach a complete remission and restores haematopoiesis in 96%-99% of children and 78-92% of adults with ALL. Standard regimen includes glucocorticoids, such as prednisone or dexamethasone as immunosuppressive agents, cytostatic drugs such as vincristine, and asparaginase with or without anthracycline. Recently, it was shown that patients with BCR-ABL1-positive ALL can benefit from tyrosine kinase inhibitors such as imatinib, nilotinib or dasatinib (Inaba, Greaves et al. 2013).

Consolidation therapy is normally more intense than induction. This phase eradicates residual leukemic blast with high-dose of chemotherapy such as methotrexate, an inhibitor of DNA synthesis, glucocorticoids and frequent pulses of vincristine (Inaba, Greaves et al. 2013). In addition to these drugs, patients receive asparaginase during 20-30 weeks uninterrupted. The consolidation therapy is a major phase of the protocols. The benefit of prolonged use intensive therapy with asparaginase and vincristin treatment has been demonstrated for patients with ALL (Pession, Valsecchi et al. 2005). Moreover, the intensive treatment improved the outcome of patient with high-risk disease (Marks, Wang et al. 2010).

After Consolidation therapy, patients need to have continuation therapy, which can stretch from 2 years or more. During this phase, patients received antimetabolic drugs, such as mercaptopurine (daily) and/or tioguanine that inhibit enzymes involved in purine metabolism (Schmiegelow, Glomstein et al. 1997) and methotrexate (weekly) that inhibit folic acid synthesis.

Methotrexate can be combined with pulses of vincristine and dexamethasone, an immunosuppressive drug.

As for AML treatment, the hematopoietic stem cell transplantation is frequently included in ALL treatment. Allogeneic hematopoietic stem cell transplantation has a major role in the management of adults with ALL in first or second complete remission. Recent large prospective donor versus no donor analyses showed that matched allografting from brother or sister yields superior outcomes compared with chemotherapy (Goldstone, Richards et al. 2008) and its use is recommended in the highest-risk patient. Allogeneic transplantation is still the treatment of choice for adults with ALL in second complete remission and for children with high risk and/or persistent disease where sibling donor allografts yield disease-free survival (Inaba, Greaves et al. 2013). Contemporary hematopoietic stem cell transplantation protocols with high resolution Human Leucocyte Antigen typing, case-based conditioning, and improved supportive care have reduced relapse-related mortality, regimen-related toxicity, and infection (Leung, Campana et al. 2011).

Chemotherapeutic drugs treatment and stem cell transplantation have markedly improved outcome of AL patients. However, there is a remaining mortality linked to treatment-related deaths, infection such as pneumonia or septicaemia and haemostatic complication. The treatment related death could be associated to adverse effects of chemotherapy such as cardio toxicity and secondary leukaemia of anthracycline or the graft versus-host disease. Even if the intensification of therapy results in an improved overall survival, it is associated with increased toxicity (Byrd, Mrozek et al. 2002). Infection is the major cause of morbidity and mortality in AL patients and it could be associated to neutropenia or to immunosuppression (Jagarlamudi, Kumar et al. 2000). However, in the early deaths related to AL, bleeding and thrombosis are major risk factors.

1.2 Haemostatic disorders associated with acute leukaemia

The development of acute leukaemia may be associated with various co-morbidities including abnormalities of haemostasis. Bleeding and thrombosis, such as haemorrhage, DIC and venous and arterial thromboembolism (VTE and ATE), occur frequently in patients with acute leukaemia (Falanga and Rickles 2003). The incidence of haemostatic complications varies according to the type of leukaemia and the phase of treatment. Patients with AML show a rate of haemorrhage of 9.9%, and 1% of lethal bleedings occurs during admission day (Ziegler, Sperr et al. 2005). Patients with ALL show a rate of non-fatal severe haemorrhages of 3.4%.

Although thromboembolic disease has been considered less characteristic than haemorrhage in patients with AL, several studies are in contradiction with this long-held wisdom (De Stefano, Sora et al. 2005, Ziegler, Sperr et al. 2005). The rates of thrombotic complications for AML and ALL range from 2.1% to 12.1%. At diagnosis days, 1.4% of ALL patients, 9.6% of AML M3, and 3.2% of others AML patients develop thrombotic disease (De Stefano, Sora et al. 2005). Among patients with acute myeloblastic leukaemia (AML), the incidence of VTE is 5.2%, and among patients with acute lymphoblastic leukaemia (ALL), the incidence of VTE is 4.5% in the first two years of disease (Ku, White et al. 2009).

Thrombotic disorders associated with ALL are strongly related to chemotherapy. Most of these events occur during remission induction therapy (De Stefano, Sora et al. 2005, Grace, Dahlberg et al. 2011). Particularly, L-asparaginase treatment is associated with an increased risk of developing VTE. ATE is also reported but the incidence is less frequent. Thrombosis in AML occurs mainly in the first 3 months and could be associated to central venous devices and older age. However, the underlying mechanisms of thromboembolisms in ALL or AML are not fully understood.

1.2.1 Physiopathology of thrombosis in acute leukaemia

Etiopathology of thrombosis in acute leukaemia is complex and multifactorial. Several factors are related to thromboembolism such as secondary infections, blood viscosity, hospitalization-related immobility, age, central venous device, chemotherapy side effects and prothrombotic factor produced by leukemic blasts (**Figure 4**).

1.2.1.1 Secondary infections

Neutropenia induced by proliferation of leukemic cells enable bacteria develop due to minor immunity response. Bacteria can activate residual monocytes, granulocytes and macrophages, which release various cytokines, nitric oxide, and tissue factor (TF). These have a direct impact on coagulation by the factors XII and VII (Levi 2010).

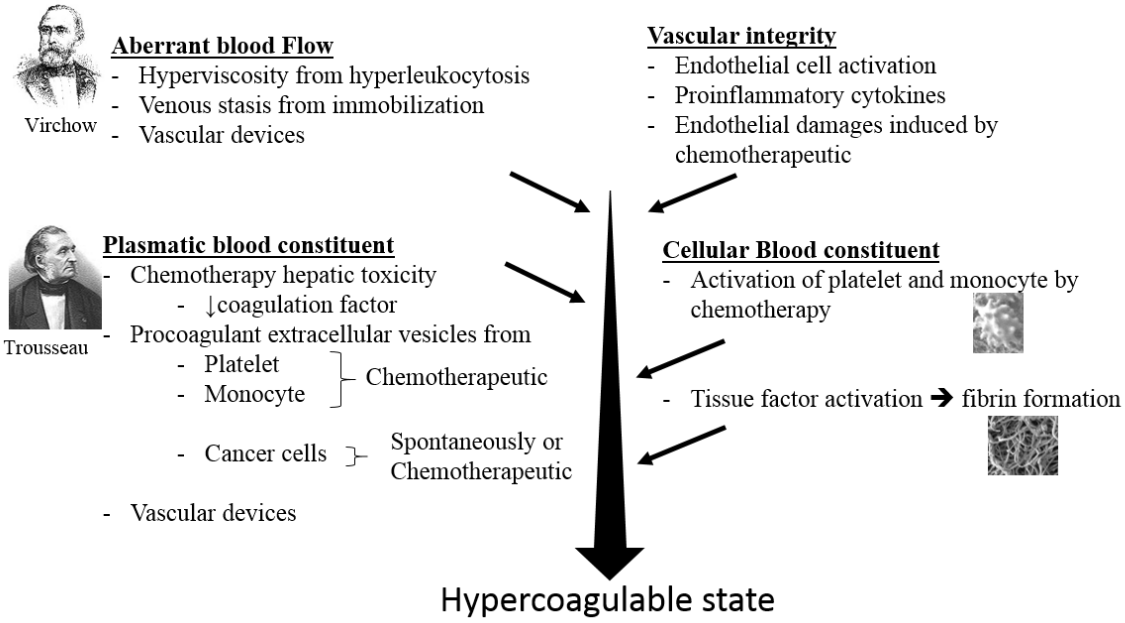

Figure 4. Hypercoagulable state in Leukaemia. Present concept of Virchow's triad (Kwaan 2007).

1.2.1.2 Hypercoagulable state

Hyperleukocytosis induced by AL creates rheological abnormalities that leads to slow blood flow and increases blood viscosity in the microcirculation. Leukostasis affects the function of brain, eyes, myocardium, lungs and kidneys. Microthrombi induced by hyperleukocytosis can spread to larger blood vessels and patient can develop venous or arterial thromboembolism. Hyperleukocytosis in AL is associated with a very high early mortality rate mostly due to respiratory failure or intracranial bleeding (Porcu, Cripe et al. 2000, Kwaan 2007). The hyper leukocytosis (peripheral blood blasts higher than 100,000/µl) complication is more frequent in AML than ALL.

Age and hospitalization-related immobility also impact on aberrant blood flow and lead to a hypercoagulable state. Several studies on thromboembolic disease in leukaemia indicate that central venous devices increases the incidence of thromboembolism (Freytes 2007). This complication remains an important limitation to the delivery of drugs and supportive care to cancer

patients. The pathogenesis of catheter-related thrombosis is multifactorial. The biocompatibility, the position of the catheter tip, the site of insertion, the puncture and thrombophilic abnormalities and catheters-related infections are often evoked (Cortelezzi, Moia et al. 2005). However, the vessel damages caused by mechanical injury play an important role in the physiopathology.

1.2.1.3 Chemotherapy effects on haemostasis

Chemotherapy increases the risk of developing thromboembolism. Several mechanisms are involved in thrombosis-related therapy.

Endothelial damages induced by drugs such as daunorubicin or cisplatin-based chemotherapy, affect coagulation by endothelial cell activation (Rickles, Falanga et al. 2007). These drugs enhance phosphatidyl serine exposure and the shedding of procoagulant extracellular vesicles (EVs). These products from activated endothelial cells directly affect the coagulation cascade and promote hypercoagulabe state (Lechner, Kollars et al. 2007, Fu, Zhou et al. 2010). ATRA can also effect vessel endothelium. ATRA increases the production of cytokines which also affect endothelial cells and induce prothrombotic function of endothelial cells (Gerber and Erdman 1981).

Some chemotherapeutic drugs such as L-asparaginase can also affect plasmatic coagulant and anticoagulant proteins by its hepatic toxicity. Synthesis of antithrombin, protein C, protein S and plasminogen is impaired by L-asparaginase treatment. Cessation of L-asparaginase therapy leads to an earlier recovery of the coagulant proteins than anticoagulant proteins. This affects the haemostatic balance and generates a hypercoagulable state (Mitchell, Sutor et al. 1995).

Membrane alteration by intercalating drugs such as cisplatin or daunorubicin induces monocyte/macrophage prothrombotic phenotype. This phenotype is due to the enhancement of TF activity already present on monocyte surface. (Walsh, Wheeler et al. 1992). Cisplatin also induces platelet activation and aggregation which contribute to vascular and thrombotic complication (Yen, Walsh et al. 1993).

Finally, the release of leukemic cell cytokines, cancer procoagulant molecules and TF in response to chemotherapy may also be important in increasing thrombotic risk (Falanga, Consonni et al. 1998).

1.2.1.4 Prothrombotic factor produced by leukemic cells

Leukemic cells are involved in hypercoagulable state induction by their molecular properties. Leukemic blasts can produce and/or express procoagulant and fibinolytic factors (Falanga and Rickles 2003, Tallman, Lefebvre et al. 2004). Several molecules such as cancer procoagulant cysteine protease and TF are found in ALL and AML (Kwaan 2007). In several cancer types, tumour cells can shed EVs which express TF and procoagulant phospholipids (Tesselaar, Romijn et al. 2007). Several studies suggest an important role of EVs derived from cancers cells in the pathogenesis of thrombosis (Bharthuar, Khorana et al. 2013, Thaler, Ay et al. 2013, Soff 2014, Thaler, Koder et al. 2014).

1.3 Extracellular vesicles

Extracellular vesicles are spherical structure limited by a membrane bilayer. EVs are used for designating all types of sub-cellular particles in plasma (Gould and Raposo 2013). EVs include exosomes and spontaneous- or activation- or apoptosis- induced microparticles/ microvesicles (MPs/MVs) (Julich, Willms et al. 2014). EVs roughly fall into the size ranges from virus (30-100nm) to platelet (1µm-5µm).

Initially, EVs were considered as waste particles or cellular debris. Currently, increasing evidence suggests a major role in many physiological and pathological states (Valadi, Ekstrom et al. 2007, Burger, Schock et al. 2013). Plasmatic EVs participate in several physiological processes such as coagulation, inflammation, cell survival and apoptosis, endothelial function, vascular remodelling, and angiogenesis (Bouvy, Gheldof et al. 2014). Circulating EVs in blood originate from different cells such as erythrocyte, leukocytes, platelets or endothelial cells. Their plasmatic levels result from the balance between their rate of release from the cell and their clearance (Dasgupta, Abdel-Monem et al. 2009). In healthy subjects, most of circulating EVs are originated from platelets and erythrocytes (Arraud, Linares et al. 2014). Some physiological and pathological conditions are associated with changes of the levels and/or the composition of plasmatic EVs (Angelillo-Scherrer 2012). EVs are implicated in inter cellular communication by harbouring or containing ligands, receptors, mRNA and miRNA. These molecules allow EVs to modulate biological properties of others cells by transfer of membrane protein and exchange of genetic information (Bouvy, Gheldof et al. 2014). In this study we mainly focus on cancer EVs and their implication in haemostasis by TF and procoagulant phospholipids present at EVs surface.

1.4 Cancer extracellular vesicles procoagulant activity

Some studies have shown that cancer cells –related procoagulant activity (PCA) mainly comes from their EVs (Davila, Amirkhosravi et al. 2008). Their PCA is linked to exhibition of TF and procoagulant phospholipids such as

phosphatidylserine (PS) (Pabinger, Thaler et al. 2013). TF is the major cellular initiator of the coagulation cascade that acts synergistically with negative charge phospholipids. Cancer cells are well known to express TF and high level of PS. By the shedding of EVs, the surface with PS and TF exposure is strongly increased and allows the binding of vitamin K-dependent Factors (FVII, FIX, FX and FII). The TF at EVs surface can cleave the FVII/VIIa and form an active complex which can activate the FX in FXa and leads to active thrombin formation (**Figure 5)** (Kasthuri, Taubman et al. 2009, Zwicker, Liebman et al. 2009).

Accumulating evidence suggests that circulating TF-bearing EVs may play important roles in venous thrombosis related to cancer due to the expression of TF by cancer cells (Davila, Amirkhosravi et al. 2008).

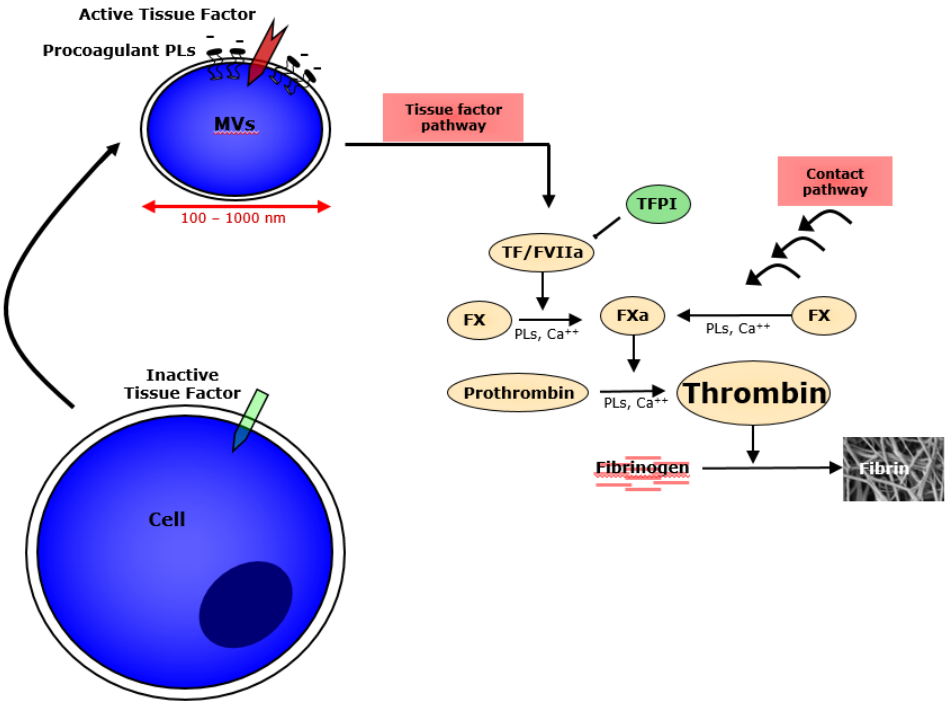

Figure 5. Activation of coagulation cascade by EVs derived from cells. Activated TF and procoagulant phospholipids on EVs surface activates tissue factor pathway of coagulation cascade. Complex TF-factor VII activates factor X which activates prothrombin. These activations need negatively charged phospholipids (PLs) and Ca^{++}. Tissue factor pathway inhibitor (TFPI) is present in plasma and inhibits the complex TF/FVIIa/FXa.

2. Aims of the study

2 Aims of the study

The early death of leukemic patient by thrombosis complication is still a major public health problem. Because of their hematopoietic disorder, their frequent thrombocytopenia and their risk of bleeding, we need to clearly understand the pathophysiology and underlying mechanisms of thrombosis in these patients. From a clinical perspective, it would be extremely helpful to identify and validate biomarkers that enable the early identification of cancer patients at risk of VTE and to target anticoagulation for primary prevention of VTE based on risk stratification. This reliable diagnosis should go through a clear understanding of the pathological mechanisms of thrombosis in leukemic patients.

The aim of our work is to investigate the hypercoagulable state in leukemic patient responsible of thromboembolic disease development. The primary objective is to characterize the prothrombotic role of plasmatic procoagulant extracellular vesicles. The secondary objective is to validate the use of EVs as potential biomarker to identify patient with high risk of thrombosis.

To reach this objective, we first developed several technics to study the EVs *in vivo* and *in vitro*. We then investigated if leukemic cells can shed procoagulant EVs and the impact of intercalating agents on EVs procoagulant activity. Finally, we studied the EVs PCA from patient plasma samples and compared this activity with clinical data

The aims of the present thesis were:

1) To evaluate the combination of thrombin generation assay, transmission electron microscopy and flow cytometry to study tissue factor bearing EVs from breast cancer cells. Breast cancer cells are well known to produce prothrombotic EVs with TF.
2) To increase the sensitivity of thrombin generation for detection of plasmatic PCA of EVs.

3) To assess the role of EVs derived from acute promyelocytic leukemia cells in the activation of coagulation cascade and the effect of DNR on EVs PCA.
4) To evaluate PCA of EVs from acute leukemic patient as biomarker in thrombotic event in acute leukemic patients.

3. Development of methods to study cancer extracellular veiscles procoagulant activity

3 Development of methods to study cancer extracellular vesicles procoagulant activity

Original article in Journal of extracellular vesicles:

Thrombin generation assay and transmission electron microscopy: a useful combination to study tissue factor-bearing microvesicles.

Damien Gheldof, Julie Hardij, Francesca Cecchet, Bernard Chatelain, Jean-Michel Dogné*, François Mullier*.

Several techniques have been described to study EVs (van der Pol, Hoekstra et al. 2010). In order to provide a complete EVs characterization, we need to combine different techniques. In this paper we work with different techniques. Thrombin generation assay gives an overview of PCA and the origin (TF or PLs) of PCA can be determined by using antibody against TF or Annexin V. Flow cytometry gives a count of EVs and allows to detect specific molecules on their surface. And transmission electron microscopy permits a precise size determination. To study the combination of these techniques, we work with a well know model in cancer derived EVs with PCA linked to TF, the breast cancer cell lines MDA-MB-231.(Davila, Amirkhosravi et al. 2008).
This study provides a characterization of cell-derived EVs, validates the sensitivity and intra/inter assay variability of TGA and provides a combination of techniques to study procoagulant EVs derived from cancer cells.

3.1 Abstract

INTRODUCTION: Patients with cancer have a 7- to 10- fold increased risk of developing venous thromboembolism. Circulating microvesicles could be a useful predictive biomarker for venous thromboembolism in cancer. Validated and standardized techniques that could be used to determine the complete microvesicle phenotype are required.

MAIN OBJECTIVES: i) to characterize tissue factor (TF) bearing microvesicles released by cultured breast cancer cells MDA-MB-231 by flow cytometry (FCM), transmission electron microscopy (TEM) and thrombin generation assay (TGA) ii) to validate the sensitivity and variability intra/inter assay of TGA as a useful method to study the procoagulant activity (PCA) of microvesicles.

METHODS: Cultured breast cancer cells MDA-MB-231 were incubated for 45 min at 37°C.

Samples were then centrifuged or not at 4,500g for 15 min and cells and MVs or MV-containing supernatants were used for TEM, FCM and TGA. In activity assays, microvesicles (i.e. cell-depleted supernatants) were incubated with anti-TF antibodies or with annexin V to assess the contribution of TF and phospholipids to the procoagulant activity (PCA). Alternatively, supernatants were filtered through 0.1, 0.22, 0.45 or 0.65µm membranes and subjected to TGA.

RESULTS: The majority of the PCA was associated with microvesicles smaller than 0.1 µm, and the mean microvesicle size estimated by TEM after 10,000 g centrifugation was 121+/-54 nm with a majority of vesicles between 100 and 200 nm. Microvesicles derived from 5,000 MDA-MB-231cells/ml were sufficient to significantly increase the thrombin generation of normal pooled plasma.

CONCLUSIONS: TEM, FCM and filtration coupled to TGA represent a useful combination to study the PCA of TF-bearing microvesicles, whatever their size. And it will be interesting to implement these techniques in patients.

Abbreviations: Venous thromboembolism: VTE; Microvesicles: MVs; Tissue factor: TF; Flow cytometry: FCM; Transmission Electron Microscopy: TEM; Thrombin Generation Assay: TGA; TFPI: Tissue Factor Pathway Inhibitor; Normal pooled plasma: NPP; Microparticles: MPs; Phospholipid(s): PLs; Procoagulant activity: PCA; Phosphate buffer saline: PBS; Standard deviation: SD; Antibody: Ab; Supernatant: Sup; Filtrate: Filt; Monoclonal antibodies: mAbs; TF bearing Microvesicle: TF-MV

3.2 Introduction

Venous thromboembolism (VTE) is responsible for 15% of deaths in cancer patients (Manly, Wang et al. 2010, Noble and Pasi 2010, Gerotziafas, Galea et al. 2011). Therefore, thromboprophylaxis for hospitalized cancer patients is recommended (Khorana, Kuderer et al. 2008). However, individual risk factors cannot identify a group of outpatients at highest risk for VTE that would benefit from thromboprophylaxis (Khorana, Kuderer et al. 2008). Several studies strongly suggest that microvesicles (MVs) harbouring tissue factor (TF-MVs) activity may have prognostic value in identifying cancer patients with increased risk of VTE (Owens and Mackman 2011). MVs are small membrane vesicles shed by most normal and/or tumour cells constitutively or following activation or apoptosis (Kasthuri, Taubman et al. 2009, Zwicker, Liebman et al. 2009). Based on the size and mechanism of synthesis, MVs are currently divided into exosomes and microparticles (MPs) (van der Pol, Hoekstra et al. 2010). The diameter of exosomes is comprised between 30 and 100 nm whereas MPs have a size comprised between 100 nm and 1 μm. However, this definition is not generally accepted due to technological limitations in size measurement (van der Pol, Hoekstra et al. 2010). MVs may present TF and negatively charged phospholipids (PL) such as the phosphatidylserine on their membrane. These elements are thought to be implicated in the procoagulant activity (PCA) (Freyssinet and Toti 2010). In several types of cancers, including breast and pancreatic cancers, TF expression is associated with a prothrombotic phenotype and correlates with grade and tumour progression (Kasthuri, Taubman et al. 2009, Zwicker, Liebman et al. 2009, Zwicker 2010). The TF is present in plasma under two major forms: a full-length TF that may be anchored at MVs surface (TF-MVs) or an alternative spliced soluble protein. TF associated with MVs is considered as the main form exhibiting PCA (Davila, Amirkhosravi et al. 2008, Mackman 2009, Manly, Wang et al. 2010). MVs are defined by size, concentration, morphology, biochemical composition, cellular origin and activity. Numerous techniques have been described to detect and/or characterize the MVs (van der Pol, Hoekstra et al. 2010). However, no technique is able to provide all MV characteristics. Consequently, the

combination of different techniques is required for a complete description of MVs (Freyssinet and Toti 2010). For example, flow cytometry (FCM), which is the most used technique to study MVs, suffers from a lack of sensitivity to small-sized MVs and gives no information about the TF and/or phopspholipid-mediated procoagulant activity. Thrombin generation assay (TGA) can be used to specifically assess the impact of TF-MV on coagulation. However, this technique is limited by absence of standardization and by the unknown sensitivity to low levels of TF due to presence of Tissue Factor Pathway inhibitor (TFPI) in plasma (Dargaud and Negrier 2010, Key and Mackman 2010).

It is therefore essential to validate and standardize a panel of techniques that could be used to determine the complete MVs phenotype and to study the effects of preanalytical steps on MVs' conformation (potential fragmentation or aggregation).

The objectives of this study were therefore i) to characterize tumour cell-derived MVs released by cultured MDA-MB-231 breast cancer cells with FCM, Transmission Electron Microscopy (TEM) and TGA and ii) to validate the intra/inter assay sensitivity and variability of TGA as a useful method to analyse MVs PCA

3.3 Material and methods

3.3.1 Cell culture

Adherent MDA-MB-231 breast cancer cells (ATCC number HTB-26) were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in RPMI-1640 medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS) (Lonza, Verviers, Belgium). Cells were maintained in a 5% CO_2 humidified atmosphere at 37°C. Natrium bicarbonate was set at 1.5g/L to control the pH. Cell viability was controlled by the trypan blue exclusion test.

3.3.2 Preparation of normal-pooled plasma and platelet-free plasma

Normal pooled-plasma (NPP) was prepared as previously described (Robert, Ghiotto et al. 2009). Platelet-free plasma was obtained by a first centrifugation once at 2,500g for 15min at room temperature (Heraeus Multifuge 1S-R, Sysmex Benelux, Etten-Leur, The Netherlands) with a light brake only, within one hour after sampling. The platelet-poor plasma was collected and transferred into a polypropylene haemolysis tube with a micropipette. Aspiration was stopped 1cm above the buffy-coat while avoiding the buffy-coat. Platelet-poor plasma was centrifuged a second time at 2,500g for 15 min at room temperature. The platelet-free plasma was collected into a fresh tube using a micropipette, while leaving about 100µl at the bottom of the tube.

3.3.3 In vitro generation of MVs

A modified version of the protocol reported by Davila et al. was performed (Davila, Amirkhosravi et al. 2008). Cells were adjusted to the desired concentrations (600,000 cells/ml for TGA and TEM) in PBS and then incubated for 45 min at 37°C without any stirring. Samples were then centrifuged or not at 4,500g for 15 min (**Figure 6**). Afterwards, cells and MVs or fresh MV-containing supernatants devoid of cells were used for TEM, FCM and TGA. PBS was used as negative control.

3.3.4 Preparation of samples for TEM observation: Tumor MVs visualisation and sizing by TEM

One ml of PBS containing 600,000 MDA-MB-231 cells and their secreted MVs was centrifuged at 10,000g for 90 min at 4°C to form a pellet (**Figure 6**). The pellet was fixed for TEM observation during 2.5 hours in 300 µl of 2.5 % glutaraldehyde in 0.1 M cacodylate buffer. Samples were then washed using 0.2 M cacodylate buffer. Osmium tetraoxide was added during 1 hour as contrasting agent. Samples were further washed with 0.2 M cacodylate buffer and dehydrated with successive baths of alcohol from 30° to 100°. Impregnation of samples in resin LX 112 was then done. After resin polymerization, samples were cut with an ultra-microtome (LKB, Bromma 8800®), mounted on a grid and stained with heavy metal salts (uranyl acetate and lead citrate). The samples were observed using Tecnai 10 TEM (FEI, Eindhoven, The Netherlands; resolution of about 5 nm at 80 kV). The MV

size determination was performed semi-automatically on high magnification TEM pictures (**Figure 7**B) with the Image J software (Maryland, USA). One hundred MVs were sized (Serda, Godin et al.). The result was expressed as mean ± S.D with range in parentheses.

3.3.5 Counting and expression of TF and MUC-1 by flow cytometry

Quantification of MVs and expressions of TF and Mucin-1 (MUC-1) on MDA-MB-231 cells and MVs were carried out by FCM as mentioned in supplementary information. MUC-1 was chosen since it has been shown that TF-MV activity of patients with breast cancer who presented with acute VTE, correlated with the presence in the blood of MV expressing the epithelial antigen MUC1 (Tesselaar, Romijn et al. 2007).

3.3.6 Measurement of the MVs procoagulant activity (PCA) by thrombin generation assay

PCA was measured by TGA according to a modified version of the method developed by Hemker (Hemker, Giesen et al. 2002). Total cell suspensions or cell-depleted *in vitro*-generated MV fractions from 600,000 cells/ml were used as the source of TF and phospholipids. 80 µl of Normal Pooled Plasma (NPP) and 20µl of cell suspensions or MV fractions were mixed in a 96-well microtiter plate (Thermo Immulon 2HB, USA) and were incubated for 10 min at 37°C. PBS was used as a negative control. Early experiments are tested with or without addition of 20µl of MP reagent, which correspond to 4µM of phospholipids (Thrombinoscope BV). The detailed protocol has been described elsewhere (Robert, Ghiotto et al. 2009).

All the experiments were performed in triplicate. The results were expressed as means ± S.D. All parameters were calculated using software thrombinoscope. The % of PCA was calculated on the lagtime reduction by MVs.

Variability of TGA was measured according to the previously described method using 80 µL of NPP + 20 µL PBS. The assays were conducted in triplicate on six independent runs with the same operator. Mean intra-assay variation and inter-assay variability for lagtime and peak were determined. Mean intra-assay variation corresponds to the mean coefficient of variation for the triplicates (n=6). Inter-assay variation corresponds to the coefficient of variation for the mean value of triplicates between the 6 independent runs.

3.3.7 Effect of MVs size on PCA

MV fractions were filtered through 0.1, 0.22, 0.45 or 0.65 µm membranes (Ultrafree-MC; Amicon, Bedford, MA, USA) following the manufacturer's instructions. Supernatant (Sup) and filtrates (Filt) were compared by TGA and FCM (1 experiment in triplicate).

3.3.8 Contribution of TF and phospholipids to MVs PCA

For contribution of TF, MVs derived from 600,000 cells/ml were pre-incubated with various monoclonal antibodies (mAbs) (10µg/ml, optimal final concentration) for 20 min at room temperature before TGA was performed.

The clone HTF-1 (BD Biosciences, Erembodegem, Belgium) was used as TF-activity neutralizing Ab (Wang, Manly et al. 2009). The clone TF9-10H10 (Merck, Darmstadt, Germany) and IgG1 (BD Biosciences, Erembodegem, Belgium) were tested as a non-function-blocking antibody (Gessler, Voss et al. 2010) and isotypic control antibody, respectively.

The phospholipid-mediated activity was blocked with annexin V (Sigma-aldrich, Bornem, Belgium) (Peng, Jiang et al. 2001). Samples were pre-incubated with annexin V (0.5 µM, final concentration) for 20 min at room temperature before TGA was performed (Davila, Amirkhosravi et al. 2008). Three independent experiments were performed in triplicate.

3.3.9 Effect of ultracentrifugation on PCA

MV derived from 100,000 cells/ml were separated from the cells by centrifugation at 4,500 g during 15 min. The collected supernatants, which contain only MVs, were ultracentrifuged at 100,000g during 90 min to pellet the MVs. MV before ultracentrifugation (Sup), pellet of MVs (Pellet UC) and supernatant after ultracentrifugation (Sup UC) were tested in TGA.

3.3.10 Effect of stirring and centrifugation on MV PCA

The standard protocol described above for the *in vitro* generation of MVs is described in **Figure 6**. In preliminary experiments, our standard protocol without stirring was compared to that proposed by Davila (Davila, Amirkhosravi et al. 2008) which includes stirring (at 1,000 rpm) on aggregometer during the 45 min of cell incubation in PBS. The cell supernatants were used for TGA.

Three centrifugation protocols to eliminate cells were also compared using TGA at 600,000 cells/ml: I) simple centrifugation at 4,500 g for 15 min (Davila, Amirkhosravi et al. 2008), II) double centrifugation at 2,500 g for 15 min and III) double centrifugation: at 1,500 g for 15 min and at 13,000 g for 2 min. Three independent experiments were performed in triplicate.

3.3.11 Effect of cell concentration on MVs PCA

To determine the sensitivity of TGA, the impact of cell concentration on TGA was assessed at 100; 500; 1,000; 2,500; 5,000; 10,000 and 20,000 cells/ml. Three independent experiments were performed in triplicate.

3.3.12 Statistics

Comparison between different conditions was performed using the Kruskal-Wallis test on Medcalc software (version 12.2.1.0). If the Kruskal-Wallis test is positive ($P<0.05$) then MedCalc performs a test for pairwise comparison of subgroups according to Conover, 1999. Conover WJ (1999) Practical nonparametric statistics 3rd edition. New York: John Wiley & Sons.

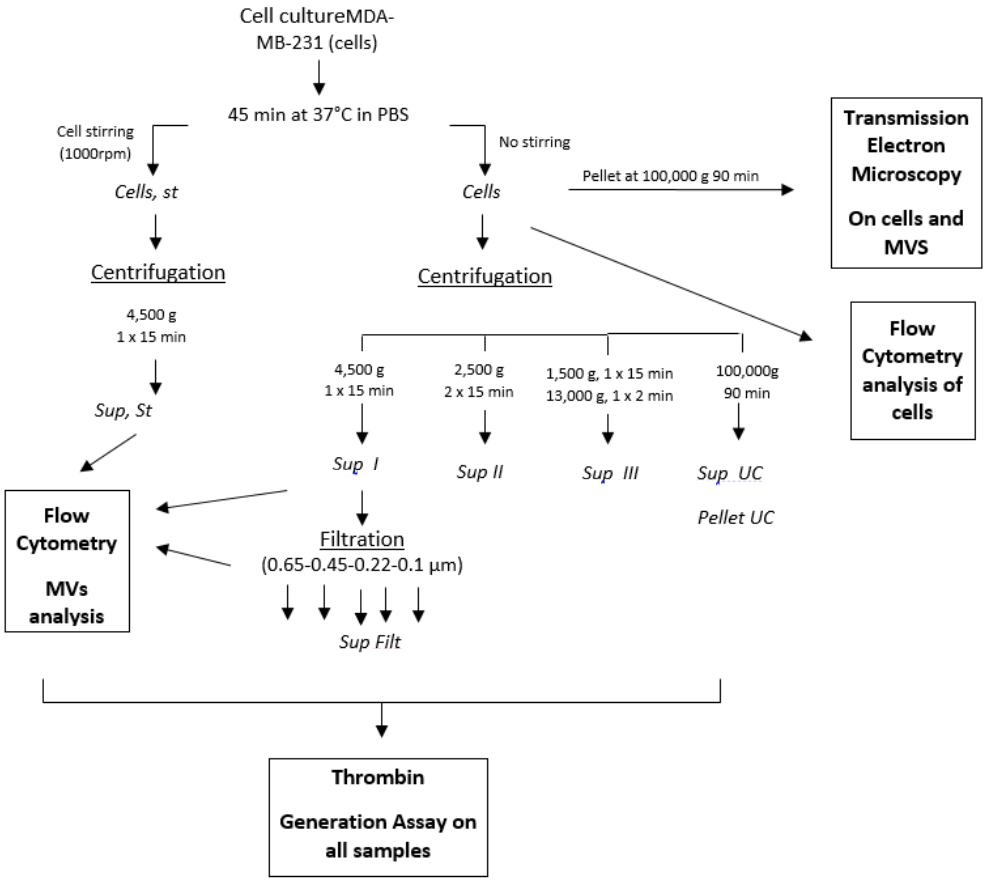

Figure 6. Sample preparation. All supernatants were freshly used for TEM, FCM and TGA. PBS: Phosphate Buffer Saline. Sup: Supernatant. Filt: Filtrate. UC: ultracentrifugation

3.4 Results

3.4.1 Characterization of MVs derived from MDA-MB-231 cells

The different experimental settings used in this work are schematized in **Figure 6**. Images of MDA-MB-231 cells with TEM after 10,000 g centrifugation showed that these tumor cells spontaneously shed small MVs from their membrane (**Figure 7**). The MVs mean size after centrifugation of the cells and MVs, was 121± 54 nm [range: 57 nm to 440 nm]. The size distribution was not statistically different when we ultracentrifuged cells with MVs at 100,000g. However, we cannot provide a picture of MVs after ultracentrifugation because of the insufficient amount of the pellets. Concerning their membrane protein surface expression, MDA-MB-231 cells were strongly positive for both MUC-1 (= CD227) and TF (= CD142). 10% of all MVs were positive for TF expression, and only 1.7% were positive for MUC1 (**Figure 8**). The final MVs concentration in "MDA" samples (cells and MVs) was 1,199 MVs/µl (Table 1).

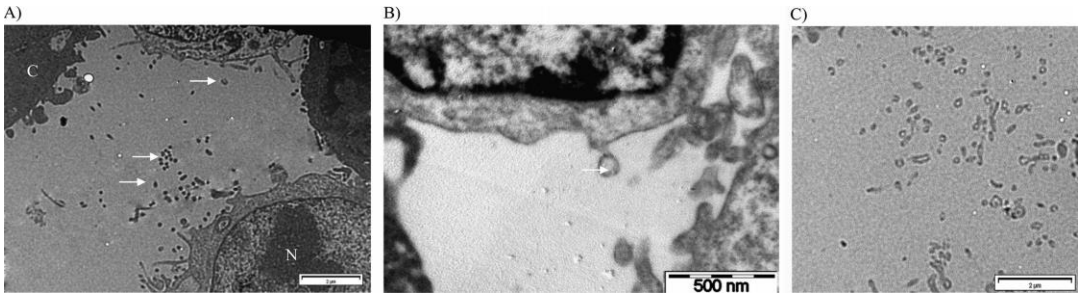

Figure 7. Transmission electron microscopy pictures of cells with derived MVs (white arrows) after ultracentrifugation at 10,000 g during 90 minutes. A) Scale bar 2 µm. N: nucleus C: cytoplasm; B) scale bar 500 nm; C) scale bar 2 µm.

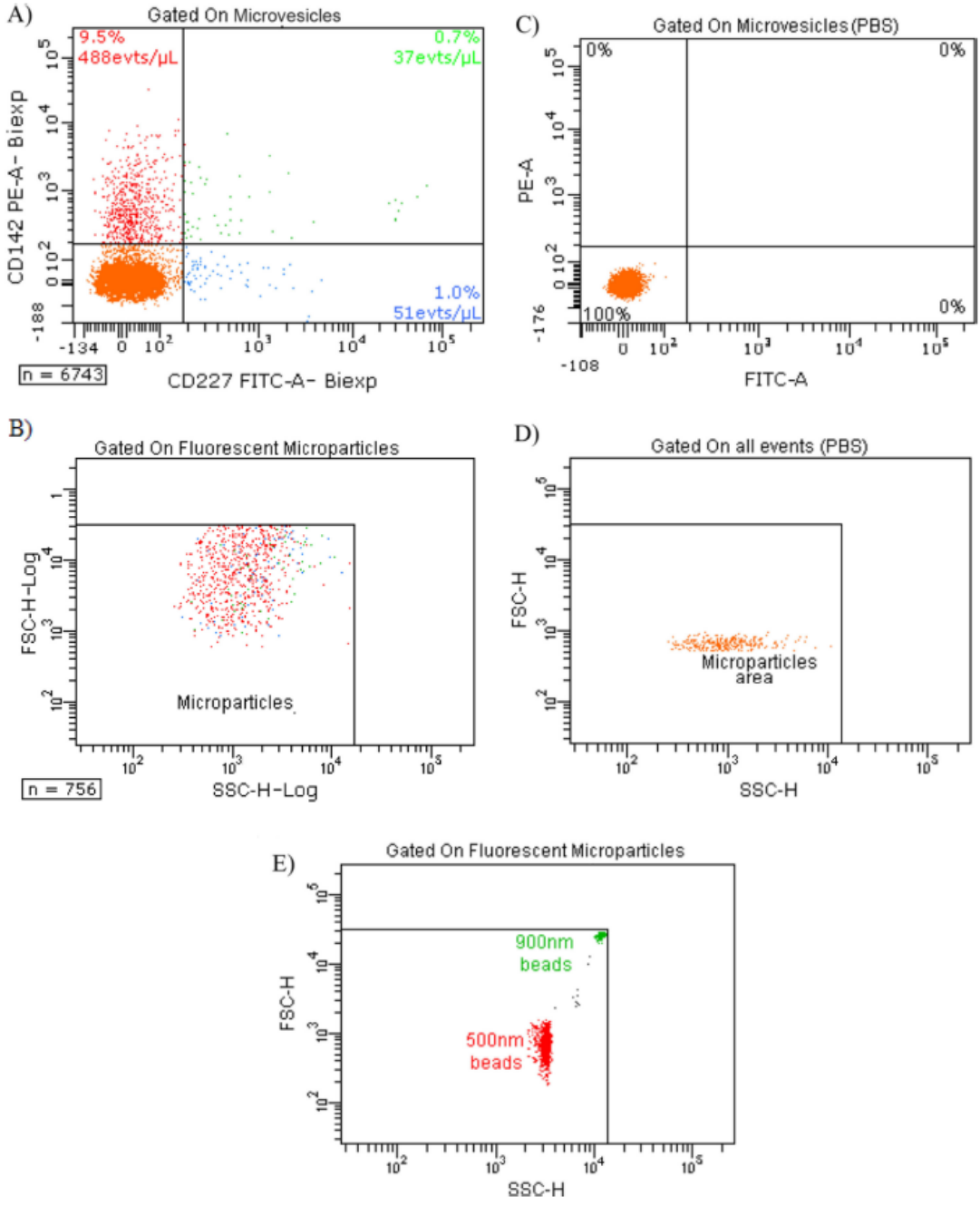
A)
Gated On Microvesicles
9.5%
488evts/µL
0.7%
37evts/µL
1.0%
51evts/µL
CD142 PE-A- Biexp
CD227 FITC-A- Biexp
n = 6743
C)
Gated On Microvesicles (PBS)
0%
0%
100%
0%
PE-A
FITC-A
B)
Gated On Fluorescent Microparticles
FSC-H-Log
SSC-H-Log
Microparticles
n = 756
D)
Gated On all events (PBS)
FSC-H
SSC-H
Microparticles
area
E)
Gated On Fluorescent Microparticles
900nm
beads
500nm
beads
FSC-H
SSC-H

Figure 8. Expression of TF (CD142) and MUC-1 (CD227) on MVs depleted of cells. A) Tumor microparticle analysis. Dual fluorescence analysis of MDA-MB-231 MVs stained with CD227 fluorescein isothiocyanate (FITC) (FL1) and CD142–phycoerythrin (PE) (FL2). CD142+ CD227+ MVs are represented as green dots, CD142+ CD227- MVs as red dots, CD227+ CD142- MVs as blue dots and background noise or other MVs as orange dots. Percentage and absolute number (/µl) of each subpopulation are indicated. B) Backgating of CD142+ CD227+ MVs (green dots), CD142+ CD227- MVs (red dots) and CD227+ CD142- MVs (blue dots) on FSC log-SSC log cytogram. C) Expression of TF (CD142) and MUC-1 (CD227) on PBS without MVs. D) FSC-SSC of control PBS without MVs labelled similarly with CD142 and CD227. E) FSC-SSC dot plot of 500 and 900nm beads used to gate on MVs.

Sample	MVs MUC-1+/TF- (/µl)	MVs MUC-1-/TF+ (/µl)	MVs MUC-1+/TF+ (/µl)
Cells	86	1014	99
Sup	44	60	30
Sup, St	39	128	15
Sup Filt 0.65µm	47	25	15
Sup Filt 0.45µm	48	21	17
PBS	0	0	0

Table 1. Flow cytometry concentration of MVs MUC-1+/TF-, MVs MUC-1-/TF+ and MVs MUC-1+/TF+ (/µl) in : cells and MPs (Cells), MDA-MB-231 supernatant (Sup), MDA-MB-231 supernatant produced with stirring (Sup, St), Sup filtered through 0.65µm and 0,45 µm membranes (Sup Filt 0,65µm and Sup Filt 0,45 µm) and PBS as control.

3.4.2 Procoagulant activity of MDA-MB-231 cells-derived MVs

MDA-MB-231 cells and their derived MVs ("MDA" sample) significantly boosted up the generation of active thrombin when compared to the PBS control curve (**Figure 9**A), as shown by the 10-fold reduction of lagtime. MDA-MB-231 supernatant ("Sup MDA" sample), which only contains MVs, also enhanced thrombin activity compared to the control as indicated by the 9-fold lagtime reduction. The difference between MDA-MB-231 cells and their supernatant was highly significant for both lagtime (P-value = 0.0091) and peak (P-value = 0.0039). FCM analysis showed a 10-fold decrease in TF+-MVs concentration between MDA and Sup MDA samples (Table 1, 1113 versus 90/µl), suggesting that the majority of MVs detected by flow cytometry carrying PCA activity remain associated to the cell surface and co-pellet with the cells after centrifugation at 4,500g. These data are consistent with the reduction of PCA between these samples.

Since FCM cannot detect vesicles smaller than 300 nm, and since TEM showed a majority of MVs smaller than 300 nm, the link between the MVs size and procoagulant activity was evaluated through filtration of the supernatant using membranes with various cut-off sizes ranging from 0.65 to 0.1 µm (**Figure 9**B). As compared to the initial supernatant ("Sup MDA"), which displayed a 9-fold reduction in lagtime, the 0.65µm- or 0.45 µm-filtered supernatant ("Sup MDA Filt 0.65µm" or 0.45 µm respectively) displayed a slightly reduced PCA, shown by a 7-fold reduction in lagtime when compared to the control. The difference between samples before and after the 0.65 or 0.45µm filtration was highly significant both for lagtime and for the peak parameter. These data are supported by FCM as filtration at 0.65 or 0.45 µm reduced by two-fold the number of TF+ MVs as compared to non-filtered supernatant (40 or 38 respectively, as compared to 90 / µl) (Table 1). Finally, lagtime was increased 2.2- and 2.5-fold after filtration through 0.22 and 0.1µm, respectively, in comparison to unfiltered supernatant, but was still 4- and 3.6-fold reduced as compared to PBS. These analyses were performed without "MP reagent", however the same observations were made with or without addition of phospholipids. We conclude that TF-MVs of different sizes participate in PCA activity, those larger than 0.65 µm displaying proportionally less activity than the MVs retained by 0.22 and 0.1 µm filters,

and that the majority of the PCA was associated with components smaller than 0.1 µm.

The use of "HTF-1", an anti-TF antibody, was associated with a strong inhibition of the PCA associated to MVs as revealed by a 6-fold increase in lagtime (**Figure 9**C). On the other hand, the PCA was not affected by a non-blocking antibody (TF9-10H10), nor by an isotypic control (IgG1). These data confirm the specificity of PCA inhibition by the anti-TF antibody.

Inclusion of annexin V during the assay was associated with a complete abolishment of PCA (**Figure 9**D).

The majority of PCA contained in supernatant was associated with the pellet after 100,000 g ultracentrifugation, but some activity remained in the post-ultracentrifugation supernatant (**Figure 9**E): this supernatant still reduced lagtime 2.5-fold in comparison to normal pooled plasma spiked with PBS, however it showed no significant increase of peak of active thrombin (**Figure 9**E).

The lagtime and peak intra-assay variation coefficients were 12% and 11%, respectively. The lagtime and peak inter-assay variation coefficients were 14% and 50%, respectively.

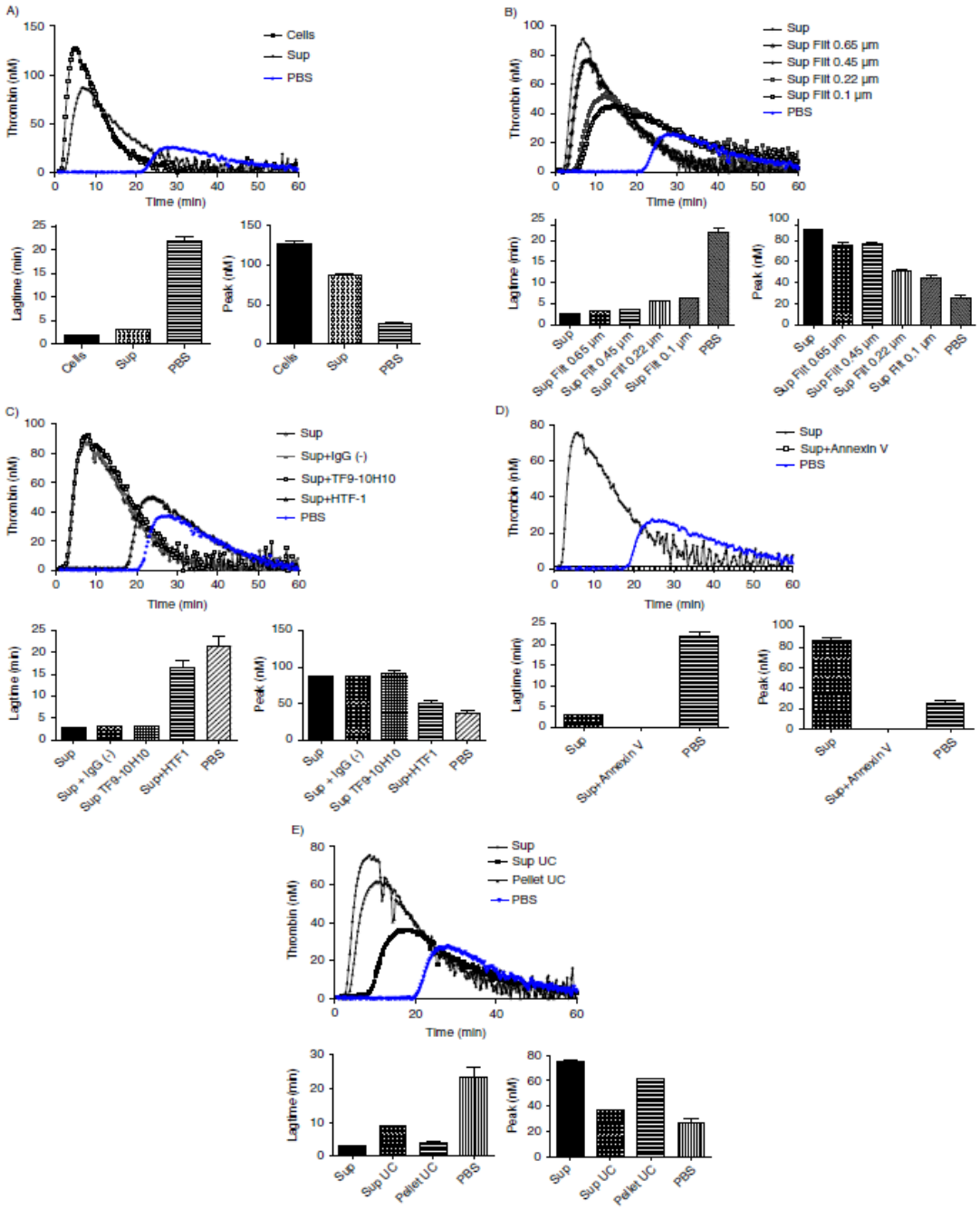
A)
Thrombin (nM)
Time (min)
Cells
Sup
PBS
Lagtime (min)
Peak (nM)
B)
Sup
Sup Filt 0.65 µm
Sup Filt 0.45 µm
Sup Filt 0.22 µm
Sup Filt 0.1 µm
PBS
C)
Sup
Sup+IgG (-)
Sup+TF9-10H10
Sup+HTF-1
PBS
D)
Sup
Sup+Annexin V
PBS
E)
Sup
Sup UC
Pellet UC
PBS

Figure 9. Curves of thrombin generation experiments and histograms of lagtime and peak parameters. In control condition, NPP is spiked with PBS. Curve data are presented as means of n=3. All the results are representative of 3 independent experiments. A) NPP spiked with MDA-MB-231 cells (Cells) or supernatant from MDA-MB-231 cells (Sup). The supernatant shows only slightly reduced PCA as compared to cell + supernatant. B) NPP spiked with supernatant from MDA-MB-231 cells (Sup), filtered or not through membranes with various sizes (Sup Filt 0.1µm/0.22µm/0.45µm/0.65µm). Filtration through 0.65 or 0.45 µm reduces only slightly the PCA, whereas filtration through 0.22 and 0.1 µm leads to stronger reduction in PCA. C) Procoagulant effect of MVs (Sup) pre-incubated with or without HTF-1 (TF-blocking Ab), TF9-10H10 ((TF-non blocking Ab) or isotypic control antibodies at 10µg/ml. HTF-1 strongly inhibits PCA of MV-containing supernatant. D) Procoagulant effect of MVs (Sup) pre-incubated with or without annexin V at 0.5 µM. AnnexinV abolishes the PCA activity of the MV-containing supernatant. E) Procoagulant effect of MVs derived from 100,000 cells (Sup), of the pellet obtained after ultracentrifugation of MVs (pellet UC Sup) and of the supernatant obtained after ultracentrifugation of MVs (Sup UC).

3.4.3 Influence of stirring and centrifugation on PCA of MDA-MB-231 cells and MVs

The stirring of MDA-MB-231 cells during MVs generation does not modify the PCA of cells ("Cells") and MVs ("Sup") derived from 600,000 cells/ml (**Figure 10**A). However, FCM analysis showed a difference between MVs concentration in samples stirred or not (Table 1).

The centrifugation protocol used to separate cells and MVs does not significantly impact TGA curves (**Figure 10**B). Indeed, curves are very similar both for the supernatant and the 0.1µm-filtered supernatant generated from 600,000 MDA-MB-231 cells/ml, using either one of protocols I, II or III described in **Figure 6**.

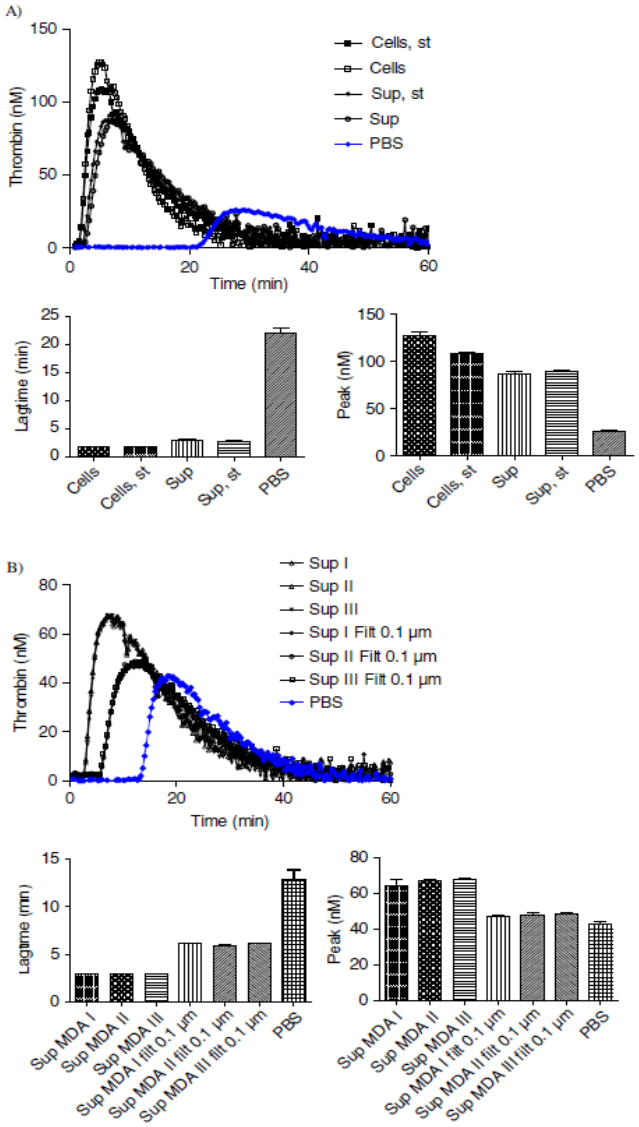

Figure 10. Effect of stirring during cell incubation and of cell centrifugation protocol on thrombin generation. A) Thrombin generation curves and histograms of lagtime and peak parameters of Normal Pool Plasma (PBS) spiked with MDA-MB-231 cells stirred (Cells, st) or not (Cells) or with their respective supernatant (Sup, st and Sup). No difference in PCA is observed between the stirred and non-stirred samples. B) Thrombin generation curves and histograms of lagtime and peak parameters of Normal Pool Plasma spiked with supernatant of cells filtered or not (Sup vs. Sup Filt 0,1 µm) and produced by three different centrifugation protocols (Sup I, II, III). In control wells, NPP is spiked with PBS. The curve is representative of 3 independent experiments.

3.4.4 Sensitivity of TGA

As shown in **Figure 11**, a dose-response relationship is observed from 100 to 20,000 cells/ml for both MDA-MB-231 cells and MVs. A threshold concentration of 500 MDA-MB-231 cells/ml is sufficient to release enough TF-MVs that significantly reduce the lagtime in comparison to control (P-value = 0.034). When the supernatant is considered, 5,000 MDA-MB-231 cells/ml is the threshold concentration required to significantly reduce the lagtime (P-value = 0.034). The coefficients of variation for Lagtime, ETP, Peak and Ttpeak were 12%, 11%, 11% and 9% in intra-assays (n=6) and 14%, 34%, 50% and 16% in inter-assay (n=6), respectively.

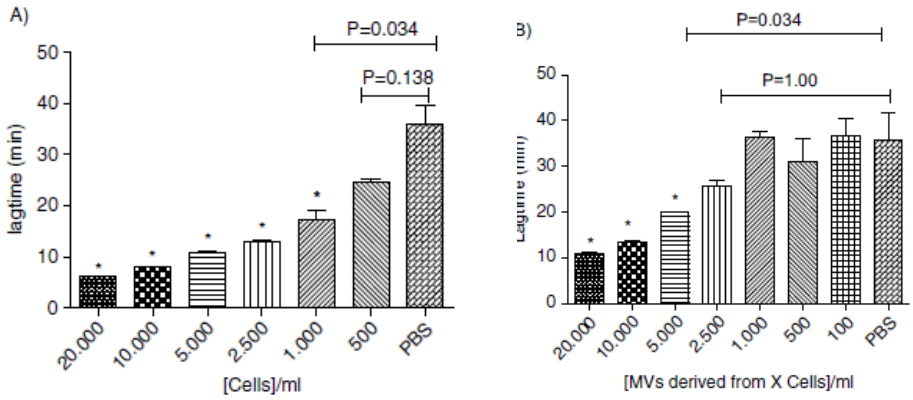

Figure 11. Procoagulant effect of cells (Cells, A) and MVs (Sup, B) derived from different MDA-MB-231 cell concentrations (0-100-500-1,000-2,500-5,000-10,000-20,000-cells/ml) on lagtime. In control wells, NPP is spiked with PBS. Results are presented as mean +/- SD. Data with significant difference in comparison to control are indicated with * ($p<0.05$).

3.5 Discussion

VTE prophylaxis during systemic chemotherapy is not recommended for ambulatory patients with cancer (Khorana, Streiff et al. 2009). Indeed, there is currently no validated biomarker presenting a thrombotic profile able to lead to an adapted prophylaxis (Khorana, Kuderer et al. 2008). The circulation of active TF associated with MVs has been considered as a promising and interesting biomarker to prevent VTE in cancer (Manly, Wang et al. 2010, Zwicker 2010, Owens and Mackman 2011). An ongoing phase III study (MicroTEC, NCT00908960) in advanced cancer patients with high levels of TF bearing MVs has been designed to evaluate the benefit of primary thromboprophylaxis and to validate the prognostic usefulness of measuring TF bearing MVs (Zwicker, Liebman et al. 2009). However, validation and standardization of techniques measuring TF-MVs are required before considering cut-off values of this biomarker in risk-stratification approaches. In this study, we compared results obtained with TEM, FCM and TGA to determine the size, the concentration and the PCA of tumour MVs. We used the well-established invasive/metastatic MDA-MB-231 breast carcinoma cell line, which is known to express high levels of TF (Davila, Amirkhosravi et al. 2008). By ***FCM***, we highlighted that the majority of cells and 10% of cell derived-MVs are positive for TF, and only a few microvesicles express MUC-1 (**Figure 8**). MUC-1 was chosen since it has been shown that TF-MV activity of patients with breast cancer who presented with acute VTE, correlated with the presence in the blood of MVs expressing the epithelial antigen MUC1 (Tesselaar, Romijn et al. 2007). Moreover, FCM allows determining TF+MVs or MUC-1+MVs concentrations. However, FCM suffers from a lack of sensitivity to small-sized MVs (i.e. MVs-size < 0.5µm) (van der Pol, Hoekstra et al. 2010) and it has been recently shown that microvesicle detection by flow cytometry is attributed to both large single vesicles and swarm detection of smaller vesicles (van der Pol, van Gemert et al. 2012, Alain R. Brisson, Sisareuth Tan et al. 2013) . Therefore, data presented in Table 1 can represent both large vesicles and aggregates of small microvesicles. Although new tools using size-calibrated beads and recent progress in instrumentation may improve detection of MVs populations of

smaller sizes, flow cytometry is the only validated technique available for MV quantification. However, unless recent improvements, FCM is only suitable for large microvesicles.(Lacroix, Robert et al. 2010, van der Pol, Hoekstra et al. 2010), Thus FCM will not allow the detection of smallest MVs and does not give any information about their TF-mediated PCA.

Using ***TEM***, we showed that there is a budding of MVs on cell surface, these results are in agreement with previous study on MV formation and show that MV constitution can be directly linked to the cell surface membrane. However, for vesicles away from cells, we cannot determine whether they bud off the cell membrane or whether they were released by another means. Moreover, by TEM, we showed that size of MDA-MB-231-derived MVs ranges from 0.05 µm to 0.45 µm in diameter, the majority of MVs is comprised between 0.1µm and 0.2µm and 36% of MVs is comprised under 100nm These results are in agreement with those of Xiong (Xiong, Aras et al. 2003). However, as slices are performed, these measures may be underestimated as the vesicles are not always cut in their equator. On the other hand, as observed with manufactured nanoparticles (Tenzer, Docter et al. 2011), MVs may associate with proteins ("corona effect") during TEM preparation leading to a overestimation of the size. Cryoelectron microscopy may represent an interesting alternative to view every MV in natural condition (Buzhynskyy, Golczak et al. 2009).
We also validated ***TGA*** to characterize the PCA of MVs. In agreement with FCM data, we confirmed that the PCA of MDA-MB-231 cells, is mainly due to MVs release. The higher PCA observed for MDA-MB-231 cells compared to their supernatant mostly resulted from a loss of the largest MVs or MVs aggregates by centrifugation, as shown by the differences in MVs concentration between cells and supernatant determined by FCM. However, our study confirmed that MDA-MB-231 cells could also support PCA (Berny-Lang, Aslan et al. 2011). Surprisingly, we also showed that the 74% of TF-activity is associated with particles smaller than 0.1 µm even if only 36% of MVs visualized by TEM are smaller than 0.1 µm. The filtration experiments also show that about 8 % of activity is associated with vesicles larger than 0.65 µm, vesicles of sizes comprised between 100 and 650 nm display 20% of MVs PCA. Thus very small vesicles display a high PCA.

By using an anti-TF function-blocking antibody (HTF-1) and annexin V which links phosphatidylserine, we confirmed that the PCA of MVs is related to the expression of active TF and negatively charged PL (Butenas, Bouchard et al. 2005). Moreover annexin V block phospholipids from MVs contained in the NPP. The anionic phospholipids are required for initiation and propagation of TF-dependent coagulation. It seems therefore likely that only TF bound in negative phospholipids membrane will support thrombin formation. This result confirms that the active form of TF mainly comes from MVs. It has been extensively reported that the presence of TF on the cell surface does not necessarily correlate with TF activity. Indeed, the majority of membrane-anchored TF molecules would be in a non-active encrypted conformation and "decryption" would thus be essential for the expression of TF function (Butenas, Parhami-Seren et al. 2009). The difference in peak between supernatant of MDA-MB-231 cells and MVs can be explained by the phospholipids provided by tumour cells (Tesselaar, Romijn et al. 2007, Davila, Amirkhosravi et al. 2008).

Because the clinical importance of MVs is increasingly being recognized, it is crucial to standardize methods to allow comparison of data across different studies. To validate the use of TGA for measuring the PCA of MVs, we assessed the impact of two pre-analytical variables, i.e. stirring and centrifugation, and determined the sensitivity of this technique. Contrary to a previous report (Davila, Amirkhosravi et al. 2008), the stirring of cells did not increase the production of MVs, as shown by MV counting by FCM and above all, by PCA measurement. In addition, no significant differences between the three centrifugation protocols were observed in TGA which is compatible with the small size of MVs released by MDA-MB-231. Moreover, ultracentrifugation did not allow abolishing completely the PCA, which is in contradiction with Davila *et al.*(Davila, Amirkhosravi et al. 2008). One hypothesis is that some small TF-MVs may not be pelleted with ultracentrifugation at 100,000g, and may require higher speed or longer time of centrifugation. Another explanation can come from the cells' release of active soluble tissue factor (Kocaturk and Versteeg 2012). Finally, 5,000 MDA-MB-231 cells/ml are sufficient to detect a lagtime reduction mediated

by TF-MVs when compared to NPP alone. This technique is more sensitive without reagent but the variability increased.
In vivo the correlation between TF-MV and thrombosis may become more or less strong depending on the type of tumour/cancer present in a patient (Kasthuri, Taubman et al. 2009, Zwicker, Liebman et al. 2009, Zwicker 2010). Therefore, additional *in vivo* studies examining the relationship between tumour/cancer progression, thrombosis and TF-MV is critical.
In addition, atomic force microscopy, a high-sensitive technique able to image biological samples in aqueous fluids was recently proposed for the detection and quantification of CD41-positive MVs (Yuana, Oosterkamp et al. 2010). This technique should be developed and validated for TF-MVs and compared with FCM, TEM and TGA.

3.6 Conclusions

This is the first study that uses FCM, TEM and TGA to characterize MVs. MDA-MB-231 releases MVs whose size was 121 +/-54 nm by TEM. TGA is a useful technique to study the PCA of tumour cell-derived MVs whatever their size. It should be combined by high-sensitive sizing and counting techniques such as AFM in order to estimate the concentration of specific MVs (i.e. TF-MVs). TGA should be validated and used to study TF-MV in plasma from healthy subjects and patients.

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4. Adapted thrombin generation assay to study procoagulant activity of plasmatic extracellular vesicles

4 Adapted thrombin generation assay to study procoagulant activity of plasmatic extracellular vesicles

Original article in Blood Coagulation and Fibrinolysis 2013:

Inhibition of tissue factor pathway inhibitor increases the sensitivity of thrombin generation assay to procoagulant microvesicles.

Damien Gheldof, François Mullier*, Bernard Chatelain, Jean-Michel Dogné*, Christian Chatelain*.

Thrombin generation assay is a useful technique to study procoagulant EVs. This technique coupled with filtration and inhibitors provide important EVs PCA information. However, this technique suffers from a lack of sensitivity for detection of EVs-TF activity due to the presence of TFPI. For the *in vitro* study this limitation can be easily overcome by a large production of EVs-TF (increased the number of cells). However, it's possible that EVs-TF levels circulating in cancer plasma patient were very low. So, we need a highly sensitive technique to study the EVs PCA in plasma from leukemic patient.
In these study, two methods are used to increase the TGA sensitivity: TFPI inhibition and plasma dilution.

4.1 Abstract

INTRODUCTION: Patients with cancer have a 7- to 10-fold increased risk of developing venous thromboembolism (VTE). Circulating microvesicles (MVs) could be a predictive biomarker for VTE in cancer. Thrombin generation assay (TGA) is a useful technique to detect procoagulant activity of MVs. However, TGA suffers from a lack of sensitivity due to the presence of Tissue Factor Pathway Inhibitor (TFPI) in plasma.

AIMS: To improve the sensitivity of TGA to tissue factor (TF) by limiting the interference of TFPI.

METHODS: Serial dilutions of MDA-MB231 cells were incubated for 45min at 37°C to generate MVs. Samples were then centrifuged and supernatants which contain MVs were used for TGA. Normal pooled plasma was incubated with inhibitor of TFPI or was diluted twice to decrease plasma level of TFPI. Lagtime was used as a surrogate marker of TGA to detect procoagulant activity of MVs.

RESULTS: i) Inhibition of TFPI decreased twice the cell concentration needed for a significant reduction of lagtime and decreased 2.4-fold the intra-assay variability. ii) Plasma dilution had no impact on the TGA sensitivity when TGA was triggered by MVs derived from MDA-MB-231.

CONCLUSIONS: Thrombin generation is a very sensitive method to study the procoagulant activity of TF-MVs. The sensitivity can be increased by inhibition of TFPI with specific monoclonal antibody against its Kunitz Domain I. A two times plasma dilution is an interesting cheaper alternative to study the procoagulant activity of MV by TGA with a good sensitivity, especially when low plasma quantities are available.

Abbreviations : microvesicles (MVs)- tissue factor (TF)- procoagulant phospholipids (PL)- tissue factor bearing microvesicles (TF-MVs)- venous thromboembolism (VTE)- thrombin generation assay (TGA)- tissue factor pathway inhibitor (TFPI)-normal pooled plasma (NPP)- endogen thrombin potential (ETP)- standard deviation (SD)- procoagulant activity (PCA)

4.2 Introduction

Association between cancer and thrombotic disorders is well described (Debaugnies, Azerad et al. , Davila, Amirkhosravi et al. 2008). Hypercoagulability in cancer is a multifactorial process that involves both physical and biochemical procoagulant mechanisms. Several substances overexpressed by malignant cells including cytokines, cancer procoagulant (a cysteine protease) and tissue factor bearing microvesicles (TF-MVs), are known to activate the coagulation cascade. MVs are considered as a key component of the haemostatic system. They are small membrane vesicles shed from normal and/or tumour cells following activation or apoptosis. They can be captured in the developing thrombus and can lead to thrombin generation by expression of procoagulant phospholipids (PL) and TF (Mackman 2004, Furie and Furie 2008). Moreover, several studies strongly suggest that TF-MVs and TF-MV activity may have prognostic value in identifying cancer patients with increased risk of developing venous thromboembolism (VTE) (Zwicker 2008, Wang, Geddings et al. 2012). Several techniques are currently used to study the procoagulant activity of TF-MVs (Key and Mackman). Two distinct types of assays for measurement of TF-MV activity in human plasma are reported using either antibody-mediated MP capture or isolation by centrifugation (Key and Mackman). However, preanalytical steps associated with these methods (centrifugation and/or washing) could impact MV size, number and activity (Yuana, Bertina et al.). When MVs could be isolated and concentrated from the patient plasma, TGA should be a great technique to observe the procoagulant activity. Thrombin generation assay (TGA) appears as a promising method to assess the impact of TF-MVs on coagulation (Dargaud, Luddington et al. 2007). Although TF activity assay like TGA is more sensitive than ELISA (Key and Mackman), TGA also suffers from a lack of sensitivity which is notably due to the presence of tissue factor pathway inhibitor (TFPI) in plasma (Yamamuro, Wada et al. 1998). TFPI is an endogenous anticoagulant protein of the serine protease inhibitor family. TFPI consists of three Kunitz type domains ; the first domain is responsible for FVIIa/TF inhibition and the second Kunitz domain is a FXa inhibitor (Shimura, Wada et al. 1996). Human

plasma level of total TFPI is 68.7 +/-14.1ng/ml and for the free TFPI 17.7 +/- 5.4ng/ml and 51.1 +/-12.0ng/ml are lipoprotein associated (Yamamuro, Wada et al. 1998).

The aims of this study were to compare the impact of the use of a TFPI inhibitor with the use of diluted plasma on the sensitivity of TGA to assess the procoagulant activity of MVs. First, we studied the impact of TFPI inhibitor or plasma dilution on thrombin generation induced by known concentrations of TF and PL (dilution of PPP low reagent). We then studied the impact of TFPI inhibitor or plasma dilution on thrombin generation sensitivity induced by MDA-MB-231 derived MVs.

4.3 Material and methods

4.3.1 Preparation of normal-pooled plasma

Normal pooled-plasma was prepared as previously described (Robert, Ghiotto et al. 2009). Platelet-free plasma was obtained from a first centrifugation at 2,500g for 15min at room temperature (RT) (Heraeus Multifuge 1S-R, Sysmex Benelux, Etten-Leur, The Netherlands) with a light brake only, within one hour after sampling. The platelet-poor plasma was collected and transferred into a polypropylene hemolysis tube with a micropipette. Aspiration was stopped 1cm above the buffy-coat while avoiding the buffy-coat. Platelet-poor plasma was centrifuged a second time at 2,500g for 15min at RT. The platelet-free plasma was collected into a fresh tube using a micropipette, while leaving about 100µl of platelet-free plasma at the bottom of the tube and pooled. The normal pooled plasma (NPP) was frozen in liquid nitrogen and stored at -80°C.

4.3.2 Cell culture

Adherent MDA-MB-231 breast cancer cells (ATCC number HTB-26) were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in RPMI-1640 medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (Lonza, Verviers, Belgium). Cells were maintained in a 5% CO_2 humidified atmosphere at 37°C. Natrium

bicarbonate was set at 1.5g/L to control the pH. Cell viability was controlled by the trypan blue exclusion test.

4.3.3 In vitro generation of MVs

A modified version of the protocol reported by Davila et al. was performed (Davila, Amirkhosravi et al. 2008). Cells were adjusted to the desired concentrations in PBS and then incubated for 45min at 37°C without any stirring. Samples were centrifuged at 4,500g for 15 min and the cell supernatants which contain MVs were used in TGA.

4.3.4 Thrombin generation assay

To study the TGA sensitivity, a modified version of the method developed by Hemker was used. The first experiments were performed with PPP low reagent which corresponds to 1pM TF and 4µM PL (Thrombinoscope BV). This activator of clotting was diluted 10-fold or 100-fold in some experiments in order to study the sensitivity of TGA with low TF concentration and low concentration of PL. In a second step of the study, cell suspensions or *in vitro* generated MV fractions from 500 to 20,000 cells/ml were used as the source of TF and phospholipids (PL). Eighty µl of NPP and 20µl of different TF and PL concentrations or MV fractions were mixed in a 96-well microtiter plate (Thermo Immulon 2HB, USA) and were incubated for 10min at 37°C. PBS alone was tested as a negative control. The next steps of the protocol were already described (Robert, Ghiotto et al. 2009). Briefly, the plasma clotting was triggered by the addition of 20µl fluorogenic substrate/calcium chloride buffered solution at 37°C. A calibration curve was also performed for each blood draw using 80µl of NPP and 20µl of thrombin calibrator and 20µl of substrate/calcium chloride-buffered solution. The reaction of substrate hydrolysis was monitored on a microplate fluorometer Fluoroskan Ascent FL (Thermo Labsystems) with a 390/460-nm filter set (excitation/emission) using the Thrombinoscope® software version 3.0 (Synapse BV). The thrombin generation curves and more relevant parameters: lagtime which correspond to the initiation of clot formation (min), endogenous thrombin potential (ETP, nM/min) and thrombin peak (nM)) were calculated using software Thrombinoscope®. The results were expressed as means ± standard

deviation (S.D.) in percent in comparison of control (CTL) which represent 100%.

4.3.5 Inhibition of TPFI

Plasma TFPI was inhibited with a monoclonal antibody (ADG4903 American Diagnostica Gmbh) against Kunitz Domain I of human TFPI. Before TGA analysis, NPP was incubated 15min at 37°C with antibody at 10µg/ml in final concentration, before performing the experiment. PPP low reagent undiluted (1pM TF and 4µM PL) or diluted 10 times (0.1pM TF and 0.4µM PL) was used. Cell suspensions or *in vitro* generated MV fractions from 500 to 20,000 cells/ml were used as the source of TF and PL.

4.3.6 Plasma dilution

NPP was diluted twice to decrease level of TFPI. PPP low reagent undiluted or diluted 10 times was used. *In vitro* generated MV fractions from 500 to 20,000cells/ml were used as a second source of TF and PL.

4.3.7 Statistics

Comparison between different conditions was performed using Wilcoxon test on GraphPad Prism® software (version 5.01). Significant results have a P-value<0.05.

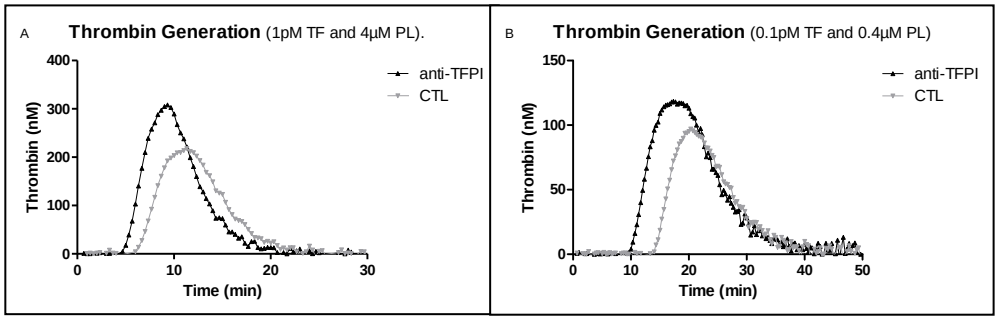

Figure 12: Thrombin generation experiments. Curve data are presented as mean with n=3. All the results are representative of the 3 independent experiments. A) Impact of antibody anti-TFPI on thrombin generation curve induced by 1pM TF and 4µM PL. CTL was NPP spiked with 1pM TF and 4µM PL without antibody. B) Impact of TFPI inhibitor on thrombin generation induced by 0.1pM TF and 0.4pM PL. CTL was NPP spiked with 0.1pM TF and 0.4µM PL without antibody (n=3).

4.4 Results

4.4.1 Effects of TFPI inhibition on TGA sensitivity triggered by 1pM TF and 4µM PL or 0.1pM TF and 0.4µM PL

Inhibition of TFPI by antibody increased the response of thrombin generation to TF and PL (**Figure 12**). A significant increase in peak and ETP and a significant decrease in lagtime were observed when NPP was incubated with anti-TFPI. The difference in lagtime and ETP was more important with 0.1pM TF and 0.4µM PL in comparison to 1pM and 4µM PL. A significant difference was only observed on the peak parameter with 1pM TF and 4µM PL (**Figure 13**).

4.4.2 Effect of plasma dilution on TGA sensitivity triggered by 0.01pM TF and 0.04µM PL

Dilution of plasma significantly increased the difference between thrombin generation initiated by 0.01pM TF and 0.04µM PL and thrombin generation without TF and PL (**Figure 14** A and B). For the lagtime mean the difference between CTL and 0.01pM TF and 0.04µM PL was 16% and insignificant when NPP was not diluted and 38% when NPP was diluted two times. For the peak mean the difference between CTL and very low TF and PL was 27% and insignificant when NPP was not diluted and 59% when NPP was diluted

two times. For the ETP mean the difference between CTL and 0.01pM TF and 0.04µM PL was 31% NPP and insignificant and 88% when NPP was diluted two times (**Figure 15**).

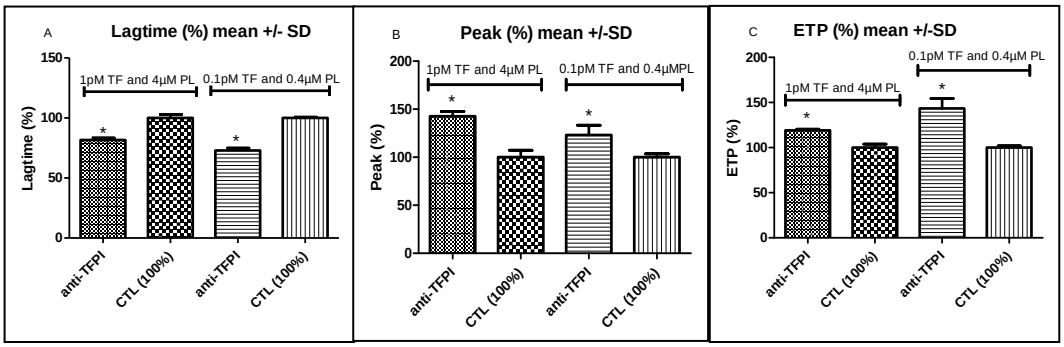

Figure 13. Thrombin generation parameters (Lagitme, Peak, ETP) expressed in percentages +/-SD. Graphs show the difference between thrombin generation curves when NPP is incubated or not with antibody anti-TFPI. Thrombin generation is triggered with 1pM TF and 4µM PL and with 0.1pM TF and 0.4µM PL. CTL use is NPP without antibody against–TFPI spiked with 1pM TF and 4µM PL or 0.1pM TF and 0.4µM PL. Significant differences ($P<0.05$) were indicated with an asterisk *.

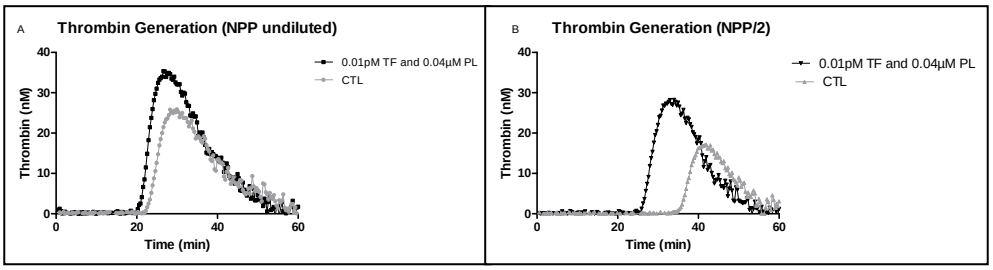

Figure 14. Thrombin generation experiments. Curve data are presented as means with n=9. All the results are representative of the 9 independent experiments. A. Thrombin generation curves with undiluted NPP. Thrombin generation is triggered by 0.01pM TF and 0.04µM PL. CTL use in is NPP undiluted spiked with PBS (no TF and PL) B. Thrombin generation curves with NPP diluted two times. Thrombin generation is triggered by 0.01pM TF and 0.04µM PL. CTL use in is NPP diluted two times spiked with PBS (no TF and PL).

4.4.3 Effect of TFPI inhibition and plasma dilution on TGA sensitivity triggered by MVs derived from different concentrations of MDA-MB-231 cells

Lagtime was the most sensitive TGA parameter when the reaction was activated by MVs derived from MDA-MB-231. The use of TFPI inhibitor decreased two times the cell concentration needed to produce enough MVs for a significant reduction of lagtime. Without TFPI inhibitor, the cell concentration required to produce MVs to significantly reduce lagtime was 5,000cells/ml. With TFPI inhibitor, only 2,500cells/ml were required to produce MVs to significantly reduce lagtime (**Figure 16**A and B). Moreover, the use of TFPI inhibitor decreased 2.4-fold the intra-assay variability.

Plasma dilution had no impact on the TGA sensitivity when TGA was triggered by MVs derived from MDA-MB-231 (**Figures 17**A and B). CTL use in is NPP diluted two times spiked with PBS (no TF and PL).

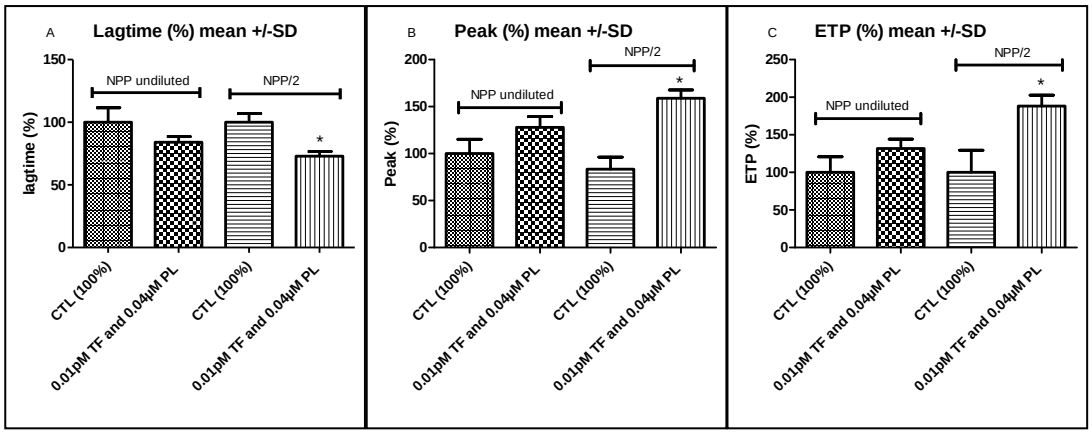

Figure 15. Thrombin generation curves parameters (Lagitme, Peak, ETP) data are presented as means +/-SD with n=9. All the results are representative of the 3 independent experiments. Graphs show the difference between thrombin generation curves when NPP is diluted or not. Thrombin generation is triggered with 0.01pM TF and 0.04µM PL. A lagtime parameter in % +/- SD. B peak parameter in %+/- SD. C ETP in % +/- SD. CTL use is NPP diluted or not unspiked with 0.01pM TF and 0.04µM PL. Significant differences ($P<0.05$) were indicated with an asterisk *.

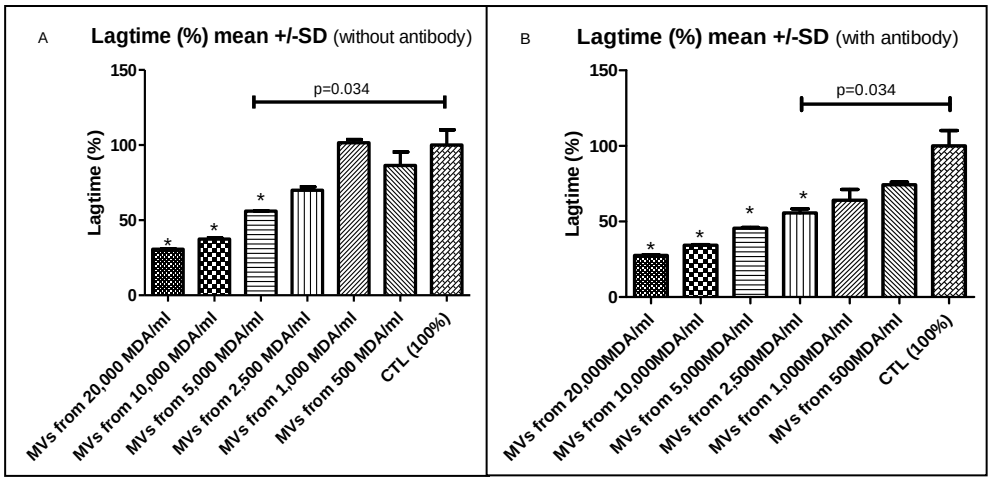

Figure 16.A. Thrombin generation curve parameter lagtime. Data are presented as means +/- SD with n=3. All the results are representative of the 3 independent experiments. A. Lagtime of NPP without antibody against-TFPI activated by MVs derived from MDA-MB-231 contained in supernatant of different cell concentrations. CTL use is NPP without antibody against TFPI spiked PBS. B. Lagtime of NPP activated by MVs derived from MDA-MB-231 and incubated with antibody against-TFPI. CTL use is NPP incubated with antibody against TFPI spiked PBS. Significant differences (P<0.05) were indicated with an asterisk *.

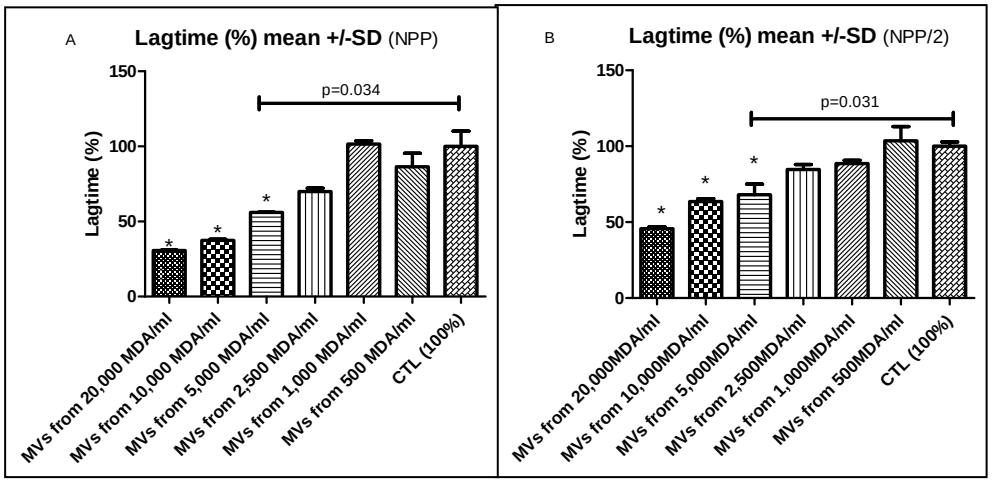

Figure 17.A. Thrombin generation curve of undiluted NPP activated by MVs derived from MDA-MB-231 at different cell concentrations. CTL use is NPP without antibody against TFPI spiked PBS. B. Thrombin generation curve parameter (lagtime) with NPP diluted two times and activated by MVs derived from MDA-MB-231 at different cell concentrations. CTL use is diluted NPP. Significant differences (P<0.05) were indicated with an asterisk * (vs CTL).

4.5 Discussion

VTE prophylaxis during systemic chemotherapy is not recommended for ambulatory patients with cancer (Khorana, Streiff et al. 2009). One of the reasons is the absence of validated biomarkers that allow the identification of a group of outpatients that would benefit for thromboprophylaxis (Khorana, Kuderer et al. 2008) The circulation of active TF associated with MVs has been considered as a promising biomarker to predict the risk of VTE in cancer patients (Manly, Wang et al. 2010, Zwicker 2010, Owens and Mackman 2011). An ongoing phase III study (MicroTEC, NCT00908960) in advanced cancer patients with high levels of TF-MVs has been designed to evaluate the benefit of primary thromboprophylaxis in reducing thromboembolic complications and to validate the prognostic usefulness of measuring TF-MVs (Menapace and Khorana , Zwicker, Liebman et al. 2009). However, validation and standardization of techniques measuring TF-MVs is required before considering cut-off values of this biomarker in risk-stratification approaches. In a previous study, we compared flow cytometry, electron microscopy and TGA to determine the size, the concentration and the PCA of tumour MVs. In this study, we focused on the optimal use of TGA to quantify the activity of TF-MVs. The aim was to compare the impact of plasma dilution and TFPI inhibition to increase the sensitivity of TGA. In the first part of the study, thrombin generation was triggered by known concentration of TF and PL. The inhibition of TFPI by blocking antibody increases the thrombin generation. The peak and the ETP are increased and lagtime is decreased when TFPI is inhibited. These results are in accordance with previous study (Gorczyca, Nair et al.). Moreover, the effect of TFPI inhibition on thrombin generation curves is more striking when system is triggered by 0.1pM TF and 0.4µM PL (**Figures 16 and 17**). These results suggest that thrombin generation is more sensitive when a diluted activator is used (Duchemin, Pan-Petesch et al. 2008). Strong activator of thrombin generation could hide some procoagulant effect. These first results led us to use weak activator of thrombin generation. The effect of plasma dilution on sensitivity was therefore measured with 0.01pM of TF and 0.04µM PL as activator. These results show that the signal of thrombin generation triggered by

0.01pM TF and 0.04µM PL in comparison of CTL (no TF and PL) is higher when plasma is diluted two times. Plasma dilution increases two times in average the response of thrombin generation assay to 0.01pM TF and 0.04µM PL. These results are in accordance with a previous study which showed that coagulation inhibition factors are most affected by plasma dilution (Duchemin, Pan-Petesch et al. 2008).

In order to assess the impact of TFPI inhibitor or plasma dilution on thrombin generation sensitivity for MV PCA detection, different MDA-MB-231 cell concentrations and cells derived MVs concentrations were used as coagulation activator. Lagtime parameter appears as the most sensitive parameter for TF-MV procoagulant activity (PCA) detection (Duchemin, Pan-Petesch et al. 2008). Thus, our results show that only MVs from 5,000 cancer cells/ml are required to significantly decrease the lagtime of thrombin generation curves. Other parameters such as ETP and Peak are only influenced by higher concentrations of MVs (MVs generated by > 10,000 cancer cells/ml). Moreover, our results show that lagtime can be significantly reduced by MVs derived from 2,500cells when TFPI is inhibited and that TFPI inhibition decreases the thrombin generation variability. The same conclusions can be drawn with the other TGA parameters (ETP, peak). Two times plasma dilution did not impact on the thrombin generation sensitivity to MVs. However, additional plasma dilution (> two times) do not give interpretable curves whatever the parameters used (lagtime, ETP, peak). This finding is of peculiar interest for TGA experiments when low plasma quantities are available (e.g. plasma from small animal experiments).

In conclusion, thrombin generation is a very sensitive method to study the procoagulant activity of TF-MVs. The sensitivity can be increased by TFPI inhibition. We recommend the TFPI inhibition with specific monoclonal antibody against Kunitz Domain I of human TFPI at 10µg/ml in final concentration for TGA experiments. It allows an augmented sensitivity of TGA and lower intra-assay variability. A two-fold plasma dilution is a valuable and cheaper alternative to study the procoagulant activity of MV by TGA with a good sensitivity, especially when low plasma volumes are available.

4.6 References

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5. Leukemic cells line can produced procoagulant extracellular vesicles *in vitro*

5 Leukemic cells line can produce procoagulant extracellular vesicles *in vitro*

Original article Thrombosis Research 2014:

Microparticle bearing tissue factor: a link between promyelocytic cells and hypercoagulable state.

Gheldof Damien, Mullier François, Bailly Nicolas, Devalet Bérangère, Dogné Jean-Michel, Chatelain Bernard, Chatelain Christian

In this study, we try to confirm that leukemic cells can shed procoagulant EVs and that these EVs have a major role in thrombotic activity of leukemic blasts. To assess this hypothesis, we use the combination of TGA, TEM and CMF to study *in vitro* EVs derived from acute leukaemia M3 subtype cell lines (HL-60 and NB4). The second part of this research is the study of anthracyclines impact on these cells and derived EVs PCA.

5.1 Abstract

Patients with haematological malignancies have a 28-fold increased risk of venous thromboembolism (VTE). Among patients with acute myelogenous leukaemia (AML), the 2-year cumulative incidence of VTE is 5.2%. Several studies suggest that microvesicles (MVs) harboring TF may play a role in VTE and disseminated intravascular coagulation (DIC) in acute promyelocytic leukaemia (APL).

The aim of this study was to assess the capacity of untreated APL cells to shed procoagulant MVs.APL cells (NB4 and HL-60 cell lines) and MVs were separated by filtration (0.1-0.22-0.45-0.65µm). The procoagulant activity (PCA) was assessed by thrombin generation assay (TGA). Alternatively, MVs were incubated with anti-Tissue Factor (TF) antibodies, with annexin V to assess the contribution of TF and phospholipids (PL) to the PCA, respectively.
NB4 cells had a high PCA mainly triggered by MVs of size under 0.45 µm. The PCA of MVs was related to the expression of active TF and PL. HL-60 cells had a weaker PCA since TF is mostly present in its inactive form. Moreover, HL-60 do not produce MVs<0.65 µm associated with PCA.

MVs could have a predicting value for VTE and DIC in patients with acute promyelocytic leukaemia and could inform physicians about the optimal use of a thromboprophylaxis.

Abbreviations: venous thromboembolism (VTE); acute myelogenous leukaemia (AML); microvesicles (MVs); disseminated intravascular coagulation (DIC); acute promyelocytic leukaemia (APL); procoagulant activity (PCA); Tissue Factor (TF); phospholipids (PL); acute lymphoblastic leukaemia (ALL); daunorubicin (DNR); normal pooled plasma (NPP); Transmission electronic microscopy (TEM); flow cytometry (FCM); thrombin generation assay (TGA); fluorescein isothiocyanate (FITC); phycoerythrin (PE); all-*trans* retinoic acid (ATRA)

5.2 Introduction

Despite the remarkable improvement of survival in patients with acute promyelocytic leukaemia for the past twenty years, a significant early mortality remains due to bleeding and thrombotic complications (Kwaan and Rego 2010). In patients with acute leukaemia, substantial activation of coagulation and resulting factor consumption result in localized thrombosis, widespread bleeding or both, hence termed the thrombohaemorrhagic syndrome (Ku, White et al. 2009). Thrombosis is a common complication of patients with malignancies (Falanga, Barbui et al. 2008). Patients with haematological malignancies have a 28 fold increased risk to develop venous thromboembolism (VTE) (Tesselaar, Romijn et al. 2007, Davila, Amirkhosravi et al. 2008). Among patients with acute myelogenous leukaemia (AML), the incidence of VTE is 5.2%, and among patients with acute lymphoblastic leukaemia (ALL), the incidence of VTE is 4.5% in the first two years of disease (Ku, White et al. 2009).

Treatment of patients with AML requires cytotoxic chemotherapy. Cytosine arabinoside and anthracyclines such as daunorubicin (DNR) are the most commonly used worldwide and are recommended as the standard of care (Fernandez 2010). Patients with AML often present with clinical or laboratory features demonstrating an activation of coagulation, such as prothrombin fragments 1 and 2, thrombin-antithrombin complexes, D-dimers, and fibrinopeptides A and B (Barbui and Falanga 2001). Ten % of APL patients die as a consequence of bleeding diathesis. These patients have increased procoagulant activity that generates the clinical syndromes often encountered, such as VTE and DIC (Ma, Liu et al. 2012).

Despite their influence on mortality and morbidity, the mechanisms inducing a hypercoagulable state are not fully understood to date. Multifactorial causes include physic immobility, chemotherapy adverse effects (Pollyea, Kohrt et al. , Schneider, Van Dreden et al.) and the overexpression of several procoagulant substances by cancer cells (cytokines, cysteine protease and tissue factor) (Davila, Amirkhosravi et al. 2008). Recent studies strongly demonstrate that cancer cells shed microvesicles (MVs) harbouring tissue

factor (TF-MVs). These TF-MVs have a primary role in thromboembolism by expression of procoagulant phospholipids (PL) such as phosphatidylserine, and TF. Tissue factor, the most potent initiator of the coagulation cascade, plays a critical role in haemostasis (Davila, Amirkhosravi et al. 2008, Petropoulou, Gerotziafas et al. 2008). TF is a transmembrane receptor which binds the coagulation serine protease FVII/VIIa to form a biomolecular complex that functions as the primary enhancer of coagulation *in vivo*. This complex activates both FX and FIX and leads to the generation of thrombin and fibrin (Kasthuri, Taubman et al. 2009, Wang, Manly et al. 2009). TF is present in blood in various forms. However, when carried by MVs, TF exerts its most intense procoagulant activity (Davila, Amirkhosravi et al. 2008, Mackman 2009, Mackman and Taubman 2009, Gheldof, Hardij et al. 2013). Microvesicles are considered as key components of the haemostatic system. They are small membrane vesicles shed from normal and/or tumour cells following activation or apoptosis. They can be captured in the developing thrombus and can lead to thrombin generation by expression of PL and TF (Mackman 2004, Furie and Furie 2008). Moreover, several studies strongly suggest that TF-MVs and their activity may have prognostic value in identifying cancer patients with increased risk of developing venous thromboembolism (VTE) (Owens and Mackman , Wang, Geddings et al. , Zwicker 2008, Zwicker, Liebman et al. 2012).

Understanding the pathogenic mechanisms of APL-related thrombosis is an important step towards developing preventive strategies against thrombotic complications of APL. The aim of this study was to assess the role of microvesicles derived from acute promyelocytic leukaemia cells, and to assess the contribution of TF and phosphatidylserine contained in MVs to activate the coagulation cascade. The effect of chemotherapeutic agents on MV procoagulant activity was also studied.

5.3 Methods

5.3.1 Design of the study

In order to confirm the role of MVs inleukemic cell lines, we assessed the cellular ability to produce MVs at the membrane surface by electron microscopy. In a second time, the expression of TF and PL on MV surface was studied by flow cytometry. Finally, MV PCA of two APL cell lines and the impact of chemotherapeutic on cells and MVs were studied by thrombin generation assay which provides information on their capacity to generate activated thrombin.

5.3.2 Cell culture

Acute myeloid leukemic cell lines (NB4 and HL-60) were cultured for 48h as previously shown (Gheldof, Hardij et al. 2013) in RPMI-1640 medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (Lonza, Verviers, Belgium). Cells were maintained in a 5% CO_2 humidified atmosphere at 37°C. Natrium bicarbonate was set at 1.5g/L to control the pH. Cell viability was controlled by the trypan blue exclusion test.

5.3.3 Preparation of normal-pooled plasma and platelet-free plasma

Normal pooled-plasma was prepared as previously described (Robert, Ghiotto et al. 2009, Mullier, Bailly et al. 2013) Blood was taken from forty-two healthy individuals by venipuncture in the antecubital vein and collected into 0.109M sodium citrate (9:1 v/v) tubes (Venosafe®, Terumo, Belgium) using a 21-gauge needle (Terumo, Belgium). The platelet-poor plasma (PPP) was obtained from the supernatant fraction of the blood tubes after a double centrifugation for 15min at 1500g at room temperature. Immediately after centrifugation, PPP from the 42 donors were brought together to obtain the normal pooled plasma (NPP) which was frozen at – 80°C without any delay. Frozen NPP samples are thawed and heat to 37°C for 5min just before experiment.

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5.3.4 Preparation of samples for Transmission electronic microscopy (TEM) observation

Samples were prepared as previously demonstrated (Gheldof, Hardij et al. 2013)**.** Briefly, cells and MVs were pelleted by ultracentrifugation. The pellet was fixed for TEM observation by glutaraldehyde and osmium tetraoxide. Samples were further washed and dehydrated by successive baths in ethanol and impregnation of samples in resin was then performed. After resin polymerization, samples were cut with an ultra-microtome and stained with uranyl acetate and lead citrate.

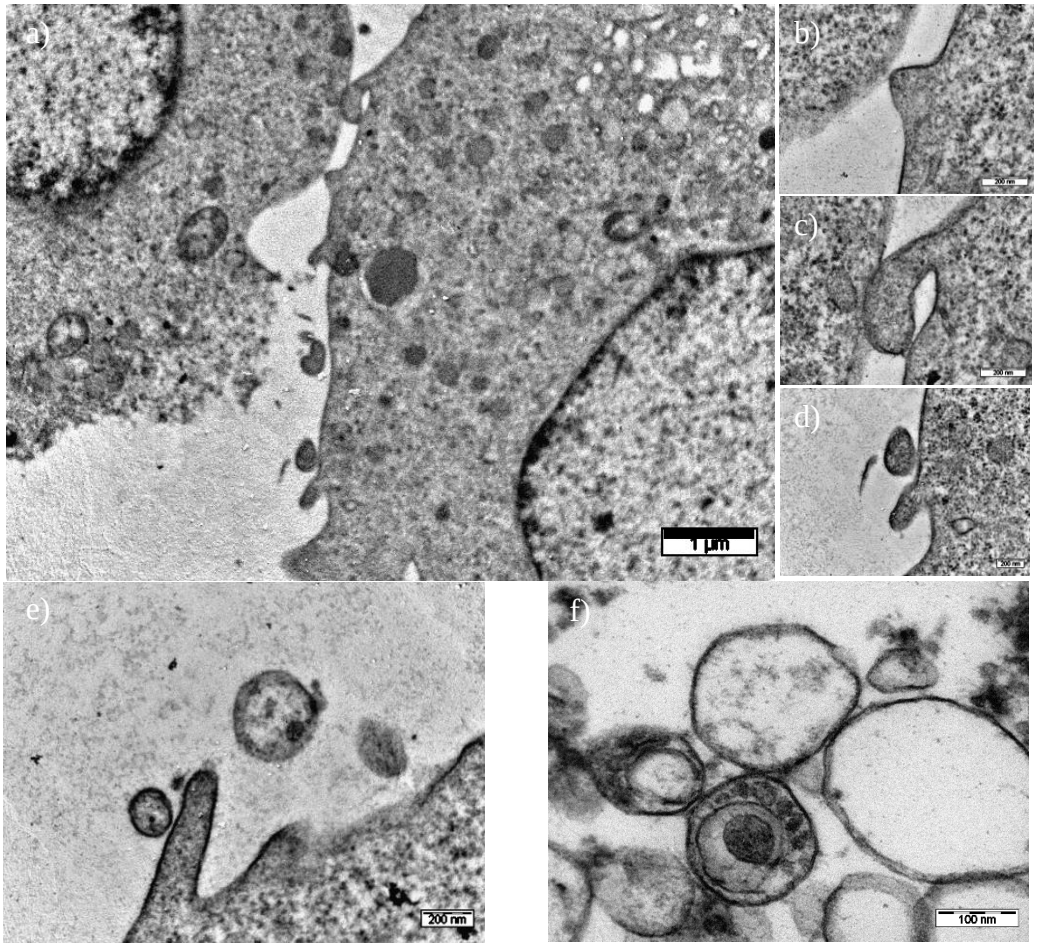

Figure 18. Transmission electron microscopy of cells and derived MVs after ultracentrifugation at 100,000g during 90min. a) Scale bar = 1 µm; b),c),d),e) Scale bar =200 nm.; f Scale bar=100nm.

5.3.5 Expression of TF and phosphatidylserine by flow cytometry (FCM)

Expressions of TF and phosphatidylserine on NB4 cells and MVs were carried out by FCM a BDIS FACS AriaI® flow cytometer (BD Biosciences, San Jose, CA, USA) with 488nm Coherent® SapphireTM solid state as light source was used for FCM measurements. Diva-6 software (Becton-Dickinson) was used for all data acquisition. MV fraction derived from cells cultured during 48h were labelled with CD142-PE and Annexin V-FITC. The size of MVs was defined using a blend of monodisperse fluorescent beads (Megamix, BioCytex, Marseille, France) of three diameters (0.5, 0.9 and 3 µm), a protocol already described (Gheldof, Hardij et al. 2013).

5.3.6 Measurement of the MV procoagulant activity (PCA) by thrombin generation assay (TGA)

PCA was measured by TGA according to a modified version of the method developed by Hemker (Hemker, Giesen et al. 2002) . In a 96-well round bottom plate, 20 µL of prewarmed trigger solution is added to one well and 20 µL of prewarmed calibrator, i.e. 600 nM alpha2M-thrombin complex to another; 80 µL of plasma is added to both wells. Ca++ is added together with the substrate at zero time (Fluka Solution). Readings are done in a microtiter plate fluorometer (Fluoroscan Ascent, Thermolabsystems, Helsinki, Finland), at 37°C, provided with a dispenser that is filled with FluCa solution. When the experiment is started, the dispenser squirts 20 µL of FluCa solution in each well at zero time. Cell suspensions or MV<0.65µm fractions from cells cultured two days were used as the source of TF and PL. 80 µl of NPP and 20µl of cell suspensions or MV fractions were mixed Culture medium was tested as a negative control. This protocol was described previously by Robert and coll. (Robert, Ghiotto et al. 2009). All the experiments were performed in triplicate.

5.3.7 Effect of MV size on PCA

Cells and MV fractions were filtered through 0.1, 0.22, 0.45 or 0.65µm membranes (Ultrafree-MC; Amicon, Bedford, MA, USA). Cells and filtrates were compared by TGA (1 experiment in triplicate).

5.3.8 Contribution of TF and PL to MVs PCA

For estimation of TF contribution to PCA, MVs < 0.65µm originating from a 2-day culture of cells were pre-incubated 10 min at 37°C with monoclonal

antibodies HTF-1 (BD Biosciences, Erembodegem, Belgium), a TF-activity neutralizing antibody (Wang, Manly et al. 2009) and the PLs-mediated activity was blocked with Annexin V before TGA was performed as previously described (Gheldof, Hardij et al. 2013). Three independent experiments were performed in triplicate.

5.3.9 Effect of daunorubicin (DNR) on MV and cell PCA

Acute myeloid leukemic cells lines were cultured 48h with 0.2 µg/ml of DNR (Sanofi-aventis, Belgium) and tested in TGA.

5.3.10 Effect of tissue factor activator on cells PCA

Acute myeloid leukemic cells lines and MVs derived were incubated for 2 minutes with 500 nM $HgCl_2$ (a potent TF activator)(Chen and Hogg 2013) in PBS before testing with TGA.

5.3.11 Statistics

Comparison between different conditions was performed using Wilcoxon test a nonparametric test that compares two paired groups on GraphPad Prism® software (version 5.01).

5.4 Results

5.4.1 Evidence for MV shedding and determination of MVs size by TEM

Figure 18 shows image of NB4 cells studied by TEM. It clearly illustrates the spontaneous release of MVs from these tumour cells. The bilayer membrane of the MVs is observed. The MV mean size after centrifugation was 337nm [range: 90 nm to 832 nm]. 52% of MVs are comprised between 200 nm and 400 nm.

5.4.2 Characterization of NB4 cells and NB4 derived MVs by flow cytometry

Figure 19 shows that MVs derived from NB4 cells are positive for tissue factor and positive for anV. A small proportion of MVs are positive for both TF and AnV.

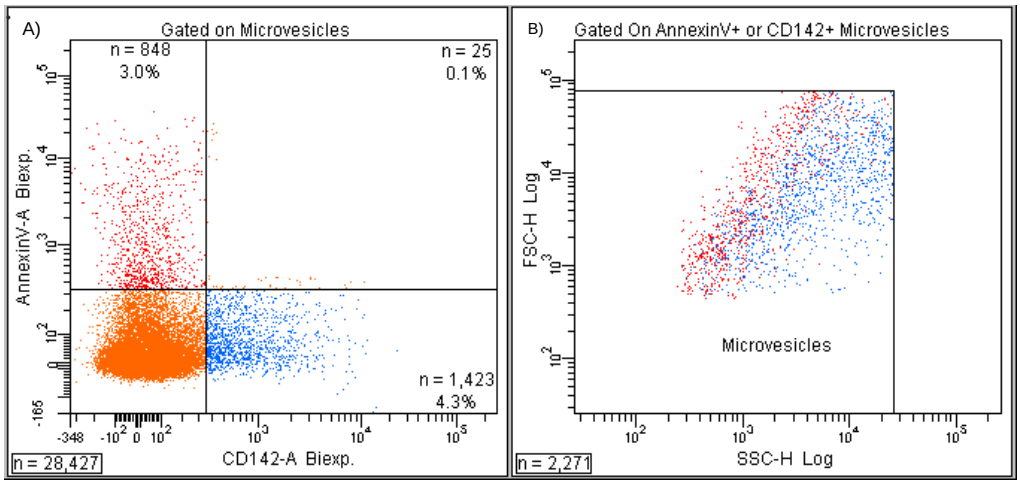

Figure 19. Expression of TF (CD142) and AnV (CD-227) on MVs<0.65µm after cell depletion. A) Analysis of tumour microvesicles. Dual fluorescence analysis of NB4 cells stained with AnV fluorescein isothiocyanate (FITC) (FL1) and CD142–phycoerythrin (PE) (FL2). CD142+ AnV+ MVs are represented as yellow dots, CD142+ AnV- MVs as red dots, AnV+ CD142- MVs as blue dots and background noise or other MVs as orange dots. B) Backgating of CD142+ AnV+ MVs (yellow dots), CD142+ AnV- MVs (red dots) and AnV+ CD142- MVs (blue dots) on FSClog-SSC log cytogram.

5.4.3 PCA Characterization of two acute promyelocytic leukaemia cells lines (NB4 and HL-60) by thrombin generation

NB4 cells and HL-60 cells can stimulate thrombin generation. HL60 cells reduce the lagtime 3.9-fold and increase the peak 1.6-fold when compared with control (**Figure 20**.B) and NB4 cells decrease the lagtime 12.6-fold and increase the peak 7.1-fold in comparison to control. No PCA is observed in HL-60 filtered with 0.65 µm membrane (no statistical difference in lagtime peak and endogenous thrombin potential (ETP)) (**Figure 20**.B). By contrast, NB4 cells can support thrombin generation activity when filtered at different sizes. MVs of sizes <0.65 and <0.45 µm decrease the lagtime 6.2- and 2.9-fold, respectively and increase the peak of thrombin 4.4- and 2.3-fold, respectively. MVs of sizes lower than 0.22 µm reduce the lagtime 1.9-, and increase the peak 1.5 (**Figure 20**.A). No procoagulant activity is associated with medium filtered through 0.1 µm. Thrombin generation activity of MVs derived from NB4 cells <0.65µm is abolished when anti-TF antibodies or AnV is added during preincubation (**Figure 20**.C).

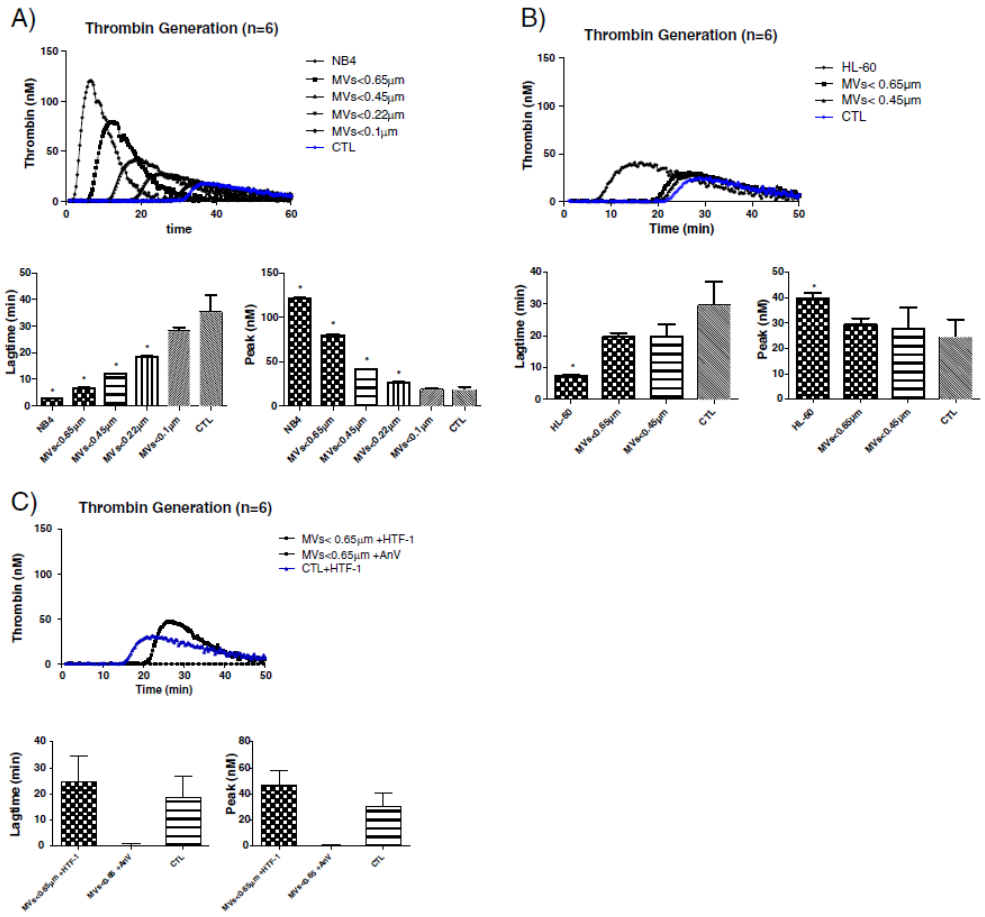

Figure 20. Thrombin generation curves and histograms of lagtime and peak parameters (n=6). A) In control condition (CTL), normal pooled plasma (NPP) was spiked with culture medium. NPP spiked with NB4 cells (NB4), and NB4 cells filtered through membranes with various sizes 0.1µm/0.22µm/0.45µm/0.65µm (MVs<…). B) Thrombin generation experiments. In control condition (CTL), NPP was spiked with culture medium. NPP spiked with HL-60 cells (HL-60), and HL-60 cells filtered through membranes with various sizes 0.45µm/0.65µm (MVs<…). C) Thrombin generation experiments. NPP was spiked with MVs derived from NB4 filtered at 0.65µm incubated with HTF-1 (MVs<0.65µm+ HTF-1) and with AnV (MVs<0.65µm+AnV). In control NPP spiked with culture medium and HTF-1. Data with significant difference in comparison to control are indicated with * ($p<0.05$).

5.4.4 PCA characterization of two acute promyelocytic leukaemia cells lines (NB4 and HL-60) treated with daunorubicin by thrombin generation

Addition of daunorubicin to cultures increases the thrombin generation by NB4 and HL-60 cells and derived MVs of <0.65µm. Lagtime of NB4 cells is slightly decreased by this treatment. No significant lagtime difference between MVs<0.65µm and MVs<0.65µm DNR is observed. The most affected parameter by DNR treatment is the peak of thrombin generation. Treated cells have a 1.4 fold peak increase and MVs derived from these cells have a 1.3-fold peak increase when compared with untreated cells. The same effect is observed on MVs<0.45 – 0.22µm since the peak is significantly increased by DNR (**Figure 21**). Lagtime of HL-60 is decreased 1.2-fold when cells are treated, and peak is increased by a 1.7-fold factor. When cells are treated with DNR, MVs<0.65 µm have a weak significant procoagulant effect as measured by either the peak or the lagtime in contrast with MVs<0.65 µm derived from untreated cells. ETP is not affected by the treatment (**Figure 22**).

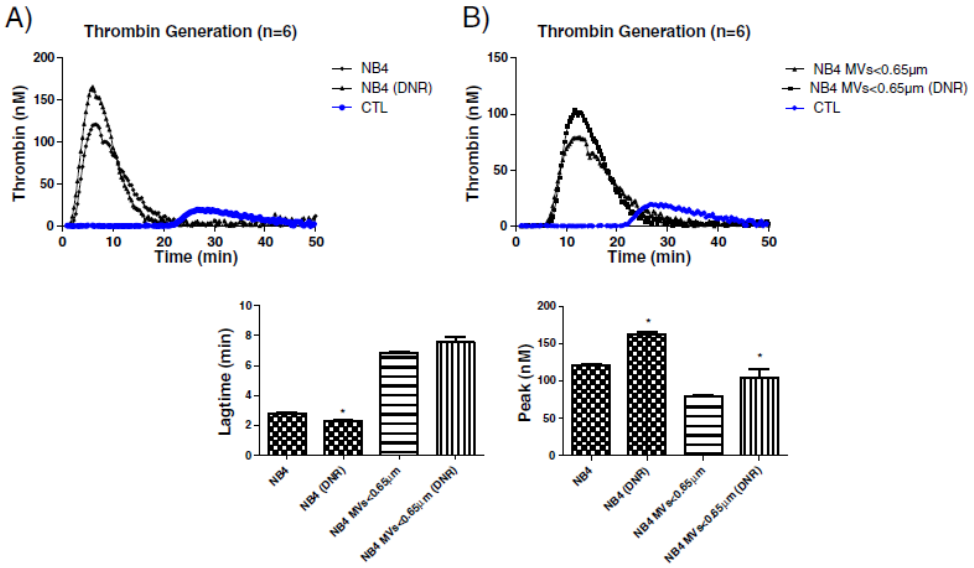

Figure 21. Thrombin generation curves and histograms of lagtime and peak parameters (n=6). A) Impact of DNR treatment on procoagulant activity of NB4 cells. B) Impact of DNR treatment on procoagulant activity of MVs<0.65µm derived from NB4 cells. Data with significant difference in comparison to untreated condition are indicated with * ($p<0.05$).

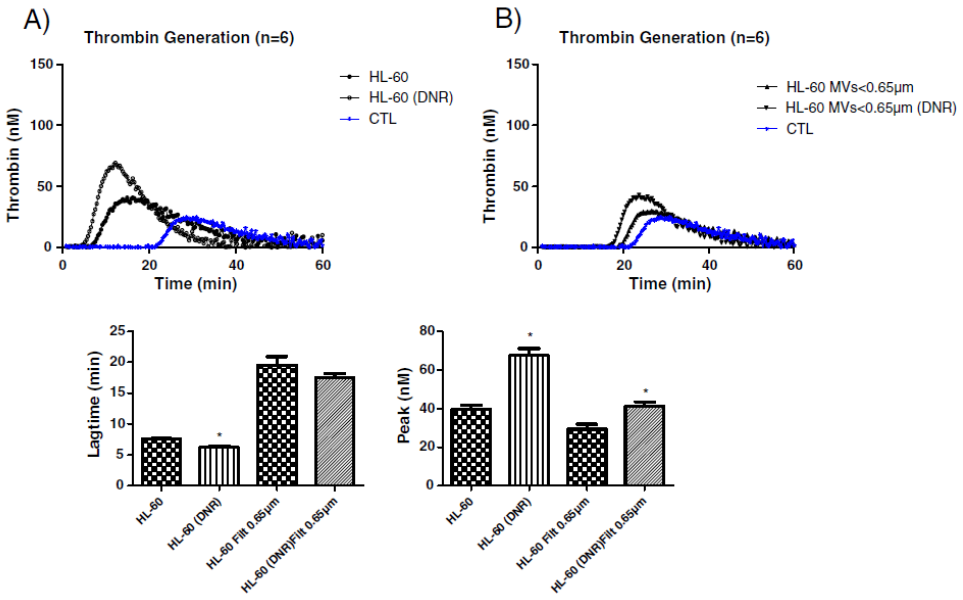

Figure 22. Thrombin generation curves and histograms of lagtime and peak parameters (n=6). A) Impact of DNR treatment on procoagulant activity of HL-60 cells. B) Impact of DNR treatment on procoagulant activity of MVs<0.65µm derived from HL-60 cells. Data with significant difference in comparison to untreated condition are indicated with * ($p<0.05$).

5.4.5 PCA characterization by thrombin generation for acute promyelocytic leukaemia cells lines (NB4 and HL-60) treated with tissue factor activator ($HgCl_2$)

As seen in **Figure 23**.A, NB4 cells have a high procoagulant activity and $HgCl_2$ does not alter this effect. **Figure 23**.B. shows that HL-60 cells have a procoagulant activity which is in turn increased by the addition of the TF activator $HgCl_2$, whatever the considered paramete. Lagtime of HL-60 treated is reduced 1.3-fold, ETP and peaks are increased 1.5- and 1.6- fold respectively (**Figure 23**.B).

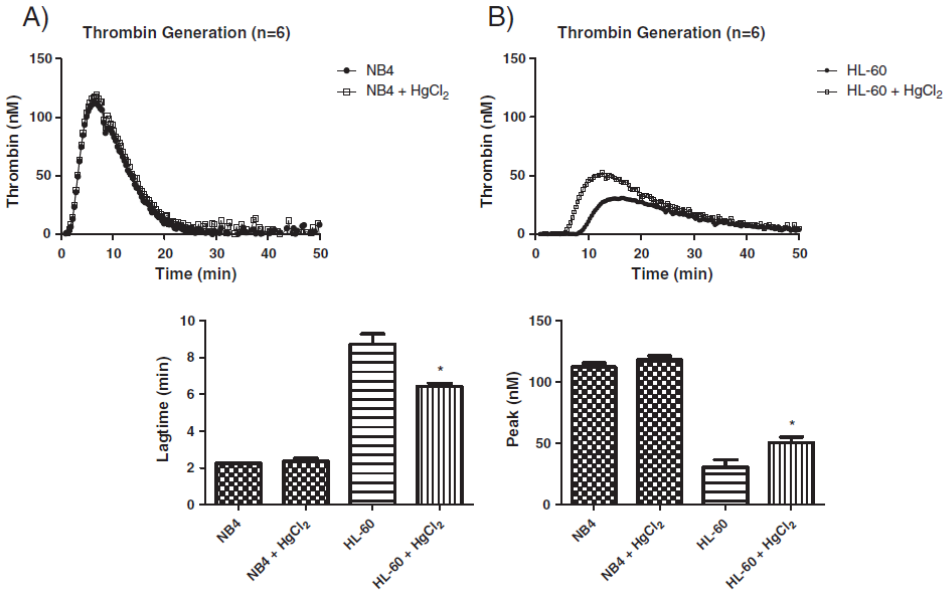

Figure 23. Thrombin generation curves and histograms of lagtime and peak parameters (n=6). A) Thrombin generation experiments. Curve data are presented as mean with n=6. Effect of TF activator ($HgCl_2$) on NB4 cells thrombin generation activity. B) Effect of TF activator on HL-60 thrombin generation activity. Data with significant difference in comparison to untreated condition are indicated with * ($p<0.05$).

5.5 Discussion

Since early mortality in leukaemia chemotherapy is often due to the high rate of bleeding complications, investigation of the pathogenesis of the haemostatic dysfunction in these diseases becomes a matter of high priority (Kwaan and Rego 2010). Cancer cells release procoagulant MVs into plasma (Haubold, Rink et al. 2009). Thrombosis and disseminated intravascular coagulation (DIC) complicate acute leukaemias either before or after chemotherapy suggesting that malignant cells release a procoagulant activity leading to increased thrombin activity. Moreover, in acute promyelocytic leukaemia, this exacerbated coagulation tends to disappear after all-*trans* retinoic acid (ATRA) therapy, a drug allowing these peculiar malignant cells to maturate. In order to check if hypercoagulation states encountered in acute leukaemias and especially in acute promyelocytic leukaemias originate from

Tissue Factor-bearing MVs, NB4 and HL60, two APL cell lines, were tested *in vitro* for their ability to produce MVs and increase TF activity and thrombin generation.

This study shows the capacity of NB4 cells to produce MVs. These MVs are shed by the plasma membrane of NB4 cells as illustrated by electron microscopy. Our data clearly illustrate the steps of MVs' generation, and confirm the bilayer ultrastructure of their membrane. MVs derived from NB4 cells have a size comprised from 90 to 832 nm with a large majority from 200 to 400 nm. Consequently, they should not be considered as exosomes, in accordance with previous studies (Castellana, Toti et al. 2010, Leong, Podor et al. 2011). However, these dimensions are measured on slices and may be underestimated since the vesicles are not always cut through their equators. On the other hand, as observed with manufactured nanoparticles (Tenzer, Docter et al. 2011), MVs may be associated with proteins ("corona effect") during TEM preparation leading to a overestimation of the size.

By flow cytometry, MVs derived from NB4 cells are characterized by their expression of TF and phosphatidylserine according to other studies (Koyama, Hirosawa et al. 1994, Marchetti, Diani et al. 2012) . Flow cytometry shows that cells and MVs express TF. NB4 cells have been previously described with a high expression of TF (Marchetti, Diani et al. 2011) By TGA we show that most of the procoagulant activity of NB4 cells comes from MVs between 100nm and 650nm. This result confirms the production of procoagulant MVs by leukemic cells. By different filtrations, and the use of inhibition of PL, we demonstrate that MVs with procoagulant activity derived from NB4 cells have a size higher than 100 nm, and that the MV procoagulant activity is linked to the expression of TF and PL mainly phosphatidylserine. Surprisingly, HL-60 cells have not the same procoagulant profile. Their procoagulant activity is associated to the cells themselves and no PCA is observed when cells are filtered through 0.65µm membrane. These cells are sensitive to all-trans retinoic acid (ATRA) therapy in contrast to NB4 cells. The treatment by DNR of both cells types increases the procoagulant activity of cells. Moreover, the procoagulant activity of MVs is also increased when cells are treated, which can strengthens our hypothesis on major implication

of MVs derived from leukemic cells in procoagulant state of patient with or without treatment. DNR treatment affects principally the TGA peak parameter, which is the most sensitive to PL. This is in accordance with another study which shows an increased procoagulant activity after DNR treatment (Zhu, Guo et al. 1999). DNR-treated cells have an increased expression of phosphatidylserine on surface and this could lead to tissue factor activation (Langer, Amirkhosravi et al. 2004). The phospholipid composition of MVs could be different when there are generated by treated or untreated cells. This increase of MVs' PCA after treatment could be due to the same causes of the increased PCA of whole cells. When comparing cells treated by DNR and by TF factor activator there is a difference only for HL-60 cells. However TF activation has a weak impact on TGA's peak. This result is in accordance with the hypothesis that the increased peak by DNR is mostly due to the overexpression of phosphatidylserine. Moreover, when HL-60 cells are treated with TF activator the lagtime is reduced after DNR addition which suggests that TF is activated by phosphatidylserine expression after DNR treatment (Langer, Amirkhosravi et al. 2004).

This study suggests the implication of TF-MVs derived from leukemic cells in inducing an increased procoagulant state. Moreover, the characterization of leukemic TF-MVs should be a useful biomarker for thromboembolism prediction (Zwicker, Liebman et al. 2012). Speculatively, it seems that MVs derived from leukemic cells could have a bad prognostic outcome since they could be related to ATRA resistance of leukemic cells for example. Szczepanski *et al.* also showed that MVs derived from cancer cells can induce an immune suppression and the demise of antitumor CD8(+) effectors T cells, thus contributing to tumour escape (Wieckowski, Visus et al. 2009, Szczepanski, Szajnik et al. 2011). These advances in MVs function comforts us in considering MVs derived from cancer cells as a key component in cancer disease.

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6. Plasmatic procoagulant extracellular vesicles from acute leukemic patients as potential biomarker of procoagulant state

6 Plasmatic procoagulant EVs from AL patients as potential biomarker of procoagulable state.

Article in preparation

Microvesicles are a potential biomarker for DIC and thrombosis in acute leukemic patients.

Gheldof Damien, Dogné Jean-Michel, Graux Carlos, George Fabienne, Sonet Anne, Chatelain Bernard, Mullier François, Chatelain Christian

Our hypothesis that leukemic cells can shed procoagulant EVs is proved *in vitro*. In this research we try to confirm this hypothesis *in vivo*. We try to confirm that patient with AL who suffer from thromboembolic disease have in their plasma an increased PCA due to the presence of EVs. We also study if the PCA of plasmatic EVs came from their procoauglant phospholipids surface or/and tissue factor anchored in EVs.

In order to study plasmatic EVs from leukemic patients, we tightly work with Biobank of CHU Dinant Godinne. We set up in January 2012 a protocol to take blood from acute leukemic patient in order to respect restrictive pre-analytic conditions associated to EVs PCA study (Mullier, Bailly et al. 2013). We work on plasmatic EVs before and after treatment induction in order to study the chemotherapy impact on plasmatic EVs PCA and in order to confirm that chemotherapy can induced a procoagulant state by increased EVs PCA.

6.1 Abstract

Background: Hypercoagulable state is a common complication of patients with malignancies. Recent studies suggest that extracellular vesicles (EVs) procoagulant activity (PCA) may have a major role in venous thromboembolism or disseminated intravascular coagulation (DIC) in acute leukaemia.

Aims: The aim is to study the impact of EVs from leukemic patient on thrombin generation and if EVs-PCA could be a potential biomarker to evaluate hypercoagulable state in patient with acute leukaemia.

Methods: Blood samples from 38 patients with acute leukaemia newly diagnosed were obtained at Day-0 (before treatment), D-3 and D-7 (3 and 7 days after treatment). Extracellular vesicles were isolated and concentrated by ultracentrifugation. EVs-PCA and EVs-tissue factor activity was measured a commercial bio-immunoassay (Zymuphen MP-TF®), respectively.

Results: Among acute leukemic patients, 3 patients have an increased EVs PCA at D-0 and 2 of them developed a thrombotic event. The remaining patients without thrombotic events do not show an increased EVs-PCA. One patient has an increased extracellular vesicles procoagulant activity at D-3 and developed a DIC at D-5. Three patients have an increased EVs-tissue factor activity (>2pg/ml), among these, two developed a thrombotic event and one had haemorrhage. A plasma from patient with induced DIC has no increased extracellular vesicles tissue factor activity from D-0 to D-7 (<2pg/ml).

Conclusions: The results suggest a strong association between hypercoagulable state and extracellular vesicles procoagulant activity. Moreover, extracellular vesicles procoagulant activity could have a predicting value for venous thromboembolism disseminated intravascular coagulation in patients with acute leukaemia and especially for excluding patient without risk of thrombotic events. .

Abbreviations: venous thromboembolism (VTE) disseminated intravascular coagulation (DIC) acute myeloblastic leukemia (AML) acute lymphoblastic leukaemia (ALL) extracellular vesicles (EVs) tissue factor (TF) procoagulant phospholipids (PL) procoagulant activity (PCA) thrombin generation assay (TGA) body mass index (BMI) before treatment (D-0) 3 days after treatment (D-3) 7 days after treatment (D-7) normal pooled plasma (NPP) ultracentrifugation (UC) standard deviation (S.D.) healthy volunteers control (CTL) tissue factor pathway inhibitor (TFPI) negative predictive value (NPV) positive predictive value (PPV)

6.2 Introduction

Despite the remarkable improvement of survival in patients with acute leukaemia for the past twenty years, a significant early mortality remains due to bleeding and thrombotic complications (Ku, White et al. 2009, Kwaan and Rego 2010). Hypercoagulable states such as venous thromboembolism (VTE) and disseminated intravascular coagulation (DIC) are highly prevalent complications of cancer and anticancer therapy (Tesselaar, Romijn et al. 2007, Davila, Amirkhosravi et al. 2008, Khorana 2013, Khorana, Dalal et al. 2013). Among patients with acute myeloblastic leukaemia (AML), the incidence of VTE is 5.2%, and among patients with acute lymphoblastic leukaemia (ALL), the incidence of VTE is 4.5% in the first two years of disease (Ku, White et al. 2009). However, the underlying mechanisms behind a hypercoagulable state are not yet fully understood. Multifactorial causes include physical immobility, chemotherapy adverse effects (Pollyea, Kohrt et al. , Schneider, Van Dreden et al.) and the overexpression of several procoagulant substances by cancer cells (cytokines, cysteine protease, tissue factor…) (Davila, Amirkhosravi et al. 2008). Previous studies have demonstrated that cancer cells and leukemic blast can shed extracellular vesicles (EVs) harbouring tissue factor (TF-EVs) (Davila, Amirkhosravi et al. 2008, Gheldof, Mullier et al. 2014). Tissue factor, the most potent initiator of the coagulation cascade, plays a critical role in haemostasis (Davila, Amirkhosravi et al. 2008, Petropoulou, Gerotziafas et al. 2008, Mackman 2009, Mackman and Taubman 2009, Diego-Garcia, Peigneur et al. 2013). EVs are considered as key components of the haemostatic system. These are small membrane vesicles shed from normal and/or tumour cells following activation or apoptosis. They can be captured in the developing thrombus and lead to thrombin generation by expression of PL and TF (Mackman 2004, Furie and Furie 2008) (**Figure 24**). The co-expression of TF and procoagulant phospholipids (PL) such as phosphatidylserine on the TF-EVs is highly prothrombotic. We and others have previously demonstrated that acute leukemic cells as promyelocytic cells generate EVs with TF-PCA (**Figure 24**) (Zhou, Shi et al. 2010, Gheldof, Mullier et al. 2014, Thaler, Pabinger et al. 2014).

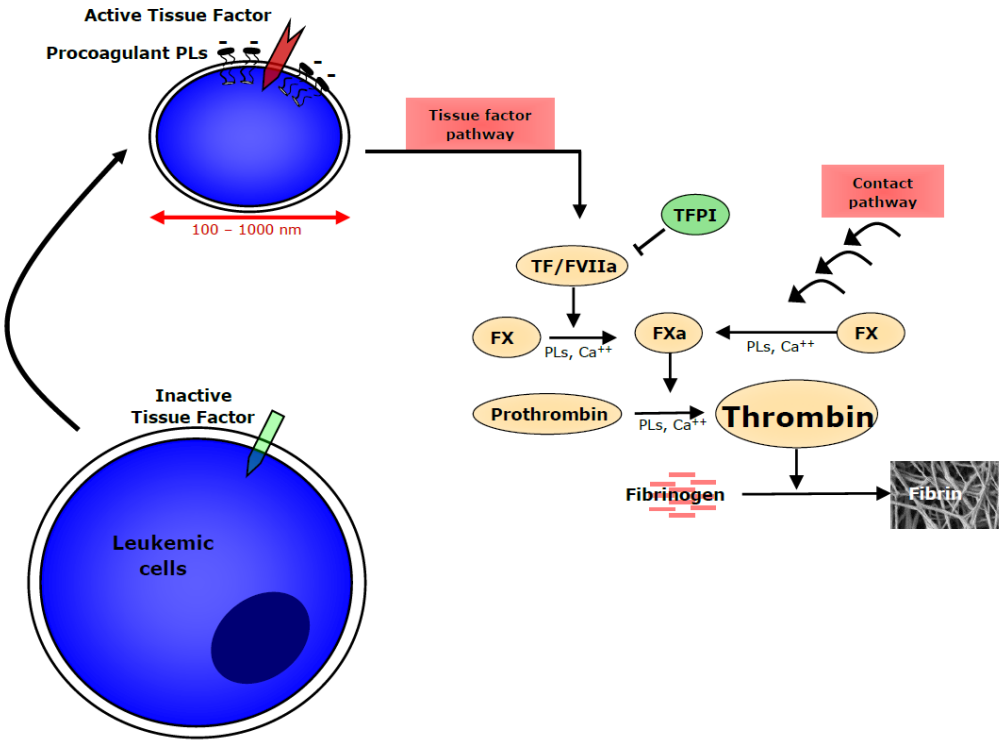


Figure 24. Activation of coagulation cascade by EVs derived from leukemic cells. Activated TF and procoagulant phospholipids on EVs surface activates tissue factor pathway of coagulation cascade. Complex TF-factor VII activates factor X which activates prothrombin. These activations need negatively charged phospholipids (PLs) and Ca^{++}. Tissue factor pathway inhibitor (TFPI) is present in plasma and inhibits the complex TF/FVIIa/FXa.

EVs concentration and surface antigens can be estimated by several analytical technics such as flow cytometry, atomic force microscopy, transmission electron microscopy and dynamic light scattering. However, they are all associated with multiple limitations reviewed elsewhere (Waisman, Danino et al. 2007, Tatischeff, Larquet et al. 2012, Issman, Brenner et al. 2013). Therefore, we mainly focused on the measurement of the EVs procoagulant activity using adapted thrombin generation assay (TGA) and a specific bio-immunoassay measuring TF-activity (Gheldof, Hardij et al. 2013, Gheldof, Mullier et al. 2013, Thaler, Pabinger et al. 2014).

The primary aims of this study was to evaluate the PCA of plasma EVs from patients with AL by thrombin generation assay and to study the correlation between EVs PCA and hypercoagulable state. The second objective was to determine the impact of chemotherapy on EV-PCA and the contribution of TF activity in EV-PCA by a bio-immunoassay measuring TF-activity (Zhou, Shi et al. 2010).

6.3 Methods

6.3.1 Laboratory and clinical data from patients with acute leukaemia

A total of 38 consecutive patients newly diagnosed with AL following the French-American-British criteria were included in this study (Walter, Othus et al. 2013). All patients signed the informed consent following the requirements of local ethical committee (Ethical committee approval number B039201212954). The study included 17 white females and 13 white males ranging from 17 to 76 years old with body mass index (BMI) between 16 and 34 kg/m^2. Patient's characteristics are summarized in table 2. The D-dimer analysis was performed on STA-Liatest DDi (LI) (Diagnostica Stago, Asnières-sur-Seine, France). Thrombin time, activated partial thromboplastin time and prothrombin time were performed on STA-R (Diagnostica Stago, Asnière sur Seine, France). Platelet count, leucocyte count and haemoglobin measurement were performed on XE-2100 (Sysmex, Kobe, Japan). The measurement of EVs-TF activity was performed with a bio-immunoassay zymuphen MP-TF (Aniara manufactured by HYPHEN BioMed, Neuville sur Oise, France). Thrombosis and haemorrhage diagnosis are performed based on routine standard clinical practices. Overt DIC diagnosis was performed according to the scoring system of the Scientific and Standardisation Committee on DIC of the International Society on Thrombosis and Hemostasis (Taylor, Toh et al. 2001, Knobl 2005).

6.3.2 Blood sampling

Blood was taken by venipuncture in the antecubital vein and collected into 0.109 M sodium citrate (9:1 v/v) tubes (Venosafe®, Terumo, Leuven, Belgium) using a 21-gauge needle (Terumo). Samples were obtained from 44 patients before treatment (D-0), 3 days after treatment (D-3) and 7 days after

treatment (D-7) (17)(Schultz, Massing et al. 1997) since persistence of blood and bone marrow blastosis at day 7 is of poor prognosis. So day 3 and day 7 represent reasonable times of estimating action of chemotherapy on blast cells and residual normal cells, before aplasia. Platelet-free plasma was obtained by a first centrifugation at 2,500g for 15min at room temperature (Heraeus Multifuge 1S-R, Sysmex Benelux, Etten-Leur, The Netherlands) with a light brake only, within one hour after sampling. The platelet-poor plasma was collected and transferred into a polypropylene haemolysis tube with a micropipette. Aspiration was stopped 1cm above the buffy-coat while avoiding the buffy-coat. Platelet-poor plasma was centrifuged a second time at 2,500g for 15min at room temperature. The platelet-free plasma was collected into a fresh tube using a micropipette, while leaving about 100µl at the bottom of the tube. Plasmas samples are frozen in liquid nitrogen and stored at -80°C.

6.3.3 Isolation and concentration of EVs for thrombin generation assay

Extracellular vesicles from patient plasma or NPP are isolated by ultracentrifugation (UC). 400µl of frozen plasma were thawed at 37°C and centrifuged at 100,000g for 90min at 4°C. The pellet of EVs is re-suspended in 60µl plasma free EVs (supernatant after UC) to concentrate EVs 6.7 times.

6.3.4 Preparation of normal-pooled plasma and platelet-free plasma

Normal pooled-plasma was prepared as previously described (Robert, Ghiotto et al. 2009, Mullier, Bailly et al. 2013). Blood was taken from forty-two healthy individuals, after information consent signed, by venipuncture in the antecubital vein and collected into 0.109M sodium citrate (9:1 v/v) tubes (Venosafe®, Terumo, Belgium) using a 21-gauge needle (Terumo, Belgium). Platelet-free plasma was obtained by a first centrifugation at 2,500g for 15min at room temperature (Heraeus Multifuge 1S-R, Sysmex Benelux, Etten-Leur, The Netherlands) with a light brake only, within one hour after sampling. Platelet-poor plasma was collected and transferred into a polypropylene haemolysis tube with a micropipette. Aspiration was stopped 1cm above the buffy-coat while avoiding the buffy-coat. Platelet-poor plasma was centrifuged a second time at 2,500g for 15min at room temperature. Platelet-free plasma was collected into a fresh tube using a micropipette, while leaving

about 100µl at the bottom of the tube. Plasmas samples are frozen in liquid nitrogen and stored at -80°C.

6.3.5 Measurement of the EVs procoagulant activity (PCA) by thrombin generation assay (TGA)

PCA was measured by TGA according to a modified version of the method developed by Hemker (Hemker, Giesen et al. 2002) . In a 96-well round bottom plate, 20µL of pre-warmed plasma EVs was added to one well and 20µL of prewarmed calibrator, i.e. 60 nM alpha2M-thrombin complex to another well; 80µL of plasma was added to both wells. Calcium (Ca^{++}) was added together with the substrate at the start of the measurement (Fluka Solution). Concentrated EVs from patient samples, healthy donors and NPP were used as the source of TF and PL. 80µl of NPP and 20µl of concentrated EV were mixed.

Readings were performed in a microtiter plate fluorometer (Fluoroscan Ascent, Thermolabsystems, Helsinki, Finland), at 37°C. Briefly, the plasma clotting was triggered by the addition of 20µl fluorogenic substrate/calcium chloride buffered solution at 37°C. A calibration curve was also performed for each blood draw using 80µl of NPP and 20µl of thrombin calibrator and 20µl of substrate/calcium chloride-buffered solution. The reaction of substrate hydrolysis was monitored on a microplate fluorometer Fluoroskan Ascent FL (Thermo Labsystems) with a 390/460-nm filter set (excitation/emission) using the Thrombinoscope® software version 3.0 (Synapse BV). The thrombin generation curves and all parameters (lagtime, peak, endogenous thrombin potential and time to peak) were calculated using software Thrombinoscope®. The results were expressed as means ± standard deviation (S.D.) in percent in comparison of PCA of EVs from healthy volunteers control (CTL) which represent 100%.

In order to increase thrombin generation sensitivity to TF-EVs PCA, plasma TFPI was inhibited with a monoclonal antibody (ADG4903 American Diagnostica Gmbh) against Kunitz Domain I of human TFPI. Before TGA analysis, NPP was incubated 15min at 37°C with antibody at 10µg/ml in final concentration, before performing the experiment. This protocol was

described previously by Gheldof *et al.* (Robert, Ghiotto et al. 2009). All the experiments were performed in triplicate.

6.3.6 Measurement of EVs-TF activity

The measurement of EVs-TF activity was performed with Zymuphen MP-TF® kit according to the manufacturer's instructions. Briefly, 20 µl of patient plasma with MP-TF-assay enhancer were introduced into the wells of a microplate coated with a murine monoclonal antibody, specific for human TF (10H10), and which does not interfere with TF activity. Following an overnight incubation and an automated washing step, washing solution was immediately introduced into the wells. Then, factor VIIa and factor X were added. The TF-FVIIa complex forms and subsequently activates factor X into activated factor X (FXa). In a third step, a specific substrate for FXa was introduced and reacts with FXa and produces a yellow color. The absorbance, recorded at 405nm on a spectrophotometer, is directly proportional to the amount of MP-TF present in the sample. Normal plasmas are found below 2pg/ml of EV-TF.

6.3.7 Statistics

Comparison between different conditions was performed using Wilcoxon test on GraphPad Prism® software (version 5.01).

6.3.8 Data Analysis

The sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) were calculated using Medcalc software (version 10.4.8) (Gent, Belgium).

6.4 Results

In this study, lagtime which corresponds to the initiation of clot formation (min) and thrombin peak which corresponds to maximal concentration of active thrombin (nM)) are the most relevant parameters to discriminate patients with or without thrombotic events (data not shown).

At D-0, 3 out of the 38 patients had no significantly increased ration in comparison to CTL (100%). All of these patients did not develop any

thrombotic event. Among the 3 patients with a significant increase of ratio, patients #11 and #17 had an hypercoagulable state and had D-Dimer levels at >6000 ng/ml and 1,370 ng/ml respectively. Patient #11 developed a DIC and patient #17 developed a venous thromboembolism in kidney. Patient #9 did not develop any clinically relevant thrombotic event. Patient with increased EVs PCA and without clinical thrombotic event had a D-Dimer value of 630 ng/mL. Only EVs from patient #9 had a reduced lagtime and increased peak with a ratio Peak%/Lagtime% over 1.7 without clinical thrombotic event (**figure 25**).

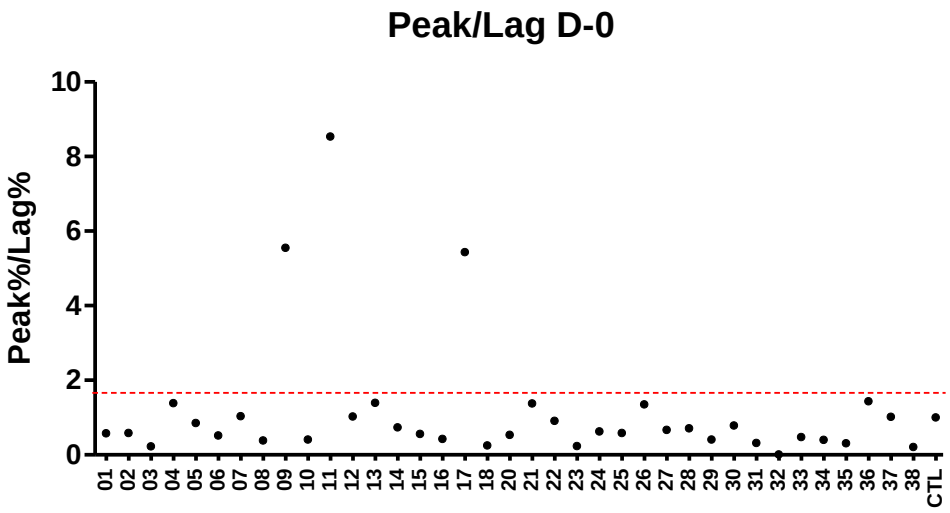


Figure 25. Procoagulant activity of EVs from patient at diagnosis (D-0) before any treatment, ratio between peak and lagtime. Peak (%) /Lagtime (%). CTL used represent 100% Peak/ 100% Lagtime.

At D-3, patient #16 had a ratio Peak%/Lagtime% of 1,76 and experienced DIC at D-5 with D-Dimer level higher than 1000 ng/ml. Patient #17 with ratio Peak%/Lagtime% at 1,77 was diagnosed with venous thromboembolism in kidney at D-0. (**Figure 26**). The combination of peak and lagtime parameter allows distinction between thrombotic and non-thrombotic populations (**Figure 26**).

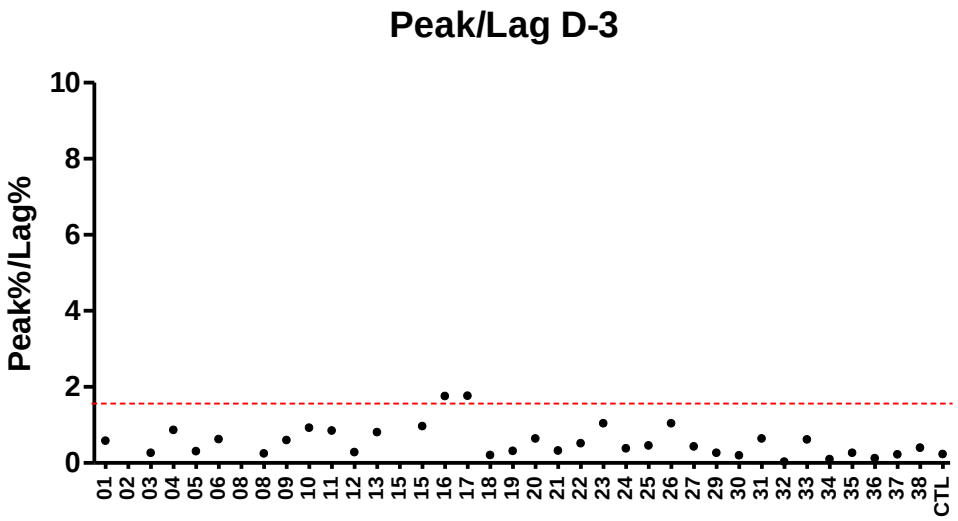


Figure 26. Procoagulant activity of EVs from patient 3 days (D-3) after induction of chemotherapy, ratio between peak and lagtime. Peak (%) /Lagtime (%).CTL used represent 100% Peak/ 100% Lagtime.

At D-7, none of the patients showed a decreased lagtime. Only patient #11 had a 38% augmentation in the peak in comparison to CTL. However the ratio Peak%/Lagtime% is under 1.7 (**Figure 27**). The NPV of this combination on the tree times points was 100% (95% CI: 89.6 to 100%) and the PPV was 80.0% (95% CI: 20.3% to 95.9%). The sensitivity and specificity were 100% (95% CI: 30.5% to 100%) and 95.72% (95% CI: 85.0% to 99.5%), respectively.

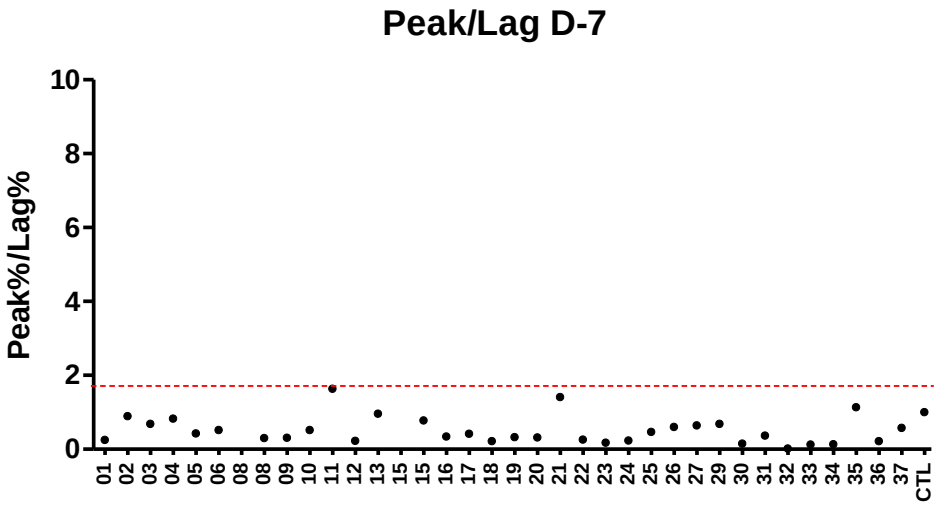
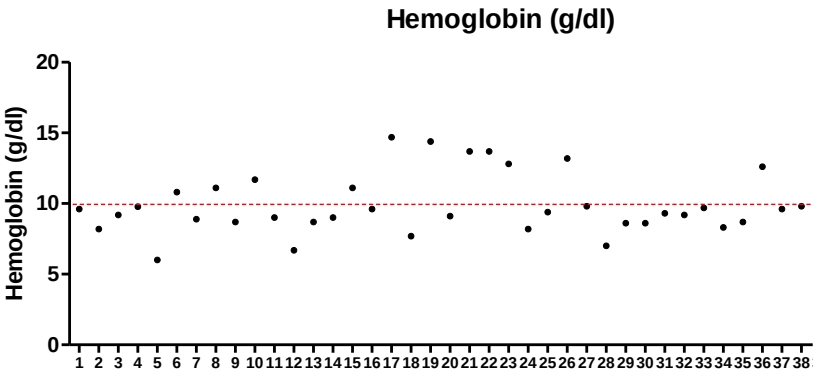
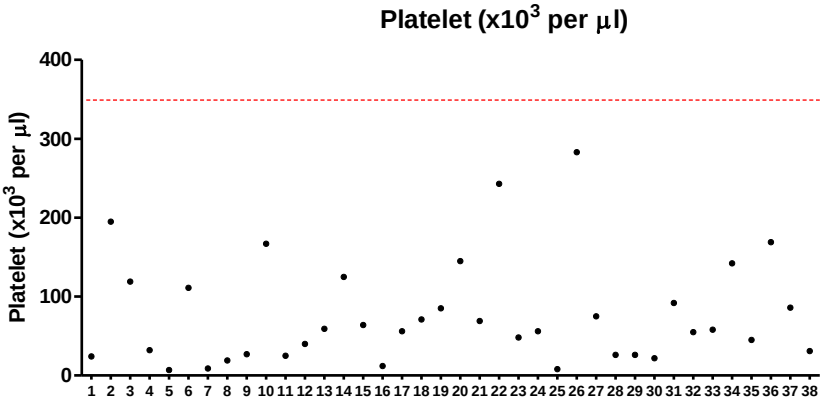
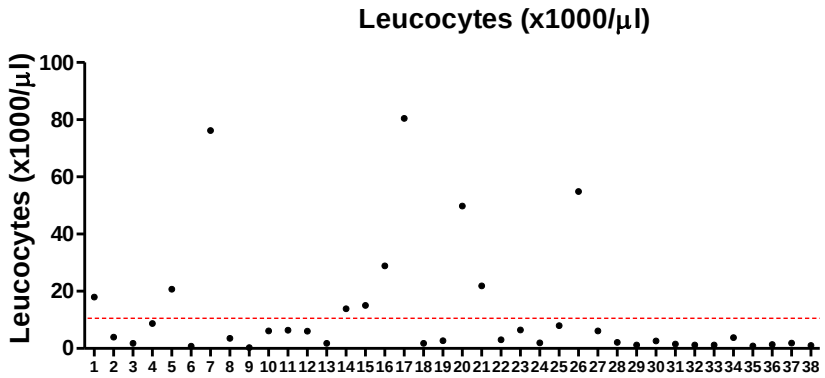


Figure 27. Procoagulant activity of EVs from patient, 7 days (D-7) after induction of chemotherapy, ratio between peak and lagtime. Peak (%) /Lagtime (%).CTL used represent 100% Peak/ 100% Lagtime.

Biological parameters used in Khorana score risk model at D-0 are displayed in **Figure 28**. Based on these parameters, patients who developed thrombotic events cannot be dissociated from patients without thrombosis. Among twenty patients, none had a platelet count higher than 350.000/µl and no one with thrombotic event had a haemoglobin concentration below 10 g/dl (Table 2). For leucocyte count, 8 patients had leukocyte count higher than 11.000/µl; the cut off proposed in the Khorana score model (Khorana and Connolly 2009). Among these patients, two had a hypercoagulable state (VTE in kidney and induced DIC five days after induction of the treatment).


Hemoglobin (g/dl)
Hemoglobin (g/dl)
0
5
10
15
20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38


Platelet (x10^3 per µl)
Platelet (x10^3 per µl)
0
100
200
300
400
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38


Leucocytes (x1000/µl)
Leucocytes (x1000/µl)
0
20
40
60
80
100
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38

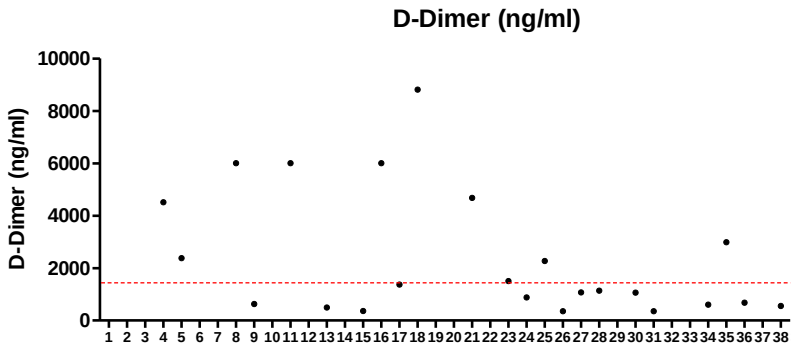


Figure 28. Biological parameters used in Khorana score risk model at diagnosis day. One point is attributed when platelets count> 350 G/l; Hemoglobin level< 100 g/l ; Leucocytes count > 11 G/k ; D-Dimer > 1.44 µg/mL

The activity of EVs-TF was determined by Zymuphen MP-TF®. These data show a high EVs-TF activity (>2pg/ml) in patient #7 who had haemorrhage (epistaxis, buccal petechiae, digestive bleeding), patient #11 who developed a DIC and #17 who developed a kidney thrombosis. Patient #16 who developed an induced DIC 5 days after treatment induction had no increased EVs-TF activity.(**Figure 29**) The NPV of this bioassay was 97.1% (95% CI: 85.3 to 99.5%) and the PPV was 66.7% (95% CI: 11.6% to 94.5%). The sensitivity and specificity were 66.7% (95% CI: 11.6% to 94.5%) and 97.1% (95% CI: 85.0% to 99.5%), respectively.

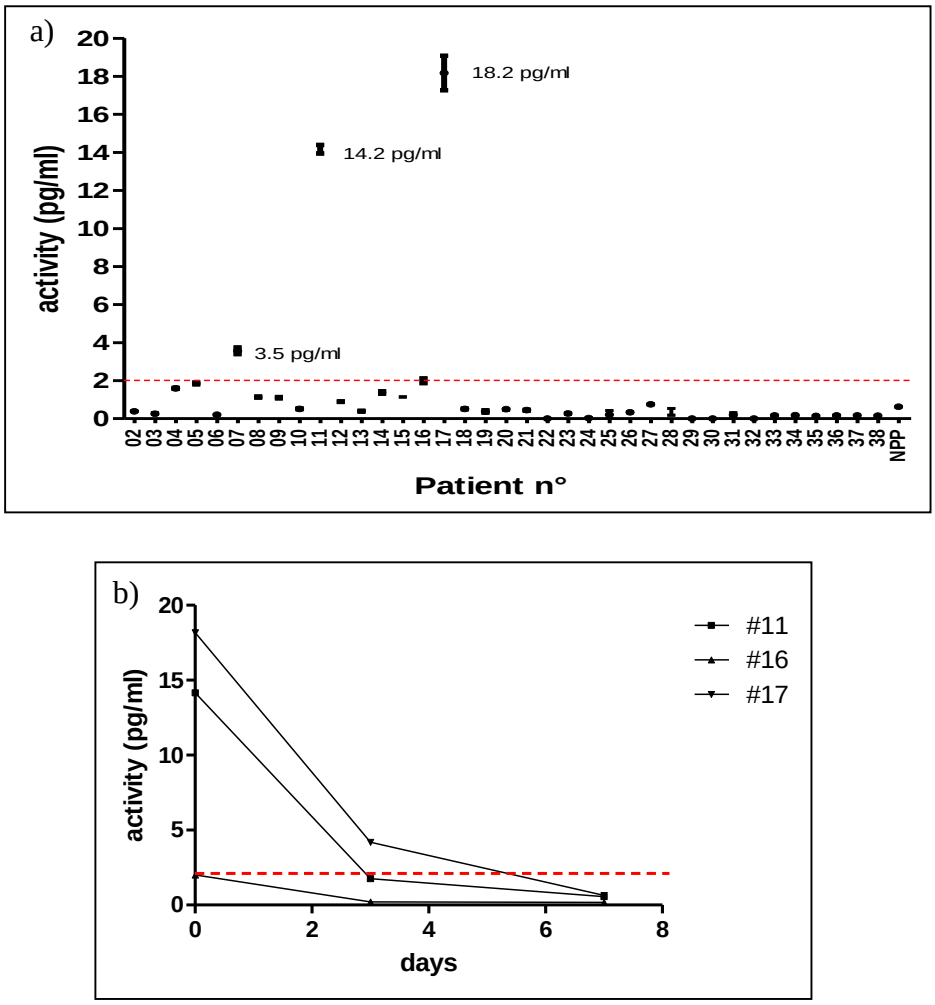


Figure 29. a) EVs TF activity in pg/ml in plasma of each patient at diagnosis day b) EVs-TF activity of patient #11, #16 and #17 with thrombotic event (DIC, induced DIC, kidney thrombosis) during seven days after induction of chemotherapy.

6.5 Discussion

Early mortality in acute leukaemia is partly due to haemostatic dysfunctions such as bleeding, thrombosis or DIC. The underlying mechanisms are still not fully understood. In this study, we aimed at investigating the implication of EVs in thrombosis. The second objective is to determine the contribution of TF activity in EV-PCA and the impact of chemotherapy on EV-PCA (Zhou, Shi et al. 2010).

Our results at diagnosis days (D-0) show that EVs from three patients have a higher potential to support thrombin generation in comparison to healthy donors. EVs from these patients have a significant impact on TGA parameters, such as lagtime reduction and increased peak (Owens and Mackman 2012). This increased PCA could be associated to an increase of EVs number and/or of PCA of EVs by expression of TF or procoagulant phospholipids (Li, Yu et al. 2010, Lima, Oliveira et al. 2011).

Interestingly, two out of these three AML patients had hypercoagulable state with DIC and thrombotic event at diagnosis. Patients #11 developed an overt DIC at study inclusion (Taylor, Toh et al. 2001, Knobl 2005), patient #17 developed a VTE in Kidney and patient #143 developed a DIC. At diagnosis, patients without thrombotic event had a similar or weaker PCA in their EVs than those from healthy controls. Only one patient (#9) had an increased PCA of EVs in comparison to CTL and does not have thrombosis.

The analysis of PCA of EVs from patients #16 at D-3 shown a slight increase of ratio in comparison to CTL and this patient developed a DIC 5 days after treatment induction. The ratio lagtime/peak shows the distinction between PCA of EVs from this patient and other patients. This increased EVs PCA can be associated to chemotherapy induction. As previously shown in several studies, chemotherapy increases procoagulant state and can rise the procoagulant activity of EVs or the EVs shedding from cancer cells (Khorana and Connolly 2009, Van Aalderen, Trappenburg et al. 2011, Gheldof, Mullier

et al. 2013). Chemotherapy could induce externalisation of phosphatidylserine on the cell surface and subsequently lead to activation of TF (Langer, Amirkhosravi et al. 2004, Tormoen, Recht et al. 2013, Langer and Ruf 2014).

Patient #11 with AML M3 has EVs PCA similar to healthy subject at D-3. This reduction of EVs PCA between D-0 and D-3 was confirmed by the bio-immunoassay and could be due to ATRA treatment at D-0. ATRA is well known to reduce the expression of TF by induction of differentiation leukemic cells (Zhang, Hu et al. 2007, Marchetti, Russo et al. 2011).

Seven days after treatment, only one patient had an increased EVs fraction PCA. This patient developed a DIC at diagnosis. This increased EVs-PCA could be due to treatment by cytarabine and idarubicine at D-3 (Tormoen, Recht et al. 2013).

The Zymuphen MP-TF® confirms the results of TGA for two patients. However, issue of patient #16 cannot be detected by this test. This suggests a lack of sensitivity for Zymuphen MP-TF® in comparison to TGA (Hellum, Ovstebo et al. 2012) (Gheldof, Chatelain et al. 2014).

This study strongly supports the role of EVs in haemostatic disorder. EVs produced by leukemic cells could have a major role in thrombosis development and hypercoagulable state in patient with acute leukaemia. In addition to their contribution in haemostatic disorder, EVs could be considered as biomarker for the risk of thrombosis. Currently, only the Khorana risk score is available to predict thrombosis in cancer patient. It is based on neutrophil count, platelet count, haemoglobin, D-dimers and body mass index is only available for patients with solid tumours (Khorana and Connolly 2009). However, as shown in Table 1 some of the parameters are irrelevant in AL since neutrophil count, platelet count and haemoglobin concentration are affected by the disease and their treatments. There is a need to develop specific biomarkers which could predict the risk of thromboembolism or DIC in AL patients.

In this context, adapted TGA with an increased sensitivity to TF-EVs PCA (Gheldof, Mullier et al. 2013) provides an estimation of PCA. This is more relevant to estimate the overall procoagulant activity of EVs rather than the more specific estimation of MP-TF activity (Gheldof, Chatelain et al. 2014). The combination of lagtime and peak parameters provides promising support for identifying high, low and perhaps no risk patients for developing with higher sensitivity, negative predictive value and specificity than Zymuphen MP-TF® (**Figures 25, 26** and **29**)

This pilot study has some limitation due to the limited number of patients and requires further confirmation on a larger population (Mullier, Bailly et al. 2013). Due to this limitation, no stratification was possible to assess the impact of the different treatment protocols.

In conclusion, PCA of EVs should be further developed as marker of the hypercoagulable state in AL and especially as a way to detect patients which can benefit from anticoagulation despite their thrombocytopenia.

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Table 2. Patient's characteristics at diagnosis. FAB: French-American-British; ALL: acute lymphoblastic leukaemia; AML: acute myeloblasitc leukemia; PLT: platelet; Hgb: Haemoglobin; Fib: FibrinogenAPTT: activated partial thromboplastin time; PT: prothrombin time; DIC: disseminated intravascular coagulation.

Patient	Age, sex	Diagnosis (FAB classification)	Hgb (g/dl)	Leucocytes (X1000/µl)	PLT (x1000/µL)	Fib (ng/µL)	APTT (sec)	PT (%)	D-dimer (ng/mL)	Haemostatic complication	Treatment
CTL			12.0-15.8	3.9-9.9	150-400	180-400	26-38	75-100	<500		
1	17, male	ALL-B	9.6	17.94	24	505	42.9	94	ND	petechiae	Daunorubicin
2	70, female	AML M2	8.2	3.92	195	434	31	100	ND	none	Aracytine+ Daunorubicin
3	76, female	AML M0	9,2	1.78	119	291	32.9	95	ND	none	VIDAZA(Raj and Mufti 2006)
4	56, female	AML M2	9.7	8.71	32	301	25.3	94	4510	none	Aracytine + Idarubicin
5	53, female	AML M2	6.0	20.69	7	608	31.4	73	2380	Haemorrhage	Aracytine + Idarubicin
6	55, male	ALL-B	10.8	0.79	111	392	26.1	81	ND	none	Idramycin + Vincristin
7	64, male	AML M5	8.9	76.24	9	315	30.2	89	ND	Haemorrhage	none

8	21, male	ALL-B	11,1	3.5	19	459	31.4	57	>6000	none	Fralle 2000(Mitchell, Lambers et al. 2010)
9	48, female	ALL-B	8.7	0.29	27	276	30.6	93	630	none	Oncovin + Asparaginase
10	49, female	AML M4	11,7	6.13	167	316	31.2	86	ND	none	Aracytine + Idarubicin
11	34, female	AML M3	9	6.36	25	84	26.4	73	>6000	DIC	ATRA D-0 + Aracytin/Idarubicin D-3 to D-9
12	47, female	AML	6.7	6.07	40	ND	34	75	ND	none	Aracytine + Idarubicin (Hovon 102)(Cornelissen, van Putten et al. 2007)
13	70, female	AML M6	8.7	1.8	59	562	28	98	500	none	POLO-AML-2 (Hartsink-Segers, Exalto et al. 2013)
14	57, female	AML M0	9	13.88	125	667	29	81	ND	Haemorrhage	Aracytine + Idarubicin
15	34, female	ALL-T	11.1	15.03	64	420	27.1	98	360	none	GRAALL 2005(Maury, Huguet et al. 2010)
16	58, male	AML M1	9.6	28.89	12	245	27.9	80	>6000	Induced DIC at D-5	HOVON 102(Cornelissen, van Putten et al. 2007)
17	47, male	AML M5	14.7	80.42	56	382	30.8	68	1370	kidney thrombosis	HOVON SAKK AML 102(Cornelissen, van Putten et al. 2007)
18	36, male	AML	7.7	1.8	71	364	30.1	73	>6000	none	HOVON-SAKK AML 102(Cornelissen, van Putten et al. 2007)

19	71, male	AML M2	14.4	2.68	85	402	34	78	ND	none	HOVON 103 AML/SAKK(Cornelissen, van Putten et al. 2007)
20	43, male	AML M0	9.1	49.78	145	887	32.9	92	360	none	HOVON 102(Cornelissen, van Putten et al. 2007)
21	60, male	ALL-B	13.7	21.86	69	520	29.2	94	4680	non	Daunorubicin
22	63. male	AML M1	13.7	3.07	243	ND	31.2	82	ND	none	HOVON SAKK AML 102(Cornelissen, van Putten et al. 2007)
23	34, male	ALL-B	12.8	6.44	48	384	38.9	87	1500	none	GRAALL 2005(Maury, Huguet et al. 2010)
24	45, female	AML M4	9.2	1.98	56	378	32	76	880	none	Aracytine+ Idarubicin
25	73, female	AML	9.4	7.94	8	280	29.2	62	2270	Haemorrhage	POLO-AML-2 (Hartsink-Segers, Exalto et al. 2013)
26	58, female	ALL-B	13.2	54.9	283	449	32	100	350	none	GRAALL 2005(47)
27	57, male	AML	9.8	6.09	75	197	23	59	>6000	none	HOVON 102(45)
28	63, male	AML M2	7	2.12	26	605	33.6	78	1140	none	HOVON SAKK AML 102(Cornelissen, van Putten et al. 2007)
29	58. female	AML M4	8.6	1.22	26	498	ND	73	ND	none	HOVON 102(45)
30	65, male	AML M6	9.3	1.58	92	375	33.1	88	350	none	HOVON SAKK AML102(45)

31	65, male	AML M6	9.3	1.58	92	375	33.1	88	350	none	HOVON SAKK AML102(45)
32	65, male	AML M6	9.2	1.22	55	552	33.4	87	ND	none	HOVON SAKK AML 132
33	41, male	ALL-B	9.7	1.24	58	ND	21.2	83	ND	none	GRAALL 2005[48]
34	44, female	AML M2	8.3	3.77	142	375	27.5	71	600	none	HOVON SAKK AML 132
35	73, female	AML M3	8.7	0.88	45	513	31.4	99	2990	none	ATRA + Idarubicin
36	73, male	AML M2	12.6	1.36	169	488	32.5	90	680	none	Hovon 103 AML/SAKK[46]
37	60, female	AML M6	9.6	1.9	86	270	27.9	77	ND	none	HOVON SAKK AML 132
38	55, male	AML	9.8	1.07	31	268	30.6	92	550	none	HOVON SAKK AML 132

7. General discussion and future perspectives

7 General discussion and future perspectives

The prevention of hypercoagulable state in cancer patient is a major goal to reduce the related death, the arrest of chemotherapy and the VTE-associated costs. As example, the mean cost of VTE management ranges from 7,700$ to 16,000$ (Bullano, Willey et al. 2005, Spyropoulos and Lin 2007, Lefebvre, Laliberte et al. 2012) with at least one half of the expenditure being attributable to hospitalization. In cancer patient, VTE associated costs can reach 20,000$ (Elting, Escalante et al. 2004).

The early death of leukemic patient by hypercoagulable complication such as VTE and DIC is still a major problem and it can be suppressed by all anticoagulant therapy currently available. Preventing thromboembolic complications in AL patients is not an easy task since the risk of haemorrhagic complications is also high due to the presence of thrombocytopenia in most cases (Guzman-Uribe and Vargas-Ruiz 2015). The only way to safely prevent thrombotic events in AL is via a better understanding of coagulation disorder related to leukaemia. The study of underlying mechanisms can lead to find new potential biomarkers to overcome the present score model for thrombosis and cancer patient, which is not suitable for AL patient.

Several studies suggest that EVs bearing TF are implicated in thrombosis related to cancer *in vivo*. (Morel, Toti et al. 2006) An extensive literature exists on the perturbation of EVs numbers and activity in various diseases. (Zwicker, Trenor et al. 2011). The circulation of active TF associated with EVs has been considered as a promising biomarker to predict the risk of VTE in cancer patients. In order to study EVs *in vitro* or to use EVs as biomarkers for clinical laboratory, several methods are required to overcome limitations of each one and provide a better characterization of EVs.

In this work we started by identifying several useful methods to study EVs. The combination of FCM, TEM and TGA allows studying antigens, the activity of TF and PLs on EVs surface, the formation and size of EVs and the size associated to PCA. Among these techniques, TGA proved to be a useful technique to study EVs-PCA origins and size.

In the second study, we aimed to optimise TGA by increasing sensitivity for TF detection, as TGA lack to study low EVs TF activity. We showed that inhibition of TFPI in NPP increased the sensitivity of EV-TF activity detection and decreased the variability of TGA.

The third study used TEM, FCM and TGA to illustrate that leukemic cells can shed EVs with PCA *in vitro*. By this combination of techniques, we assessed that leukemic cells can shed PCA EVs and this EVs came from TF and PLs on EVs surface. This study strongly supports the implication of EVs in thrombotic disease in leukemic patient.

The last study of this thesis showed that EVs PCA could be a promising biomarker to prevent thrombosis in AL patient. Only one score model is validated to predict thrombosis in cancer patient, the Khorana risk model. However, it is available only for solid tumors (Khorana and Connolly 2009). Some of the parameters included in this risk model are irrelevant in AL since neutrophil count, platelet count and hemoglobin concentration are affected by the disease and their treatments. These data show that we must find biomarkers not affected by the pancytopenia and highlight the used of new potent biomarkers such as P-selectin or EVs-TF (Connolly and Khorana 2010). Activity tests are more relevant than antigen assays and quantification by FCM (data not shown) to identify patients who developed thrombosis and to exclude patient without thrombotic event. This could be due to the multiple alternative spliced TF and the availability of Ab against TF (Bogdanov, Balasubramanian et al. 2003). Electron microscopy is not relevant to study EVs as biomarker. This technique cannot quantify EVs-TF and is too long in comparison to activity tests.

7.1 Clinical perspectives

Thrombin generation assay is a sensitive technique to evaluate the procoagulant activity from plasmatic EVs. However this technique should be coupled with an ultracentrifugation and with the use of antibody against TFPI which increase the TAT and the cost. The development of similar test

practical in clinic laboratory should be done with less time and cost expenditure.

A rapid and low cost EVs-TF activity test should be assessed on a large cohort of cancer patient. Cancer patients and their EVs-TF activity should be followed for a long time to study and correlate their outcomes (such as thrombosis (arterial or venous), thrombosis related death, thrombosis related to treatment and relapses) to EVs-PCA. Moreover, patients with thrombotic diseases not related to cancer (i.e DIC) could be included in the validation of EVs-PCA biomarker (Delabranche, Boisrame-Helms et al. 2013).

In parallel, a prospective clinical study could be proposed to assess the efficacy of anticoagulant to prevent blood clots in the veins in participants who have hematologic cancer and a high levels EVs-TF activity.

7.2 Biological perspectives

Vascular endothelial activation probably contributes to the procoagulant phenotype in haematological malignancies. Indeed, some *in vivo* results suggest an endothelium activation in haematological malignancies. Increased levels of circulating P-selectin and VWF have been observed in patients' blood compared to controls and would reflect this endothelial activation (Blann, Gurney et al. 2001). A procoagulant activity of endothelial cells after anthracyclines treatment has also been highlighted (Swystun, Shin et al. 2009) and an increased release of procoagulant endothelial EVs was observed after daunorubicin treatment. EVs generated by cancer cells could contribute to this endothelium activation by transfer of procoagulant protein, or by transfer of miRNA. This new insight into endothelial activation mechanism in cancer might be an interesting field of investigation (Bouvy, Gheldof et al. 2014).

EVs are also known to be a pathway for cell communication and are able to impact remote target cells' phenotype by carrying and transfer protein and nucleic acid (mRNA, miRNA) (Ratajczak, Wysoczynski et al. 2006). This impact of phenotype could be a way for drug multiresistance spreading

among tumor cells. This hypothesis is currently under investigation by our group in lymphoma and could be extended to other cancers. First result seems promising to confirm the transmission of multidrug resistance from resistant HL-60 to sensible HL-60 by EVs.

8. References

8 References

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