Faculty of Medicine
Department of Pharmacy

GIGA - Research - I[3]
Unit of Medical Chemistry

Characterisation of graft-versus-host disease biomarkers in plasma

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for the degree of
«Docteur en Sciences Biomédicales et Pharmaceutiques»

Academic year: 2012 - 2013

Abstract

Introduction of hematopoietic stem cell transplantation (HSCT) as an immunotherapy provided a new option to improve survival of patients affected by haematological malignancies and congenital immunodeficiency syndrome. However, acute graft-versus-host disease (aGVHD) remains a major life-threatening complication after HSCT, limiting its application. As no plasma biomarkers available in clinical laboratory for aGVHD diagnosis, observation of clinical manifestations and histological examination of organ biopsies constitute the current diagnostic approach. Thus, to optimize management of aGVHD and reduced therapy-related toxicity, an early specific, rapid and non invasive diagnosis is needed. As proteomic approaches are useful tools for the rapid screening of protein content in complex samples, it makes them attractive for the discovery of new disease biomarkers.

In the present work, we used up-to-date proteomic approaches with the aim to find new plasma biomarkers for the diagnosis and the early detection of acute GVHD. To deal with the high dynamic range of plasma protein concentrations, different sample preparation methods were firstly investigated. A method based on combinatorial peptide ligand affinity beads that allows detection of more information with good reproducibility, was selected. In addition, to extract a maximum of information from patient plasma samples we used three complementary proteomic approaches, namely 2D-DIGE, SELDI-TOF-MS and 2D-LC-MS^{E}. The differentially expressed proteins between patients with and without aGVHD indicate a significant increase of the inflammation response and disturbance in the coagulation cascade. Interestingly, the early variation of these proteins 15 days before aGVHD diagnosis suggests the detection of the disease before symptoms appearance. In addition, we found that measurement of IL-10 levels, in the first month after HSCT allows the prediction of subsequent aGVHD onset while it is not the case for IL-7 and IL-15 levels. Finally, multivariate analysis provided a combination of biomarkers comprising fibrinogen, fragment of fibrinogen beta chain, SAA, prothrombin fragments, apolipoprotein A1 and hepcidin that can optimally distinguish controls and aGVHD samples (AUC 94.7). In conclusion, a combination of proteomic approaches allows us to discover a new panel of biomarkers that may help for the diagnosis of aGVHD.

Résumé

L'introduction de la transplantation de cellules souches hématopoïétiques a fourni une nouvelle option thérapeutique afin d'améliorer la survie des patients atteints d'hémopathies malignes et de syndromes d'immunodéficience congénitale. Cependant, la maladie du greffon contre l'hôte (GVHDa) demeure une complication majeure, potentiellement mortelle après la greffe. Actuellement, en l'absence de biomarqueurs plasmatiques dans les laboratoires cliniques pour le diagnostic de la GVHDa, l'observation des manifestations cliniques et l'examen histologique des biopsies d'organes constituent la seule approche diagnostique. Pour optimiser la prise en charge des patients atteints de la GVHD aigüe, un diagnostic précoce, spécifique, rapide et non invasif est nécessaire. Le développement d'outils protéomiques permettant une analyse rapide du contenu protéique d'échantillons complexes, a rendu cette technologie intéressante pour la découverte de nouveaux biomarqueurs de maladie.

Dans ce travail, nous avons utilisé plusieurs approches protéomiques dans le but de trouver des nouveaux biomarqueurs plasmatiques afin d'améliorer le diagnostic et la détection précoce de la GVHDa. Dans un premier temps, pour pallier au problème lié à la gamme très étendue de concentration protéique dans le plasma, différentes méthodes de préparation d'échantillons ont été étudiées. Une méthode, basée sur un principe de liaison par affinité à des ligands peptidiques, a permis la détection d'un plus grand nombre d'informations avec une bonne reproductibilité et a donc été sélectionnée. En outre, pour extraire un maximum d'informations à partir des échantillons de plasma de patients, nous avons utilisé trois approches protéomiques complémentaires ; le 2D-DIGE, le SELDI-TOF-MS et la 2D-LC-MS^E. Les protéines différentiellement exprimées entre les patients avec et sans GVHDa indiquent une augmentation significative de la réponse inflammatoire et une perturbation de la coagulation liée à la pathologie. De plus, la variation de la concentration de ces protéines 15 jours avant le diagnostic de la GVHDa, suggère que la maladie pourrait être détectée avant l'apparition des symptômes. D'autre part, nous avons constaté que la mesure du niveau d'IL-10 dans le premier mois après la greffe permet de prédire l'apparition ultérieure de la GVHDa alors que ce n'est pas le cas pour l'IL-7 et IL-15. Finalement, une analyse statistique multivariée a permis de générer une combinaison de biomarqueurs comprenant le fibrinogène, un fragment de la chaine beta du fibrinogène, la SAA, des fragments de la prothrombine, l'apolipoprotéine A1 et l'hepcidine permettant de distinguer de façon optimale les échantillons contrôles et les

échantillons GVHDa (AUC 94,7). En conclusion, une combinaison d'approches protéomiques nous a permis de mettre en évidence un nouveau panel de biomarqueurs, qui pourrait aider au diagnostic de la GVHDa.

Remerciements

Je ne peux évidement terminer ce travail sans remercier toutes les personnes qui ont contribuées toutes ces années à cette longue aventure aussi bien professionnelle qu'humaine.

Je tiens tout d'abord à remercier mes promoteurs, le Pr. Marianne Fillet et le Pr. Yves Beguin de m'avoir donné l'opportunité d'effectuer ce travail.
Un tout grand merci Marianne pour ta disponibilité, ton soutien, tes encouragements et ta compréhension tout au long de ces années. En plus de m'avoir apporté tes connaissances et ton expérience dans le domaine scientifique, tu as eu la patience de m'apprendre à toujours voir le côté positif des choses et à persévérer malgré les difficultés.
Merci au Pr. Yves Beguin pour la mise en place de cette étude, que ce soit du coté administratif (comité d'éthique) et organisationnel (instauration des protocoles de prélèvement en clinique, contact avec d'autres centres universitaires) afin de me permettre d'obtenir les échantillons nécessaires à ce travail. Merci également pour la relecture des articles.

Je tiens à remercier le Dr Frédéric Baron pour ses réponses à mes questions cliniques, et surtout celle qu'il a surement le plus entendue « Y a-t-il une GVHD aigue aujourd'hui ? ». Merci également pour ta participation active à l'étude sur IL-7 et IL-15 ainsi que pour les corrections de l'introduction.
Je remercie toute les personnes liées au service d'Hématologie Clinique comprenant l'unité stérile, l'hôpital de jour et le service d'hospitalisation. Merci au Pr G. Fillet pour son aide dans l'organisation des prélèvements. Merci aux médecins et particulièrement au Dr E. Willems pour la collecte des informations concernant les patients, qui m'ont été d'une grande aide. Merci également à Sophie pour ton aide ces dernières semaines et d'avoir pris le temps de répondre à mes questions. Je remercie les membres du personnel infirmier pour leur participation au bon déroulement du suivi des prélèvements. Merci à Mme Leurquin pour le temps passé à imprimer les dossiers des patients. Je tiens également à remercier les patients, qui ont donné leur accord pour participer à cette étude.
Un merci tout particulier à Olivier pour son aide, sa bonne humeur, sa gentillesse et ces longues discussions pendant les heures d'« aliquotage » du lundi et du vendredi matin.

Je tiens à remercier vivement les membres du jury d'avoir consacré du temps pour la lecture et l'évaluation de ce travail. Je remercie également les membres de mon comité de thèse pour leurs conseils et leurs encouragements.

Ce travail n'aurait pu aboutir sans l'aide des différentes plateformes du GIGA : la plateforme « protéomique » pour les identifications de protéines et l'analyse des échantillons par LC-MS^{E}, la plateforme « Imagerie et cytométrie de flux » pour les analyses CBA et la plateforme bioinformatique

pour les analyses biostatistique. Merci aussi à Laurence Seidel du département « Informatique Médicale et Biostatistique » pour les analyses statistiques multivariées.
Je tiens tout particulièrement à remercier le Dr Pierre Leprince pour son aide à la réalisation de l'analyse 2D-DIGE ainsi que pour son expérience et ses conseils. Merci également à Azeddine et Sabrina pour leur aide et leur bonne humeur.

Je remercie vivement tous les membres du département de « Chimie médicale » de m'avoir accueillie dans leur laboratoire afin de pouvoir réaliser mes recherches.
Je remercie profondément toutes les personnes que j'ai côtoyé au quotidien pour leur aide, leur soutien, leurs conseils, tous ces bons moments et ces discussions toujours très « scientifiques », ces personnes sans qui ce doctorat n'aurait pas été le même : Merci à Anne-Françoise, Anne, Ayman, Félicia, Kateryna, Magali, Pierre, Serkan, Sophie, T-Lan, Xin et Zheshen. Merci Gaël pour les services rendus, ta gentillesse et tes conseils. Je tiens à remercier tout particulièrement Aurélie, Céline et Jessica qui ont été mes piliers jusqu'au bout. Merci pour tous ces chouettes moments passés avec vous (bien qu'il y en aura encore plein d'autres j'espère) et surtout merci pour votre soutien, votre positivité et tout simplement d'être des amies.

Merci Dominique pour avoir partagé ton expérience, tes conseils aussi bien professionnels que humains et ta « positive attitude ».
Merci France, Amélie et Alexandra pour votre bonne humeur.
Merci également à toutes ces personnes que l'ont croise dans les couloirs, avec qui on échange des idées, des conseils et des encouragements.

Ce travail n'aurait pu être réalisé sans les soutiens financiers du FNRS, du Télévie, du Fond Leon Fredericq, du CHU et de l'université de Liège.

Je remercie mille fois mes parents et mon frère d'avoir toujours été là pour moi, d'avoir été patients, de m'avoir toujours encouragé et cru en moi. Merci papa pour l'élaboration de la base de données qui m'a énormément aidée. Merci aussi à mes beaux-parents, Christophe et Justine ainsi qu'à mes amis pour leur présence.

Xavier, je te remercie infiniment pour m'avoir soutenue, supportée pendant les moments les plus difficiles et d'avoir toujours été compréhensif. Merci d'être là pour moi !

Et pour finir, à tout ceux que j'ai oublié de citer : MERCI !

List of Abbreviations

2D-DIGE: two dimensional - difference in gel electrophoresis

ACN: Acetonitrile

aGVHD: Acute graft-versus-host disease

ALC: Absolute lymphocyte count

Apo A1: Apolipoprotein A1

Apo C1: Apolipoprotein C1

APP: Acute-phase protein

ATG: Anti-thymocyte globulin

AUC: Area under the curve

BM: Bone marrow

CB: Cord blood

CE: Capillary electrophoresis

cGVHD: Chronic graft-versus-host disease

CMV: Cytomegalovirus

CR: complete remission

CRP: C - reactive protein

CSF: Cerebrospinal fluid

DLI : Donor lymphocyte infusion

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-Linked Immunosorbent Assay

ESI: Electrospray ionisation

F 1+2: Prothrombin fragments 1 and 2

F13B: Coagulation factor XIII beta chain

FDA: Food and drug administration

FGB: Fibrinogen beta chain

G-CSF: Granulocyte colony-stimulating factor

GVHD D0: Day of aGVHD diagnosis

GVHD D-15: 15 days before aGVHD diagnosis

GVL: Graft-versus-leukemia

GVT: Graft-versus-tumor

HAMP: Hepcidin antimicrobial peptide

HGF: Hepatocyte growth factor

HLA: human leukocyte antigens

HPE: Homeostatic peripheral expansion

HPLC: High performance liquid chromatography

HRG: Histidine-rich glycoprotein

HSC: Hematopoietic stem cells

HSCT:Hematopoietic stem cell transplantation

IEF: isoelectric focusing

IFN: interferon

Ig: immunoglobulin

IL: Interleukin

IMAC: Immobilized Metal Affinity Capture

ITIH4: Inter-alpha-trypsin inhibitor heavy chain H4

LC-MS: Liquid Chromatography – Mass Spectrometry

LPS: Lipopolysaccharides

MALDI-TOF: Matrix-Assisted Laser Desorption/Ionisation - Time-Of-Flight

MHC: Major histocompatibility complex

MiHAs: minor histocompatibility antigen

MMF: Mycophenolate mofetil

MNC: Mononuclear cells

MS/MS: Tandem mass spectrometry

MS: Mass spectrometry

MSC : Mesenchymal stem cell

MSE: Mass spectrometry-based expression

MTX: Methotrexate

NK: Natural Killer

NKT: Natural Killer T

NRM: Non-relapse mortality

OR: Odds ratio

PBS: Phosphate buffer saline

PBSC: Peripheral blood stem cells

PE: Phycoerythrin

PLG: Plasminogen

PLGS: ProteinLynx global server

PMF: peptide mass fingerprinting

PSA: Prostate specific antigen

PTM: Post-translational modifications

Q: Quadrupole

QC: Quality control

RIC: Reduced-intensity conditioning

ROC: Receiver operating characteristics

RSD: Relative standard deviation

SAA: Serum amyloid A

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SELDI-TOF-MS: Surface-Enhanced Laser Desorption/Ionisation – Time-Of-Flight - Mass Spectrometry

SOP: Standard Operating Procedures

SPA: sinapic acid

SVM: Support Vector Machine

TBI: Total body irradiation

TCD: T-cell depleted

TCR: T-cell receptor

TFA: Trifluroacetic acid

TIC: Total Ion Current

TNF: Tumor necrosis factor

TOF: Time of flight

TRC: Transplant-related complication

UPLC: Ultra Performance Liquid Chromatography

WB: Western Blot

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INTRODUCTION

I. Allogeneic stem cell transplantation

1. Definition

Hematopoietic stem cell transplantation (HSCT) is a procedure based on intravenous hematopoietic stem and progenitor cells infusion to restore normal hematopoiesis and treat malignancies (Thomas et al., 1975; Weissman, 2000; Baron et al., 2004; Giralt et al., 2009). Allogeneic transplantation, in opposition to autologous, refers to a graft which hematopoietic stem cells (HSCs) are provided by a donor genetically different than recipient. In adult somatic tissues, multipotent cells are unspecialized cells that have the ability of self renewal and of differentiation into a variety of specialized cells. They have the physiological role to maintain tissue homeostasis by replenishing mature cell populations of the given tissue, and to respond to stress by repairing damaged tissue. HSCs, as multipotent cells, can provide all differentiated blood cells from the myeloid (monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, platelet) and lymphoid lineages (T- and B-cells, natural killer (NK) cells) (Figure 1). Different sources of HSCs are available: bone marrow, peripheral blood or cord blood.

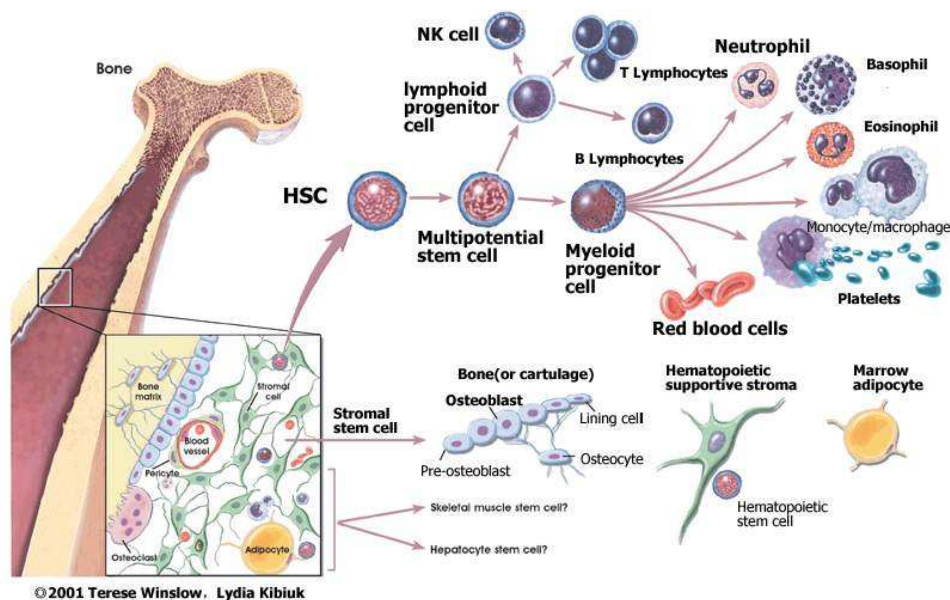


Figure 1: Development of blood cell types from hematopoietic stem cells to mature cells

Historically, origin of HSCT development results from clinical observations of myelosuppressive effects of radiation exposure following Hiroshima and Nagasaki nuclear bombs explosions (Clark et al., 1952). Moreover, further studies demonstrated that shielding the mice's spleen with lead or bone marrow cells infusion could prevent marrow lethality (Jacobson et al., 1951;

Lorenz et al., 1951). However, success of HSCT was hampered by the onset of lethal secondary disease (graft-versus-host disease: GVHD) and graft rejection which resulted from histoincompatibility between donors and recipients. Thus, the subsequent determination and understanding of the major histocompatibility complex (MHC) and human leukocyte antigens (HLA) as the major determinants of graft failure as well as strategies to prevent fatal graft-versus-host disease significantly improve its clinical application (Van Rood et al., 1958; Wilson et al., 1963; Storb et al., 1970). Now, allogeneic HSCT has become a standard treatment option for a variety of hematologic malignancies including leukemia, lymphoma, myeloproliferative disorders, myelodysplasia as well as congenital immunodeficiency or defective hematopoietic states. In 2009, more than 26000 allogeneic HSCT were carried out worldwide (Deeg et al., 2010). Curative potential of HSCT is based on two key aspects: 1) conditioning regimen which induces a cytoreduction of malignant cells and additionally leads to recipient immunosupression preventing graft rejection and ensuring engraftment 2) hematopoietic cell infusion from healthy immunocompatible donor allowing residual malignancy suppression via graft-versus-tumor effect mediated by donor immunocompetent cells (mainly donor T-cells in the HLA identical setting). The process of HSCT is resumed in Figure 2.

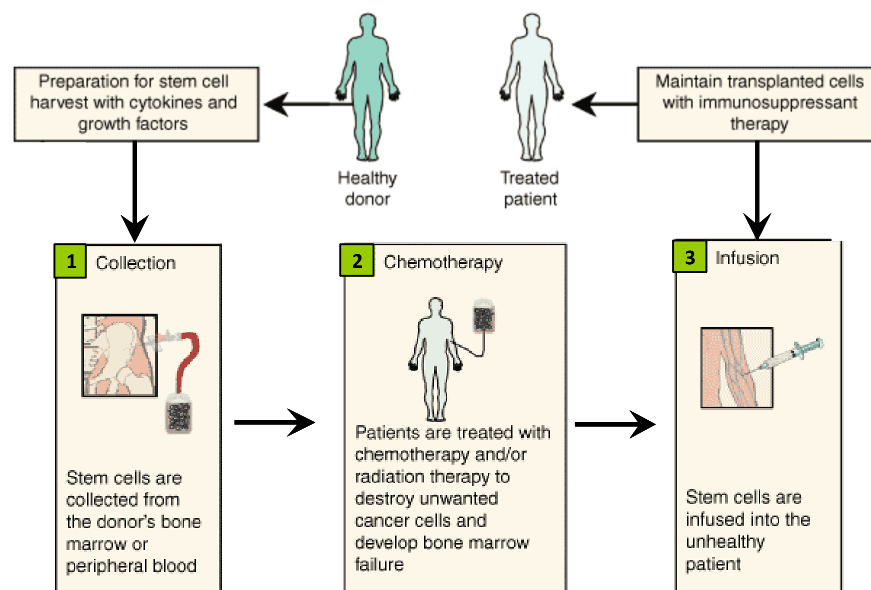


Figure 2: Procedure of allogeneic stem cell transplantation

1.1. HSCT as immunotherapy: graft-versus-tumor effect

The existence of graft-versus-tumor (GVT) effect was first reported by Barnes et al. after they observed a lower incidence of relapse in irradiated mice receiving allogeneic marrow transplants compared to those receiving syngeneic transplant from identical twins (Barnes et

al., 1956; Appelbaum, 2001). Further investigations in human allograft showed increased rate of leukemia relapse after T-cell depleted graft infusion and GVT effect was associated with the occurrence of graft-versus-host disease demonstrating that donor immune cells play a role in HSCT efficacy (Weiden et al., 1979; Horowitz et al., 1990). Morever, donor lymphocyte infusion (DLI) given after transplant induced complete remissions in numerous patients who had relapsed their malignancies after the transplantation (Kolb et al., 1995). Donor lymphocyte CD4+, CD8+ and natural killer (NK) cells have been reported as mediators of GVT effect, using direct cytotoxic Fas-dependent apoptosis and perforin degranulation or indirect cytokine-mediated pathway to eradicate malignant cells (Ringden et al., 2009). GVHD and GVT effect are similar and interrelated reactions differing by the target antigen inducing alloreactive T-cell activation. If the antigen is specific to the host and is widely expressed on recipient cells, GVHD occurs; but if the antigen is specific to tumor cells, than only malignant cells will be target and thus lyse. Although it is not essential, extensive chronic GVHD (cGVHD) is associated with decreased risk of relapse or progression after all types of allogeneic transplantation (Weiden et al., 1979), and was associated with increased probability of progression-free survival in a number of studies investigating nonmyeloablative HSCT (Baron et al., 2005; Baron et al., 2012). However, this is not the case for acute GVHD (aGVHD) which is also associated with a lower risk of progression but also with increased risk of nonrelapse mortality.

2. Main components of HSCT

2.1. Conditioning regimen

The first step of HSCT is the administration of preparative regimen (conditioning) that has three main objectives: antitumoral action, recipient immunosuppression and “creation of space” to favour engraftment of donor HSCs. Myeloablative or “high-dose” regimen consists of supralethal dose administration of chemotherapy, commonly alkylating agents (e.g cyclophosphamide, busulfan, etoposide, cytosine arabinoside) and/or radiation, as total body irradiation (TBI) at doses varying between 800 and 1440 cGy (Giralt et al., 2009), sometimes added with monoclonal antibodies (antithymoglobulines – ATG). However, high morbidity and mortality associated with these regimens have limited their application to younger patients with good performance status while median ages for patients with various haematological malignancies ranged from 65 to 70 years (Molina et al., 2000). Therefore with the better understanding of GVT and DLI therapy, less toxic reduced intensity and truly non-myeloablative

conditioning regimens were developed, in which immune-mediated GVT effect is the major key of tumor eradication (Giralt et al., 1997; Slavin et al., 1998; McSweeney et al., 2001; Servais et al., 2011). Nonmyeloablative conditioning means 1) temporary persistence of host hematopoiesis 2) prompt hematologic recovery (<4 weeks) without cell infusion and 3) presence of mixed chimerism (coexistence of hematopoietic cells of host and donor origin) upon engraftment. Thereby, efficacy of HSCT after nonmyeloablative therapy depends nearly exclusively on the GVT effect while engraftment and graft rejection has to be under pharmacological control. Most reduced intensity conditioning regimens combine purine analogs, such as fludarabine, and alkylating agents, usually busulfan, melphelan or cyclophosphamide (Baron et al., 2004). Combination of fludarabine with low-dose TBI (200 cGy – 400 cGy) can be used as nonmyeloablative regimen. In the case of high risk of graft rejection, polyclonal antibodies targeted T-cell globulin (ATG) is used to facilitate donor cell engraftment (Gratwohl et al., 2012). Diverse conditioning regimens and their toxicity are presented in Figure 3.

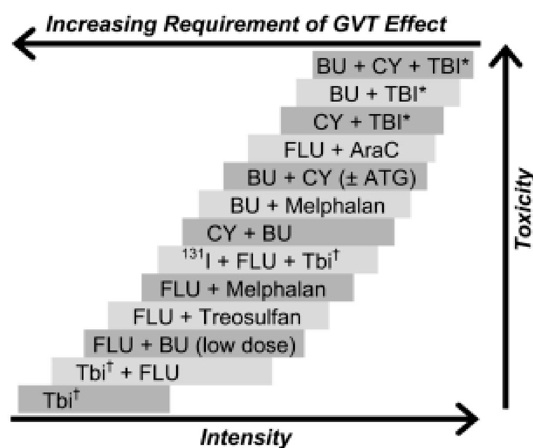


Figure 3: Different dose intensities and toxicities of conditioning regimens.
BU: busulfan; CY: cyclophosphamide; TBI: total body irradiation; Flu: fludarabine; AraC: cytosine arabinoside; ATG: antithymocyte globulin and 131I: anti-CD45 antibody conjugated to ^{131}I. * High dose TBI (800-1320 cGy) and † Low dose TBI (200-400 cGy).

Choice of preparative regimens was determined by type of disease, patient age and comorbid conditions. Currently, older patients and patients with comorbidities will generally be offered HSCT using reduced/ low-intensity conditioning (Deeg et al., 2010). Due to their higher relapse rate in some disease categories (Martino et al., 2006), HSCT with reduced-intensity conditioning was not applied to cure aggressive diseases not in control at the time of transplantation (acute leukemias, high-grade lymphoma or Hodgkin disease). However, probability of HSCT success

with these diseases can be increased in patients undergoing primary therapy and in complete remission at time of transplant.

2.2. Stem cell sources

HSCs niche, defines as the microenvironment that provides the HSCs and their descendants with regulatory signals that are essential for their quiescence, self renewal, proliferation and differentiation, in order to produce appropriate numbers of mature cells throughout life, is localized in the bone marrow (Filipovich, 2012). However, HSCs can be mobilized out of the bone marrow into circulating blood. Therefore, different sources of stem cells are available: bone marrow, peripheral blood or cord blood. Bone marrow (BM) as the central site of hematopoiesis and hematopoietic stem cells (HSCs) was the first source of stem cells exploited for HSCT. However, due to invasive procedure under anesthesia and potential complications related to stem cell puncture from donor bone marrow, peripheral blood mononuclear cells (PBSCs) became a more suitable source. PBSCs are "mobilized" from the marrow into the peripheral blood after injection of recombinant granulocyte colony-stimulating factor (G-CSF). Peripheral blood hematopoietic stem cells are used in approximately 60-70% of allogeneic HSCT (Gratwohl et al., 2006). The use of peripheral blood hematopoietic cells is associated with an accelerated recovery of hematopoiesis, a decreased relapse rate in hematologic malignancies and an improvement in overall and disease-free survival in patients with late-stage disease when compared to traditional BM transplantation (Powles et al., 2000; Bensinger et al., 2001; Group, 2005). However, a higher risk of extensive cGVHD is also associated with PBSC infusion (Couban et al., 2002; Korbling et al., 2011), as well as a higher risk of grade III-IV aGVHD (Bittencourt et al., 2009). Later, cord blood (CB) has been also considered as a source of stem cells. As T-cells in CB are less abundant and immature, it allows a greater HLA disparity without an increased rate of GVHD (Rocha et al., 2001). However, due to the low number of available cells, the use of CB in adult recipients is associated with a slower engraftment and thus a higher risk of graft failure (Gluckman et al., 1997; Rocha et al., 2004).

2.3. Donor selection

The choice of the donor and human leucocyte antigen (HLA) compatibility are determinant elements contributing to the success of HCT as well as in preventing acute GVHD. HSCs can originate from different sources: the patient itself (autologous HCT), a genetic identical twin

(syngeneic) or in the case of allogeneic transplantation: a sibling/ familial donor or an unrelated donor. The HLA system, the human analog of the multigene system of major histocompatibility complex (MHC), is necessary for the recognition of the “self” from the “non-self”, and thus determines the immune signature of each individual. Donor stem cells thus have to express similar class I and II MHC molecules than those of the recipient to avoid rejection reaction. The most influential HLA molecules in the context of allogeneic HSCT are encoded by 5 distinct loci: 3 producing the protein of the class I MHC (locus HLA-A, -B or –C) expressed at the surface of all nucleated cells and 2 producing classe II MHC (locus HLA-DR and -DQ) mainly expressed in antigen-presenting cell surface. These genes are highly polymorphics (hundred to thousand variant alleles) and a single combination of MHC alleles adjacent on a chromosome and transmit together (described as a haplotype), is inherited from each parent, resulting in HLA pairs. On the basis of the typing of HLA-A, -B, and –DRB1 allele for related donor and additionally HLA-C and –DQB1 for unrelated transplantation, donor can be full matched (6/6 or 10/10), haploidentical (5->8/10) or mismatched for one (9/10) or multiple alleles (in the case of cord blood transplantation). Mismatch(es) can be in the GVHD direction, meaning that a MHC allele is present in the host but not in the donor, in the graft rejection direction (MHC allele is present in the donor but not in the recipient) or both directions. HLA-matched sibling donor is generally the preferred donor source because of a reduced risk of graft rejection and GVHD. However, as one MHC haplotype is inherited from the mother and the other from the father, siblings have a 25% chance to be fully compatible while first-degree relatives (child - mother or father) have one common haplotype. As in the population, only 30% of HSCT candidate have a HLA-matched sibling donor, alternative solutions include matched unrelated donor, mismatched donor, haploidentical family donor or cord blood donor (Gluckman, 2012). As GVHD is not only mediated by MHC incompatibility but also by recognition of peptides derived from polymorphic proteins exclusively present in the recipient (called minor histocompatibility antigens (MiHAs)), a matched related donor for PBSC allograft will be preferred to a matched unrelated donor (Welniak et al., 2007). Morever, better survival was observed when patients received T cell-depleted haploidentical transplant from the mother after myeloablative regimen, probably due to an immune tolerance developed during pregnancy (Stern et al., 2008). In the case of unrelated donor, the best choice is male, young, ABO matched and CMV negative if the recipient is negative or CMV positive if the recipient is positive (Gluckman, 2012). More recently, some authors propose, in complement of HLA typing, the genotyping of

Killer-cell immunoglobulin-like receptors (KIR) genes, encoded for NK cell protein surface recognising class I HMC molecule as an inhibitory signal for lysis activation in case of T-cell depleted haplo-identical transplantation(Sun et al., 2005; Miller et al., 2007; Ludajic et al., 2009).

3. Immune reconstitution after HSCT

Myeloablative conditioning for allogeneic HSCT, used as the primary tool to eradicate malignant disease, is followed by a long-lasting defect of cell-mediated immunity. Although myelosuppression is milder after nonmyeloablative regimen, the depth and extent of lymphodepletion tend to be similar, with prolonged periods of immune incompetence mainly induced by the significant therapeutic immunosuppression facilitating the engraftment and preventing graft rejection (Maris et al., 2003; Mackall et al., 2009; Seggewiss et al., 2010).

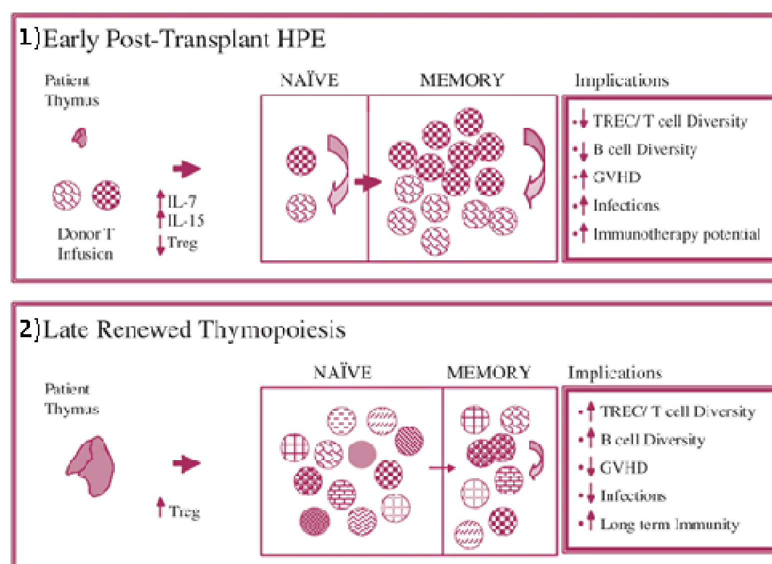
The curative property of allo-HSCT therapy for haematological malignancies, especially in the case of nonmyeloablative regimen, is based on the graft versus leukemia reaction mediated by donor lymphocytes, mainly T-cells and perhaps NK cells. Immune reconstitution after HSCT is thus the key aspect for the success of the therapy. In addition, profound and long-lasting immunodeficiency following transplantation increases the risk of severe post-transplant infections and thus mortality.

Therefore, to estimate capability of newly generated immune system, reconstitution of the different lymphocyte populations (B, T, NK, NKT) and antigen presenting cells (monocytes, macrophages and dendritic cells) should be considered not only quantitatively but especially qualitatively, in terms of functional subsets (Krenger et al., 2011; Toubert, 2012).

Following myeloablative HSCT, the diverse immune cell populations recover with different kinetics after the conditioning and cell infusion procedure. After a pancytopenic phase induced by the preparative regimen, the innate immune system is primarily reconstituted (neutrophils, monocyte and NK-cells) and often normalized within 2-4 weeks post-HSCT, followed by subsequent recovery of adaptative immune system represented by T- and B-cells. The rapidity of neutrophil restoration depends of the source of stem cells; time of recovery approximate 2 weeks with G-CSF mobilized PBSC and is longer for BM and cord blood grafts (Mackall et al., 2009). NK cell population acts early in the immune response against infection and tumour-transformed cells. They are the first lymphocyte subpopulation to be reconstituted in all graft settings, usually within 3 months (Toubert, 2012). B-cell reconstitution, representing humoral

immunity is generally slow and immunoglobulin levels are reduced after transplantation. Early after transplantation there is a restricted B-cell repertoire, with limited BCR diversity that recovers only slowly (Velardi et al., 1988; Shimabukuro-Vornhagen et al., 2009). The reconstitution of an effective B-cell compartment may take up to 2 years after HSCT (Seggewiss et al., 2010).

T-cell reconstitution has a central role after HSCT due to its involvement in aGVHD pathology as well as in mediating graft-versus-tumor effect. Immune recovery of a functional T-cell population after HSCT is a prolonged process and can be separate in two mechanisms (Williams et al., 2007; Krenger et al., 2011)(Figure 4):



<u>Figure 4</u>: Mechanisms of immune reconstitution after allo-HSCT.

1) *Thymic-independent pathway*: Naïve and memory T-cells derive by homeostatic peripheral expansion (HPE) from donor T-cell clones present in the non T-cell depleted graft in response to lymphopenia and antigenic stimulation. These cells have low tolerance for the recipient environment and thus can induce alloreactive reaction such as the beneficial GVL but also GVHD. They can also originate from host T-cells that have survived the conditioning regimen, often in the case of RIC or nonmyeloablative regimen (Baron et al., 2005; Williams et al., 2007). This processus assures a first line of adaptative response against infection during the first 100 days posttransplant before long-term recovery of complete immune response. These T-cells respond quickly against previously encountered pathogens, but their repertoire is limited by the starting repertoire of the mature T-cells after HSCT.

2) *Thymic-dependant pathway*: after 100 days postransplantation, precursor T-cells deriving from HSCs imported from bone marrow are subjected to the thymic maturation. In the thymus cortex, immature triple negative (TN: $CD3^-$ $CD4^-$ $CD8^-$) T-cells proliferate and subsequently acquired CD4 and CD8 protein surface, referred as double positive (DP) thymocytes. Recombinant rearrangements of variable (V), diversity (D) and joiner (J) genes of T-cell receptor (TCR) provide de novo naïve T-cells with a diversified TCR repertoire and they are subjected to positive thymic selection. According to the restriction of their TCR to recognize either MHC class I or II molecules, DP thymocytes differentiate into CD8 or CD4 single positive (SN), respectively. Afterwards, SN cells migrate to the medulla where they are subjected to the negative thymic selection in order to eliminate self-reactive cells. After exit from the thymus, mature T-cells are exported via blood circulation to peripheral lymphoid tissues. Thus, this process provides de novo naïve T-cells that are able to respond to a large spectrum of pathogens. However, it is particularly delayed or nonexistent in eldered patients due to thymic atrophy.

In myeloablative HSCT, cytotoxic T lymphocyte (or CD8+ T-cells: expressing the surface protein CD8) subsets recover more rapidly and efficiency by peripheral homeostatic expansion than T-helper cells (or CD4+ T-cells: expressing the surface protein CD4), even often reaching supranormal levels within 2-8 months after HSCT (Mackall et al., 2009). This results in an abnormal and chronically reduced CD4+/CD8+ ratio. CD4+ T cells rely more on thymic production of naïve T-cells. In addition, it has been shown that another population of T cells, the invariant NKT cells capable of regulatory functions, recovered in the first 90 days post-HSCT and a low iNKT level is associated with a higher risk of GVHD (Rubio et al., 2012).

3.1. Factors influencing immune reconstitution

IL-7 and IL-15 are cytokines that signal through receptor complexes containing the common γ chain receptor subunit and both play a role in lymphocyte homeostasis. IL-7 is a key element for lymphocyte reconstitution acting as a growth and antiapoptotic factor for T-cells as well as a major player in the regulation of peripheral T-cell homeostasis (Fry et al., 2005; Ma et al., 2006)(Figure 4 (1)). Indeed, IL7 -/- and IL-7Rα mice exhibit severe lymphoid hypoplasia including deficiencies of both B and T cells (Peschon et al., 1994; von Freeden-Jeffry et al., 1995).

Moreover, the IL-7 Rα -/- mice show a more severe lymphopenia than that of IL-7 -/- mice potentially due to the ability of thymic stromal lymphopoietin to bind to IL-7Rα and support T cell development (Pandey et al., 2000). Circulating IL-7 is mainly secreted by stromal cells in lymphoid organs and bone marrow environment (Kim et al., 2011) and IL-7 levels increase in case of lymphopenia partly because of reduced IL-7 consumption. Indeed, according to Mazzucchelli and al, production of IL-7 mRNA by stromal cells is constant (Mazzucchelli et al., 2007). The maintenance of cell survival by IL-7 signaling results from the balance between pro-apoptotic and antiapoptotic members of the B-cell lymphoma 2 (BCL-2) family (Khaled et al., 2002; Mazzucchelli et al., 2007). In the thymic dependent pathway, IL-7 is involved in at different stages of T-cell proliferation, differentiation and positive/negative selection. (Abdul-Hai et al., 1996; Fry et al., 2002; Ponchel et al., 2011). DN cell progression in the thymus and TCR γ chain rearrangement also require IL-7 (Mazzucchelli et al., 2007). In addition, IL-7 signaling is also involved in the development of NK-T cells (Boesteanu et al., 1997) and B-cells via the V(D)J recombination (Mazzucchelli et al., 2007; Ponchel et al., 2011).
As IL-7 is the key regulator of T-cell pool after HSCT, IL-15 also plays an important role in the homeostasis of memory CD8+ T-cells as well as in the development and function of NK and NK-T cells. Indeed, selective losses of memory CD8+ T cells, NK cells and NKT cells were observed in IL-15−/− and IL-15Rα−/−, indicating that IL-15 signals provide essential positive homeostatic functions for these subsets of cells (Lodolce et al., 1998; Kennedy et al., 2000). A relevant source of IL-15 is members of the monocyte/macrophage lineage while cells associated with hematopoiesis such as BM stromal cell line, primary human BM stromal cell line, thymic and intestinal epithelium are also produced IL-15 (Fehniger et al., 2001). IL-15 activates the proliferation of CD56bright NK cells in a dose dependent fashion via the activation of IL2/IL15Rβ (Carson et al., 1994; Fehniger et al., 2001).

The dosage of donor T-cells and the intensity of conditioning constitute the most important parameters that influence expansion (Baron et al., 2006; Krenger et al., 2011). Other factors as recipient age, sex, genetic difference between donor and recipient, use of immunosuppressive agent (e.g. ATG), source of stem cell as well as GVHD or infections affect immune recovery after HSCT (Maury et al., 2001; Fallen et al., 2003; Toubert, 2012). Thymic-dependant recovery can be delayed due to advanced age, myeloablative conditioning or GVHD episode (Krenger et al., 2011). Indeed, cytoreductive conditioning can induce tissue damage to the epithelial cells of the

thymus and a decreased ability to produce IL-7 (Chung et al., 2001) while thymus T-cell production, especially CD4+ cells diminishes with increased age .

4. HSCT-related complications

Most transplant complications have an association with conditioning regimen- or GVHD prophylaxis-related toxicities, alloreactivity between donor and recipient cells, immunodepressed status or relapse. GVHD represents a major limiting factor of allogeneic HSCT. Acute form of this complication is the object of this work and will be detailled in section 2. Chronic GvHD occurs most commonly between 100 days and 2 years after transplantation in 20-50% of long term survivors (Giralt et al., 2009). Others complications are bacterial, fungal and/or viral infection, renal failure, veno-occlusive disease, graft rejection and relapse. In the case of myeloablative conditioning, additionally to the immunodeficiency, risk of infection in the early post transplant period is increased with the mucosal damages induced by the conditioning in the intestinal tract.

II. Acute Graft-versus-Host Disease (aGVHD)

1. Definition

Graft-versus-Host Disease is an immune allo-reaction of donor immunocompetent cells present in a genetically disparate environment (recipient). Historically, in the first experiment of HSCT on murin models, a secondary disease resembled to GVHD (named rodent disease) has been observed. Later translation of HSCT to human showed a more severe GVH reaction limiting the first application of HSCT in human. Three key criteria for the development of GVHD were established by Billingham in the 1960s namely (Billingham, 1966):

- The presence of immunocompetent cells in the donor graft,
- The inability of the recipient to adequately reject the donor cells,
- The immunological disparity between host and donor

Originally, two forms of GVHD are defined and distinguished by the time of onset after HSCT: acute GVHD (aGVHD), generally occurring within the first 100 days post-transplant and chronic GVHD (cGVHD), generally occurring after 100 days post-transplant. However, with the emergence of reduced-intensity and non myeloablative regimen, a late-onset aGVHD occurring after 100 days postgraft but with typical signs and symptoms arise. Morever, another form of GVHD named, “overlap syndrome” in which concomitant aGVHD and cGVHD features coexist, was also observed. Thus, acute and chronic GVHD are preferably distinguished by differences in clinical manifestations (Filipovich et al., 2005). Indeed, both involve distinct pathological processes: aGVHD has strong inflammatory components, whereas chronic GVHD displays more auto-immune and fibrotic features (Blazar et al., 2012).

2. Risk factors of aGVHD

Moderate to severe aGVHD occurs in approximately 40% of all recipients of allogeneic HSCT but ranges from 10 to 80% according to several risk factors. As GVHD is an alloimmune reaction, the major risk for occurrence is the presence of antigen disparity. Antigen disparity can be at level of the major histocompatibility complex (MHC) whose gene of chromosome 6 encodes the HLAs, but also at level of minor histocompatibility antigens (mHAs) encoded outside of the MHC locus. The severity of aGVHD is directly related to the degree of MHC mismatch (Flomenberg et

al., 2004). Other risks increasing aGVHD onset are the use of unrelated donors, certain conditioning regimens, donor/recipient gender disparity and prophylactic regimen (Devergie, 2008; Flowers et al., 2011). Regarding conditioning, use of total body irradiation is strongly associated with acute GVHD due to the generation of tissue injuries, especially in the gastrointestinal tract. Concerning gender disparity, transplant from a female donor to a male recipient particularly show a higher risk of aGVHD. Indeed, proteins encoded by the polymorphic Y chromosome genes are recognized by female T-cells as MiHAs and thus can induce immune reaction. Some other criteria are controversial such as donor and recipient age (ref), graft source (peripheral blood stem cells versus marrow) or underlying disease (Eapen et al., 2007; Flowers et al., 2011; Jagasia et al., 2012).

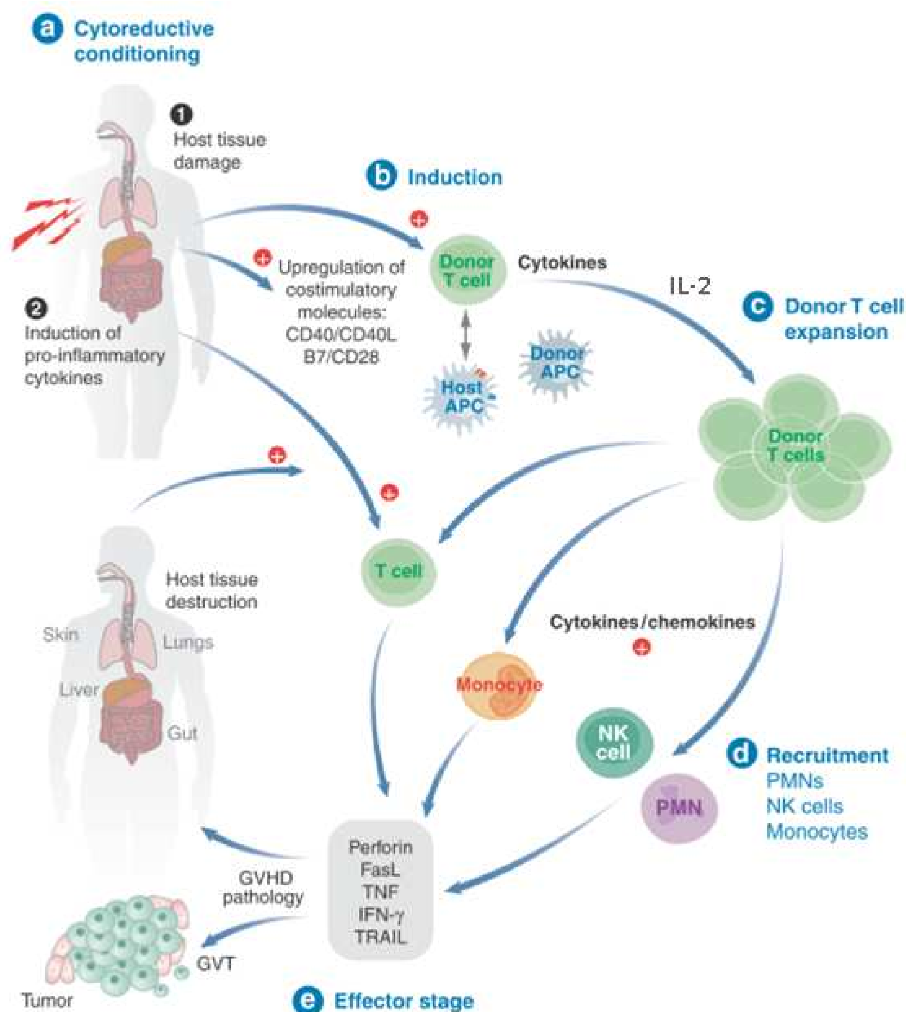
More recently, many genetic variants such as single-nucleotide polymorphism (SNPs) and other polymorphisms of genes encoding for regulatory elements of immune system (e.g. chemokines and T-cell surface proteins) have been reported to influence the risk of aGVHD. The most studied are IL10, TNF and NOD2 polymorphisms but variable results have been observed. In a recent study comprising 1298 recipient and donor samples, Chien et al. evaluated the influence of 14 gene SNPs polymorphisms previously published and associated with aGVHD (Chien et al., 2012). They confirmed the previous results associating aGVHD with a donor/recipient specific IL-6 SNPs genotype, responsible of higher circulating IL-6 levels. An association of IL-2, CTL4, HSPE, MTHFR SNPs and aGVHD risk was also reported in this study. However, conclusions on SNPs polymorphisms and aGVHD occurrence remain inconsistent between studies, maybe due to heterogeneity of studied populations (Dickinson, 2012).

3. Biology of aGVHD

Physiopathology of aGVHD is well established and widely reviewed (Reddy et al., 2003; Welniak et al., 2007; Socie et al., 2009; Paczesny et al., 2010; Blazar et al., 2012). Involvement of mature donor T cells, actors of adaptative immunity and the fundamental cellular mediators of GVHD, was first reported by Korngold and Sprent after observation in a mice model that removal of T-cells from inoculum prevents GVHD (Korngold et al., 1978). Morever, donor lymphocyte infusion, used for GVL activation in case of relapse, causes aGVHD in 60% of DLI recipient (20% of severe grade III-IV aGVHD) (Kolb et al., 1995). In addition, before activation of T-cell-mediated response, innate immune system is an important component of the induction of aGVHD (Penack et al., 2010) as a critical role of host (Shlomchik et al., 1999) and donor antigen-

presenting cells (APCs) (Matte et al., 2004) was established. Moreover, released cytokines, acting as immunoregulators and immune response mediators play a key role in the initiation and expansion of GVH reaction.

According to Ferrara and Antin, GVHD can be considered as a 3-step complex immune response with positive feedback loops that perpetuate the process (Figure 5): 1) damages induced by conditioning and underlying diseases, and subsequent antigen-presenting cells (APCs) activation (Figure 5a and b) 2) T-cell activation and costimulation, expansion, differentiation and trafficking (Figure 5b and c) and 3) cellular and inflammatory effector phase inducing destruction of target tissues (Figure 5d and e). However, pathophysiology of aGVHD is more complex than a sequential spatiotemporal process. This has to be more considered as an overlapping of these different processes, which influence severity and maintenance of GVHD.



<u>Figure 5</u>: Different steps of aGVHD physiopathology

1) APCs activation following damages induced by conditioning and underlying disease

Damages to host tissue induced by chemo- and radiotherapeutic regimen, previous infection and underlying disease lead to transient release of inflammatory cytokines by injured host cells. In addition, TBI-induced epithelial cell damage in gastrointestinal site play an important role in amplifying GVH reaction due to its direct contact with bacteria and microbial products and thus favour systemic translocation of bacterial products such as lipopolysaccharides (LPS) (Hill et al., 2000). This causes a primary reaction modulated by the innate immune system after recognition of pathogen-associated molecular patterns (PAMPs) and activation of Toll-like receptor (TLR), present on the macrophage and APCs surface leading to an amplification of cytokine secretion. In the clinical setting, polymorphisms of the genes encoding TLR and NOD-like receptors (NLRs) are associated with a higher GVHD incidence (Penack et al., 2010; Shin et al., 2011; Blazar et al., 2012). All these elements result in a "cytokine storm", which self-amplifies. Secretion of danger signals by injured tissues such as IL-1, TNF-α, IL-6, IFN-γ and other damage-associated molecular patterns (DAMPS: e.g. proteolytic products, S100 protein, ATP,…) act to upregulate the expression of adhesion molecules as well as MHC antigens and costimulatory molecules on host or donor APCs. APCs provide the critical signal for activation of the alloreactive donor T-cells, the mediators of aGVHD.

2) Donor T-cell activation

Activation of donor T-cell inducing aGVHD can be mediated via either direct presentation of host alloantigens by host APCs or via indirect presentation of host alloantigen by donor APCs. Classically, presentation of intracellular alloantigen linked to MHC class I molecules will activate specifically CD8+ while extracellular alloantigens linked to MHC class II molecules activate CD4+ cells (Korngold et al., 1982; Korngold et al., 1985). However, interaction between alloantigen/ MHC complex on the APC surface and TCR of donor T-cell is not sufficient for complete activation. A second signal provides by danger signals secreted in the first step and costimulatory factors are required for APC/ T-cell interaction and thus subsequent T-cell activation and proliferation. Co-stimulatory signal can be an activating signal favouring T-cell proliferation (e.g. CD28, ICOS/B7 interaction) or a negative signal inhibiting the T-cell activation (e.g. CTLA-4, PD-1/ B7 interaction). Thus, the second signal determines the outcome of the first signal that leads to either complete, partial activation or anergy of T-cells. In response to APCs, activated donor T-cells proliferate, differentiate mainly into T-helper 1 cells (Th1) or into Th2

cells (as defined by cytokine production) and increase gene transcription of cytokines and their receptors. Activated Th1 cells secrete proinflammatory cytokines, such as IL-2 and IFN-γ while Th2 release immunomodulatory cytokines like IL-4 and IL-10. More recently, the involvement of Th17 (Serody et al., 2012) and regulatory T-cells (Treg) in GVHD pathophysiology has been reported but their precise action and regulation remain to be elucidated. Th17 cells generate pro-inflammatory cytokines such as IL-17A, IL-17F, IL-21, IL-22, TNF and chemokines. On the contrary, Treg cells have the ability to induce immune tolerance and prevent autoimmunity as well as aGVHD (Edinger et al., 2009). The balance between Th1/Th2 cell cytokines (Krenger et al., 1996) Treg cells and probably Th17 cells is a critical factor in the development of aGVHD and may play a role in the severity and tissue distribution (Yi et al., 2009; Teshima, 2011).

3) Cellular and inflammatory effectors

After expansion and differentiation, activated T-cells migrate into target tissues and recruit other effectors such as NK cells and cytotoxic lymphocytes (CTLs), leading to direct tissue damages. Classical Fas/Fas ligand and perforin/granzyme pathways are utilized by NK and CTLs cells to lyse target cells. Cytokines and chemokines produced by local injured tissues, T-cells and macrophages enhance the effectors recruitment and amplify the disease process. Subsequent destruction of tissues and related inflammation perpetuate and amplify the “cytokine storm”.

4. Clinical manifestations, grading and diagnosis

aGVHD is a multiorgan inflammatory syndrome affecting most frequently skin followed by upper and lower gastrointestinal tract and liver. The targets of immune response in aGVHD are the epithelial cells including basal and suprabasal cells of the epidermis, the intestinal epithelium and the biliary duct epithelium. Main features of manifestations are erythematous skin reaction, maculopapular rash, cholestatic liver disease and gastrointestinal dysfunction (nausea, anorexia, diarrhoea). Less reported, immune and hematopoietic system dysfunctions, such as thrombocytopenia or long-lasting immunodeficiency delaying immune recovery and favouring opportunistic infections, are also related to GVHD pathogenesis (Teshima, 2012). The diagnosis of aGVHD is mainly based on clinical manifestations and biopsies of target organs. However, it is not always straightforward and can be confounding with competing causes (infection, drug toxicity) which must be excluded. Diagnosis of aGVHD is often confirmed after histological examination of injuried tissues.

4.1. Skin

The skin is the earliest and most frequent target in acute GVHD, affecting 81% of patients at the onset (Ferrara et al., 2009). Characteristic skin aGVHD is maculopapular, sometimes pruritic or less often painful resembling a sunburn reaction (Ball et al., 2008). Histological features of skin aGVHD reveals inflammatory process in the dermal and epidermal layers including apoptosis at base of epidermal rete pegs and variable numbers of lymphocytes arranged in a linear fashion along the basement membrane zone. The hallmark change is satellite cell necrosis consisting of apoptotic keratinocytes with tightly adherent lymphocytes observed in the epidermis with associated vacuolar interface changes (Figure 6) (Chavan et al., 2011) (2012 BCSH, ferrara 1991, goker 2001). However, these observations remain poorly specific, can result from cytotoxic therapy and share with other HSCT complications such as drug hypersensitivity reaction or bacterial/viral infections.

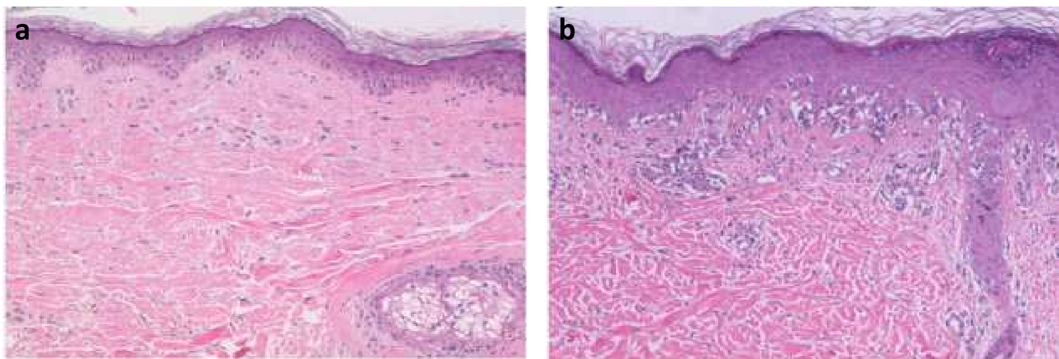


Figure 6: Histological analysis of skin biopsies stained with hematoxylin and eosin:
a) No evidence of GVHD and b) grade II GVHD

4.2. Gastro-intestinal tract

Common gastrointestinal tract manifestations are cramp abdominal pain with or without anorexia, nausea, vomiting and diarrhoea. Evaluation of volume and aspect of stools (watery, bloody) determine severity of gut involvement. As other HSCT complication such as infection or drug toxicity might also present similar symptoms, the gold star of gut diagnosis is histological examination although imaging with CT-scan, MRI or TEP might be helpful. The most important signs of intestinal aGVHD are crypt cell apoptosis and the presence of crypt loss (Figure 7A). Currently, diagnosis of intestinal aGVHD combines the assessment of clinical symptoms with histological examination and microbiological analysis. In order to improve intestinal aGVHD diagnosis, Kreisel and al. developed and evaluated criteria (named “Freiburg criteria”) based on macroscopic findings obtained during ileocolonoscopy for diagnosis of grade ≥ 2 acute

intestinal GVHD, grade requiring intensification of therapy (Kreisel et al., 1994; Kreisel et al., 2012) (Figure 7B). Criteria are presence of spotted erythema, aphthous lesions and more severely ulceration and complete denudation of the mucosa. After microbiological examination of stool and exclusion of infection cases, they obtained good sensibility and specificity when compared to standard histology. Although this diagnosis approach allows saving 1 day of treatment, it remains an invasive procedure and may be associated with health hazards in patients with risk of bleeding.

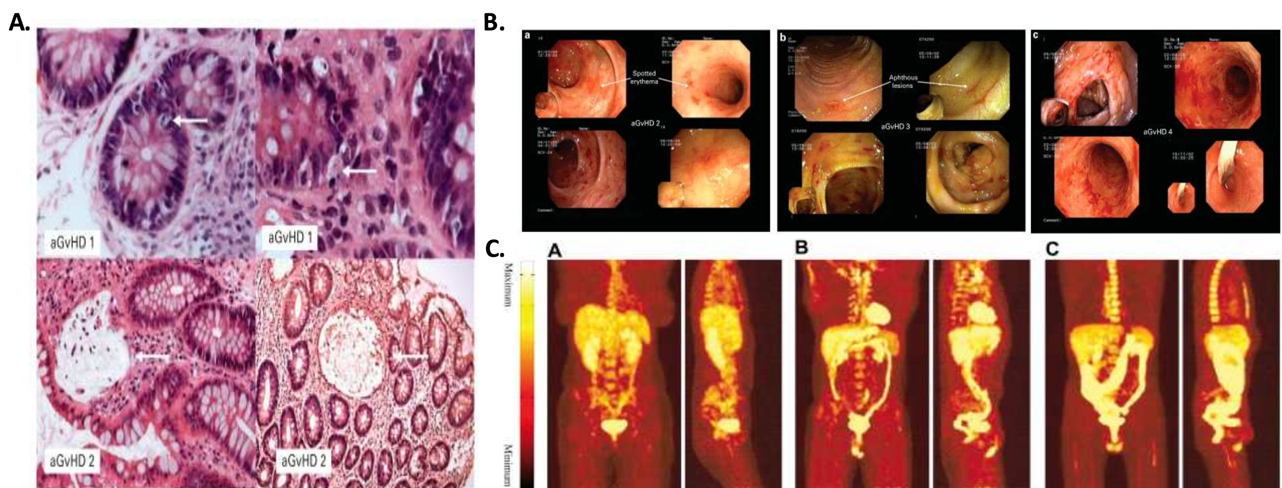


Figure 7: Diagnostic methods of intestinal aGVHD
A. Biopsies from intestinal tract
B. Colonoscopy (a) grade 2 aGVHD, b) grade 3 aGVHD and c) grade 4 aGVHD)
C. FDG-PET (A. no GVHD, B. grade 2 aGVHD and C. grade 3 aGVHD)

Thus, other non invasive diagnosis approach based on 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) (Stelljes et al., 2008) and computed tomography (CT) scan (Shimoni et al., 2011) were evaluated. The first method consists of the observation by PET scan of the FDG uptake in the intestine bowel, showing intense FDG accumulation associated to donor lymphocytes infiltration and GVHD-related inflammation (see Figure 7C). However, this procedure has to be validated in a larger cohort and, specificity and early prediction remain to be evaluated. Evaluation of abdominal CT imaging may show bowel wall oedema, vascularity and fluid-filled dilated loop, which may be associated with GVHD (Benesch et al., 2008; Tuncer et al., 2012). However, these findings are not solely specific to aGVHD and can also be observed in CMV infection.

4.3. Liver

Hepatic involvement in aGVHD is manifested by a cholestatic syndrome. Hyperbilirubinemia is a typical symptom of liver failure associated with aGVHD. Elevations of alkaline phosphatase and transaminase levels may also be observed (Benesch et al., 2008). However, other confounding disorders related to HSCT, such as hepatic veno-occlusive disease, infections or drug/preparative regimen toxicity have similar features. Thus, examination by liver biopsies is the most definitive method to confirm the aGVHD diagnosis. Histological observations of hepatic GVHD occurrence are bile-duct destruction with lymphocytic infiltration of small bile ducts and epithelial cell apoptosis (Shulman et al., 1988; Tuncer et al., 2012).

4.4. Grading

According to the severity and organ involvement in the aGVHD process, aGVHD curative therapy varies. Thus, to propose the most successful therapeutic approach of aGVHD, staging have to be correctly defined. The severity of acute aGVHD is clinically graded from I to IV using a standardized system that evaluates the extent involvement of the three principal target organs. The first classification of aGVHD was developed by Glucksberg et al. in 1974 (Glucksberg et al., 1974) and modified by Thomas in 1975. First, the degree of involvement of the different organs is assessed by objective criteria (Table 1). Then, overall stage from grade I (mild) to grade IV (very severe) is based on a combination of organ stages (Table 2). Severe GVHD has poor prognosis, with 25% long-term survival (4 years) for grade III disease and 5% for grade IV (Cahn et al., 2005; Ferrara et al., 2009).

Table 1: Stages based on organ involvement

Stage	Skin based on maculopapular rash	Liver based on serum bilirubin	Gastrointestinal tract based on quantity of diarrhoea
1	<25% of body surface	20- 30mg/mL	500 - 1000 ml/day or biopsy-proven upper gastrointestinal involvement
2	25 - 50% of body surface	30 - 60mg /mL	1000 - 1500 ml/day or bloody diarrhoea
3	> 50% body surface	60 – 150 mg/mL	>1500 ml/day
4	Generalised erythroderma with bullae and desquamation	> 150 mg/mL	> 2000 cm3/day or severe abdominal pain with ileus

Table 2: Overall grading based on organ staging

Stage	Skin	Liver	Gastrointestinal tract	Performance status
I	1-2	0	0 or isolated nausea/vomiting	0-1
II	1-3	1	1	2
III	2-3	2-3	2-3	3
IV	2-4	2-4	2-4	4

Grading of acute GVHD based on Glucksberg classification used in the clinical setting at CHU of Liège

Following a consensus conference in 1994, Glucksberg classification was modified and the subjective criterion of clinical performance was removed. Moreover, this grading system includes the percentage of skin involvement (Przepiorka et al., 1995; Dignan et al., 2012).

Another grading system was proposed by the International Bone Marrow Transplant Registry (IBMTR), classifying aGVHD from the A to D grade but it showed no evidence of aGVHD diagnosis improvement (Cahn et al., 2005).

5. Prophylactic and therapeutic strategies of aGvHD

As grade III-IV aGVHD has an extremely poor prognosis despite therapeutic intervention, prophylactic strategies remains the best therapy for aGVHD. First, HSCT has to be planned by limiting factors with high risk of GVHD incidence.

One of the difficulties of preventing and avoiding GVHD is the close relation between the undesirable GVH reaction and the indispensable GVT effect, necessary for complete eradication of malignant cells. Indeed, while donor T-cell activation against recipient tumoral cells is beneficial, T-cell activation in response to healthy host tissue recognition is a life-threatening complication.

5.1. Prevention strategies

Given that donor T-cells have the central role in development of aGVHD, prophylactic agents are inhibitors of T-cell function administrated through the peri- and post-transplant time. Prophylatic strategies based on drug combination give less incidence rates of grade II-IV aGVHD and higher overall survival compared to drug alone (Storb et al., 1986). Prevention therapy

usually consists in an association of an inhibitor of calcineurin (ciclosporin or tacrolimus) and an antimetabolite (methotrexate or mycophenolate mofetil) (Storb et al., 2010).

Ciclosporin and tacrolimus specifically inhibit the CD4+ signal transduction pathway leading to the synthesis of interleukin-2, necessary for the proliferation of T-cells. These agents bind to their intracellular receptor, immunophilin, forming a complex that block the phosphatase activity of calcineurin. This blockade results to the inhibition of the cytoplasmic NF-AT dephosphorylation and then its translocation to the nucleus hindering the activation of their regulated genes (Ho et al., 1996; Hamawy et al., 2003). However, ciclosporin and tacrolimus are associated with nephro-, neuro- and hepato-toxicities and many side-effects due to the widespread presence of calcineurin. Paradoxally, use of calcineurin inhibitors, decreasing IL-2 production leads to a defect in Treg proliferation and function (Zeiser et al., 2006). Tacrolimus is often administrated as continuous infusion at dose of 0.03-0.04 mg/kg/day with switch to an oral preparation (0.15 mg/kg/day) in order to maintain a blood level of 15 ng/mL or below (Benesch et al., 2008). Although combination of tacrolimus-MTX may be more effective than ciclosporin-MTX association for grade II-IV aGVHD prevention, there is no significant difference in terms of overall survival (Ram et al., 2009; Jagasia et al., 2012). Indeed, the use of such therapy increases risk of toxicities and infections.

Methotrexate (MTX) used in combination with calcineurin inhibitors, is an analogue of aminopterin, an antagonist of folic acid. It inhibits the reductase dihydrofolate, the intracellular enzyme responsible of the conversion of folic acid to its reduced form. First known and studied as a chemotherapeutic agent due to its antimetabolite role, mechanism of methotrexate as immunosuppressant is not well established. It was thought to be cytotoxic to the rapidly proliferating activated T-cells. In the case of aGVHD prevention, MTX is administrated under a short-course MTX scheme on days 1, 3, 6 and 11 posttransplant. Due to the important toxicity of MTX especially in the gastrointestinal tract (stomatis) and delayed time of engrafment, use of mycophenolate mofetil (MMF), the inactive form of mycophenolate acid is sometimes preferred especially for patients receiving reduced intensity conditioning or those given cord blood transplantation (Sandmaier et al., 2009). Acting as an antimetabolite, MMF inhibits inosine monophosphate deshydrogenase, blocking de novo purine synthesis, which results in an inhibition of both T- and B-cell proliferation as well as in antibody production (Franklin et al., 1969; Suthanthiran et al., 1996; Deol et al., 2011). Compared to MTX, MMF combined with a

calcineurin inhibitor fastens hematopoietic engraftment and causes less toxicity, especially stomatitis with similar incidence of aGVHD and overall survival after HLA-identical sibling donor RIC transplantation (Pinana et al., 2010).

An additional strategy to pharmacological agents is cellular therapy by manipulating graft to deplete specific T-cell subsets (T-cell depleted: TCD) or select positively CD34+ stem cells as well as mesenchymal stem cell or Treg co-infusion. Although TCD decreased the rate of aGVHD, infusion of CD4+, CD8+ T-cell depleted graft or CD34+ stem cell graft is controversial due to the higher risk of graft failure, relapse and infection associated to slower immune recovery (Wagner et al., 2005; Ho, 2011).

Another drug used in aGVHD prophylaxis is antithymocyte globulin, antibody allowing *in vivo* T-cell depletion. In the clinical setting, ATG was used as a prophylactic agent in order to decrease incidences of grade III-IV aGVHD and extensive cGVHD, which are more elevated in patients receiving PBSC instead of BM cells (Couban et al., 2002; Bittencourt et al., 2009; Korbling et al., 2011). Indeed, according to a recent systemic review of all phase III randomized control trials comparing ATG and control, use of ATG in prevention significantly decrease the incidence of grade III/IV aGVHD but not grade I/II aGVHD (Kumar et al., 2012). However, overall survival was not modified with ATG administration (Baron et al., 2012; Kumar et al., 2012) and may be even lower due to a higher rate of relapse (Soiffer et al., 2011). In addition, a recent randomized trial including patients undergoing HSCT following myeloablative conditioning, demonstrated a lower incidence of extensive cGVHD in the group with use of ATG Fresenius compared to controls without ATG prophylaxis (Socie et al., 2011). After RIC HSCT, similar observations of reduced incidence of grade II-IV GVHD and cGVHD were also made with administration of alemtuzumab (Campath), a monoclonal antibody directed against the surface protein CD52 present on mature lymphocytes (Soiffer et al., 2011; Baron et al., 2012). However, disease-free survival was lower with alemtuzumab and ATG compared with T-cell replete regimen (Soiffer et al., 2011).

MSCs are multipotent cells that have the ability to differentiate in a variety of cell lineage (such as bone, cartilage, muscle or adipose tissue) under specific conditions as well as supporting survival, proliferation and engraftment of HSCs in bone marrow niche. Moreover, they exhibit immunosuppressive abilities in vitro and in vivo on alloreactive T- and NK-cells as well as may promote immune tolerance that make them good candidate as solution for GVHD prevention (Baron et al., 2012). Some authors observed in pilot studies a decrease of grade II-IV aGVHD

incidence after co-infusion of MSCs with BM or PBSC graft compared to those without MSCs infusion (Ning et al., 2008; Baron et al., 2011). However, the real advantage of this therapy in GVHD prophylaxis and impact on overall survival (graft rejection, relapse) remains to be demonstrated in randomized clinical studies.

Another cellular therapy is based on infusion or in vivo induction of regulatory T-cells (Tregs) for suppressing alloreactivity. Tregs have the potential to suppress aberrant immune responses and to regulate peripheral T-cell homeostasis by inhibiting division, expansion and differentiation of donor T cells to alloantigenic stimulation (Sakaguchi et al., 1995; Taylor et al., 2001; Prinz et al., 2012). Although optimal dose, conditions of cell expansion and best-suited cell surface phenotype to identify Treg remain to be clearly defined, first studies in humans showed that co-infusion of T-reg with conventional T-cells after conditioning may prevent lethal GVHD and enhance engraftment in the important clinical context of haploidentical HSCT (Di Ianni et al., 2011; Di Ianni et al., 2011). This approach has the advantage to potentially avoid GVHD without affecting GVL effect (Edinger et al., 2003).

5.2. Treatment

Choice of management strategy is dependent of aGVHD grade and target organ. Grade I aGVHD, affecting only the skin, generally does not require treatment other than topical steroids and adjustement of calcineurin inhibitor dose. First line of standard treatment for management of grade II-IV aGVHD is addition of glucocorticosteroids, prednisone or methylprednisolone to the initial prophylaxis regimen (Messina et al., 2008; Dignan et al., 2012; Martin et al., 2012). If prevention therapy was previously stopped, restarts of calcineurin inhibitors administration can be considered. The dose recommended by the BCSH/BSBMT guidelines is 1mg/kg/day or 2mg/kg/ day of IV methylprednisolone for patients with grade II aGVHD and grade III/IV aGVHD, respectively (Dignan et al., 2012). Duration of systemic corticosteroid has not been well defined. Usual practices begin gradual reduction in dose after 7-14 days of treatment (Apperley et al., 2012) while a recent report of the American Society of Blood and Marrow Transplantation recommend to start the tapering of steroid doses as soon as aGVHD manifestations show major improvement (Martin et al., 2012). Indeed, many side effects are associated with corticosteroid treatment and thus can impact on survival of recipient.

Use of additional “nonabsorbable” steroids such as beclomethasone or budenoside in patients suffering of intestinal aGVHD was reported as more efficient than systemic corticosteroids

alone (McDonald et al., 1998) and may allow the reduction of systemic steroids treatment (McDonald, 2007). Complete responses occur in 25% to 40% of patients, and clinically relevant improvement, defined as regression of skin rash or decrease in the volume of diarrhea and the extent of liver function abnormalities, in 40% to 50% of patients with grades II to IV acute GVHD (MacMillan et al., 2002; Deeg, 2007). Patients with more severe aGVHD are less likely to respond to treatment (MacMillan et al., 2002). In a randomized phase 2 trial, the addition of a second drug (etanercept, denileukin or pentostatin) to corticosteroid for initial therapy failed to significantly improve response rates although that addition of MMF seems to be promising with a day-28 response rate of 60% and a 9-month overall survival of 64% (Alousi et al., 2009). Promising efficacy of MSC infusion with initial corticosteroid therapy was observed in a pilot clinical study by Kebriaei et al. Indeed, 77% and 16% of patients with grade II-IV aGVHD showed a complete or partial response by day 28 respectively, without sign of infusional toxicities (Kebriaei et al., 2009). Unfortunately, these results have not been confirmed in a recent (still unpublished) phase III randomized study.

Second line of treatment has to be considered in case of refractory recurrence after initial dose reduction or response failure, defined as progression over 3 days of standard treatment, if no clinical or biochemical changes was observed after 7-14 days depending of aGVHD severity or GVHD relapse during/after corticosteroid tapering. The most frequent choice of second line therapy involves immunosuppressive drugs such as MMF, rapamycine, pentostatin, one or more monoclonal antibodies, or ATG (Apperley et al., 2012). However, ATG administration is associated with side effects such as acute febrile reaction, hypotension or thrombocytopenia as well as higher risk of infections. Other possibilities are extracorporeal photopheresis, mycophenolate mofetil, anti-TNF antibodies (e.g.: infliximab, etanercept) or antibodies against IL-2 receptor (daclizumab). According to a systematic review of Martin et al. (Martin et al., 2012), none of these therapies show evidence of a higher rate of aGVHD complete remission or increased estimate of 6 months survival.

Recently, cellular therapies consisted of donor mesenchymal stem cell infusion (Le Blanc et al., 2004) or Treg expansion were considered as steroid-refractory aGVHD treatment. First reports of MSC infusion are promising but these results remain to be confirmed (Le Blanc et al., 2008; Baron et al., 2012). Administration of sirolimus, an inhibitor of mammalian target of rapamycin kinase (mTOR) used among others to promote Treg expansion, appears to be a promising agent for steroid-refractory aGVHD treatment (Ghez et al., 2009; Hoda et al., 2010).

In all cases, as aGVHD and the classical therapy are associated with a higher risk of bacterial, viral or fungal infections, increased monitoring and prophylaxis for such infection is recommended.

III. Proteomics and biomarker discovery

1. Definition

The word "proteome" was first introduced in 1996 by Wilkins et al. and comes from the combination of words "protein" and "genome" (Wilkins et al., 1996). The idea of proteomics emerged with the previous development of two-dimensional gel electrophoresis and the need of the closer understanding of in vivo biological regulation mechanisms. Although genome sequencing is a good starting point to understand the function of an organism, the wide variety of proteins deriving from a given genome exhibits a more complex system. Indeed, the genome is a static entity present in all cells while the proteome is a dynamic entity which can change with time and under different conditions and localization. Moreover, all proteomes comprise a number of entities that exceed by far the number of genes (Wilkins et al., 1996). The estimated number of proteins encoded by the human genome comprising ~ 21 000 coding genes is two or three orders of magnitude higher. Several diverse mechanisms can result in the expression of many protein variants from the same gene locus in one species such as single nucleotide polymorphisms (SNPs), alternative splicing of pre-mRNA, post-translational modifications or proteolytic cleavage of the protein (Rappsilber et al., 2002). Thus, the aim of proteomics is the exploration of changes in all proteins expressed by an organism genome (cells or tissue) under a specific state (namely proteome). It includes study of sequence, quantity, state of modification, interactions with other proteins, activity, subcellular distribution and structure (Patterson et al., 2003). Proteomic technologies can be used to study fundamental cellular mechanisms or to better understand physiological modifications induced by a disease or a drug for example. Thereby, clinical proteomics is a sub-discipline of proteomics that involves the application of proteomic technologies and strategies to the field of medicine.

2. Searching for new diagnostic biomarkers

Biomarker is defined by the Biomarkers Definition Working Group as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers Definitions Working Group, 2001). Thereby, they may be used in diagnosis, prognosis, disease monitoring, and/or evaluation of therapeutic response (Gillette et al., 2005). Many biological signatures can be grouped under the term biomarker: gene mutations, RNA transcripts, proteins abnormally

expressed, unusual post-translational modifications and unexpected metabolites (Boschetti et al., 2012).

Traditional method for new disease clinical marker discovery consists in the evaluation of single disease-related molecule (e.g. protein) levels between disease and non diseased patients, generally using targeted antibody-based approach. However, tested proteins are typically selected on the basis of current knowledges of disease mechanism and pathophysiology. On the contrary, proteomic approaches allow the simultaneous evaluation of hundreds to thousands proteins in a blind manner without a priori, increasing the potential number of candidate biomarkers. Based on the assumption that a combination of biomarkers is more specific and sensitive for disease diagnosis than single marker (Etzioni et al., 2003), proteomic technologies became very attractive and widely applied for clinical investigation in the last decade. Indeed, large variety of diseases among others several cancer types (ovarian, breast, protate, etc) (Hu et al., 2006), liver diseases (Uto et al., 2010), autoimmune diseases (Hueber et al., 2006), inflammatory bowel diseases (Vaiopoulou et al., 2012), psychiatric disorders (Patel, 2012) has been studied using proteomic approaches in order to identify new disease-specific biomarkers.

However, the pipeline between discovery of candidate biomarkers and clinical implementation is long and challenging, requiring a large amount of clinical samples. Indeed, in spite of the use of various proteomics technologies that has yielded a large number of candidate biomarkers, only few have been validated (Anderson, 2010).

As presented in the Figure 8, the clinical biomarker research should be made up of several key steps: 1) study design and discovery process 2) qualification and verification of candidate biomarkers 3) validation and clinical assay development and 4) clinical assay implementation (Rifai et al., 2006). To complete a biomarker discovery project successfully, a close communication between clinicians, researchers and biostatisticians is a key (Mischak, 2007). The first step determines the clinical question to answer, the appropriate sampling size and controls, the experimental workflow and a standardized sample collection/ storage procedure (Good et al., 2007). Then, typical proteomic studies include the quantitative comparison of samples from “different biological conditions” with the underlying assumption that the proteins showing different abundance are functionally related to the processes affected by the applied conditions (Domon et al., 2010). In the case of disease biomarker discovery using proteomic technologies, the process consists of the differential protein expression analysis comparing

diseased and adequate control samples, avoiding "contamination" by other diseases or confounding conditions. In order to evaluate protein abundance, analytical process requires the use of reproducible sample preparation and proteomic methods as well as quality controls.

As there is no single technology platform that can satisfy all of the desired proteomic measurement (Patterson et al., 2003), a combination of different proteomic approaches is recommended.

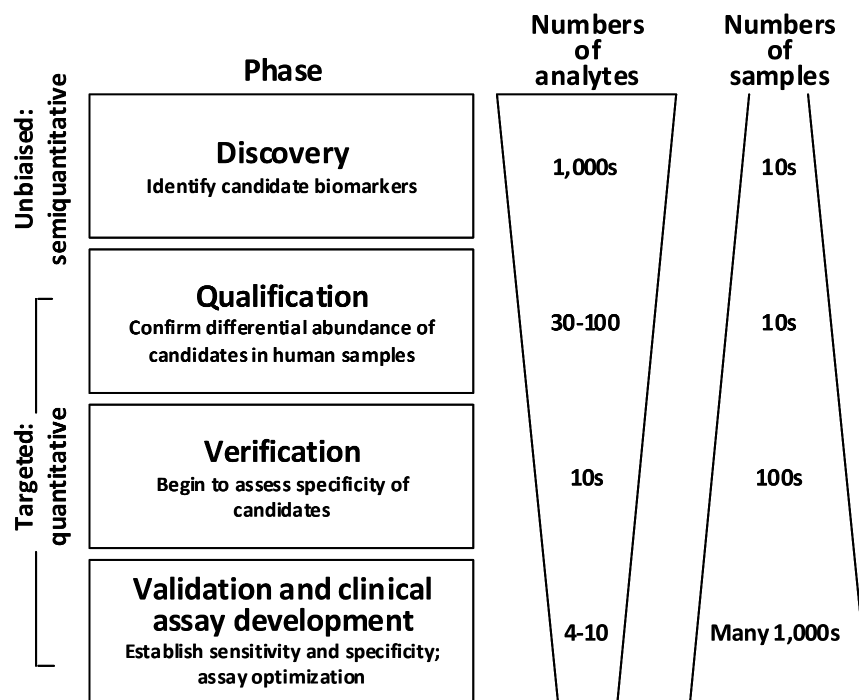


Figure 8: The four main steps necessary to develop new biomarker assay for disease diagnosis.
Discovery phase allows the identification of a high number of candidate biomarkers from a limited number of samples. Subsequent steps require a large sample cohort in order to select the most robust biomarkers.

Afterwards, in order to select the best discriminative candidate biomarkers exhibiting significant difference in abundance between studied sample groups, subsequent univariate and/or multivariate statistical analyses should be applied. In the case of proteomics study, statistical analysis is challenging due to the inherent high-dimensionality of acquired data sets compared to few samples analysed. Thus, application of adequate multivariate predictive models by biostatisticians with state-of-the-art computational expertise is necessary. Subsequent identification of candidate biomarkers, preceded by a purification step should be then performed by mass spectrometry using peptide mapping or sequencing.

After biomarker discovery process, "qualification" step confirms that differential abundance is inherent to biological alteration and not a false positive, by testing the most promising candidate biomarkers on the same sample cohort using alternative targeted and quantitative

approaches. To evaluate consistency of association between markers and disease, marker sensitivity (the likelihood that a diseased sample will test positive) and specificity (the likelihood that non-diseased sample will test negative) are estimated. Then, "verification" step (or pre-validation) evaluates the candidate biomarkers on a larger sample cohort comprising hundreds samples with larger environmental, genetic and biological variations (Rifai et al., 2006). Finally, validation process tests the robustness of the candidate biomarkers against a level of biological variability that more accurately reflects the variability in the target population with a representative incidence of the disease in the population. Then, validated biomarkers may be selected for clinical implementation, in which the method is refined to meet the rigorous standards required for clinical tests.

3. Biomarker discovery process

3.1. <u>Type of samples</u>

Besides the clinical question to answer and patient selection, the choice of the sample type to be analysed is an important point (for more details concerning study design, please refer to our review in appendix). Different biological samples are available for investigation of new clinical biomarkers: body fluids such as serum, plasma, urine, saliva, cerebrospinal fluid or synovial fluid as well as tissue biopsies (Hu et al., 2006). The selection of the biological samples determines the nature of discovered biomarkers and depends of their future application (diagnosis or pathological mechanisms) and the studied diseases. In the case of new assay development for disease diagnosis, serum and plasma are the preferred biological fluids as a source of circulating biomarkers. Indeed, they are a representation of all body by transporting messages from all tissues and besides, are easily accessible. However, the high number of proteins in such complex biological sample and its important concentration dynamic range extended over a factor of 10^{11} is challenging. Indeed, available methods for protein discovery have typical dynamic ranges of only 10^2 - 10^4, forcing the improvement of sensibility and specificity of proteomic approaches. Thus, development of sample preparation and prefractionation methods is necessary and always under investigation.

3.2. <u>Sample fractionation</u>

After centrifugation of blood, plasma and its coagulated form, serum, contain 60-80 mg/ml protein, including at least 10,000 protein species (Anderson et al., 2002). However, only 22

abundant proteins comprise approximately 97% of the protein content of serum, the remaining 3% present in low concentration is considered as the deep proteome (Figure 9). The most abundant proteins are albumin and immunoglobulins, representing 60-70% of total protein content. Different categories of proteins are present in the plasma and can be classified according to their function or origin into different groups such as classical plasma proteins (e.g. albumin), immunoglobulins, receptor ligands (e.g. hormones), tissue leakage products, aberrant or foreign secretion (i.e. from tumors or infectious organism)(Anderson et al., 2002). Albeit classical plasma proteins can bring some indications on a pathological state, it can be supposed that tissue leakage products are more specific candidates as disease biomarkers. Thus, methods to improve the detection of low relative abundance proteins are investigated as they are considered as disease-specific biomarkers.

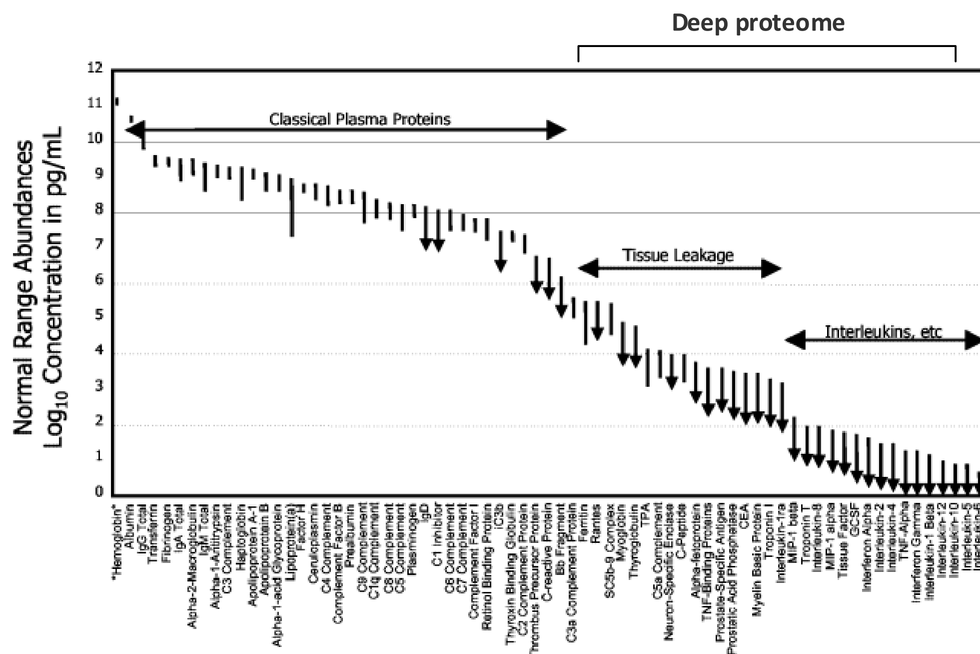
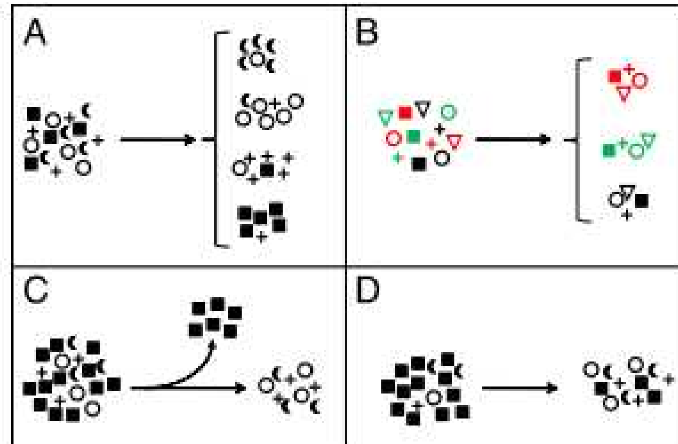


Figure 9: Human plasma proteome
Dynamic range of protein concentrations in human plasma is extended over 11 orders of magnitude; from albumin (~40g/dL) to interleukin-6 (1-5 pg/mL). Deep proteome comprising tissue leakage and interleukins represents only 3% of the total plasma protein content.

Current approaches to extend accessible concentration range and deep proteome detection are mainly based on separation of proteins according to their physicochemical or functional properties. Hydrophobicity degree, ionic dominant charges, isoelectric point, molecular mass and propensity to interact with metal ions as well as affinity for specific antibody are among the general discriminating properties of proteins (Issaq et al., 2002; Righetti et al., 2005; Luque-Garcia et al., 2007; Ly et al., 2011; Boschetti et al., 2012). Fractionation methods include

immunoaffinity substraction, gel or capillary electrophoresis (CE), chromatography, ultracentrifugation, isoelectric focusing or precipitation (Finoulst et al., 2011). As presented schematically in Figure 10, solid-phase chromatographic separation methods can be based on different fractionation principles:



<u>Figure 10</u>: Schematic representation of the main principles of solid-phase chromatographic fractionation.

A) separation according to physicochemical properties; for instance based on the protein charge (ion-exchange chromatography), on the hydrophobicity (reverse phase chromatography), on the affinity for metal (IMAC) or on the molecular weight (size exclusion chromatography).

B) separation according to the presence of a specific functional group in the protein using affinity ligands (e.g. glycosylation, phosphorylation).

C) depletion of most abundant proteins by immunoaffinity; albumin and immunoglobulins are the most popular target proteins. In addition, kits depleting the 6, 12, 14 or 20 most abundant proteins are also available.

D) Simultaneous dilution of high abundance proteins and concentration of low abundance species using ligand libraries (ProteoMiner®). This process leads to the equalization of protein concentration (Thulasiraman et al., 2005; Righetti et al., 2006).

Ideally, sample preparation methods should be high-throughput, reproducible and preserve quantitative information. In addition, they should avoid loss and dilution of samples as well as to be compatible with downstream analytic techniques. In order to enlarge dynamic range and thus peptide/protein coverage, several complementary techniques of preparation can be combined.

3.3. Differential proteomic analysis

Over the last decade, the panel of proteomic strategies for biomarker discovery was enlarged and is always in progress. However, all proteomic analyses in the case of clinical biomarker discovery require common steps: sample preparation (as described in the section 3.3.1.), protein abundance evaluation followed by statistical analyses to compare protein levels between sample groups and subsequent protein identification.

Evaluation of the protein levels in studied samples by proteomic approaches can be either quantitative or semiquantitative. Typically, absolute quantification of proteins requires the use of one or more external standard peptides to generate a calibration curve for specific peptides from the protein (Silva et al., 2006). So, absolute quantitative proteomics is dedicated to the evaluation of targeted proteins in the qualification and validation steps. Given the large number of proteins to be screened in each sample in the discovery phase, non-targeted approaches are rather semiquantitative methods. Non-targeted proteomic technologies applied in the biomarker discovery process can be divided into two main categories: 1) gel-free and 2) gel-based methods. Gel-free technologies involve mass spectrometry-based methods and antibody-based microarrays, although this latter strategy cannot be considered as a “blind” approach even though hundred of individual protein can be evaluated simultaneously (Wingren et al., 2006). Gel-based methods are mainly represented by approaches based on 2D-gel electrophoresis. We will only present in this introduction the technologies used in the context of this work.

3.3.1. Gel-free analysis

The most popular gel-free technologies are mainly based on chromatographic separation coupled with mass spectrometry analysis. The basic principle of mass spectrometry (MS) is to generate ions, to separate these ions by their mass-to-charge ratio (m/z) and to detect them qualitatively and quantitatively by their respective m/z and abundance. Therefore, mass spectrometry can be used to identify and quantify protein components in complex samples (Gross, 2011). Classically, a mass spectrometer is composed by an ion source that produces ionized components in a gas phase, a mass analyzer that allows the separation of components according to their mass/charge ratio in an electromagnetic field and a detector that records ions enabling the generation of the mass spectra (Figure 11). Moreover, given the large amount of acquired data, data interpretation requires state-of-the-art computers with intelligent data

processing, search programs and relevant database. A large number of possible combinations of ionization methods, mass analysers and detectors are available and recommended for specific applications.

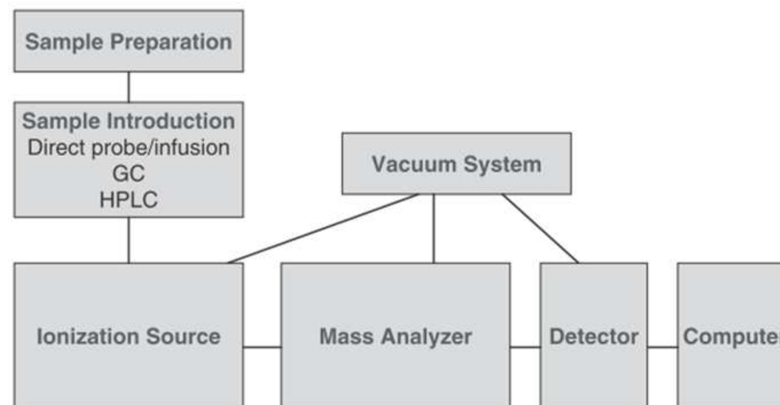


Figure 11: Classical instrument configuration of a mass spectrometer

Soft ionisation methods are commonly used for proteomic analysis and include matrix-assisted laser desorption/ionisation (MALDI) and electrospray ionisation (ESI).

Mass spectrometers are commonly coupled online with separation technologies such as liquid or gaseous chromatographs as well as capillary electrophoresis. In the chromatographic column, the molecules are partitioned between the stationary phase and the mobile phase according to their affinity for one or other and introduced in the mass spectrometer depending on their retention time. Tandem mass spectrometry consists of two mass spectrometers in series connected by a chamber known as a collision cell. The sample to be examined is essentially sorted and weighted in the first mass spectrometer, then fragmented in the collision cell, and a fragment or fragments sorted and weighted in the second mass spectrometer.

Two strategies are classically used for protein quantification by MS: the use of stable-isotope coding method and the label-free quantification (Elliott et al., 2009). The first approach is based on the introduction of a chemically equivalent differential mass tag that allows the comparative quantitation of proteins in one sample to another. Label-free technologies include SELDI- and MALDI-TOF-MS as well as label-free shotgun LC-MS/MS.

Surfaced-enhanced laser desorption/ionisation – Time of flight MS (SELDI-TOF-MS)

This technique combines sample prefractionation by retentate chromatography and mass spectrometry in a high-throughput format. Various chromatographic surfaces (ProteinChip)

with different binding properties (named spot) are available: either chemically (cationic, anionic, immobilised metal ion, hydrophobic, etc…) or biochemically (preactivated surface for antibody, DNA binding, etc…) treated surfaces.

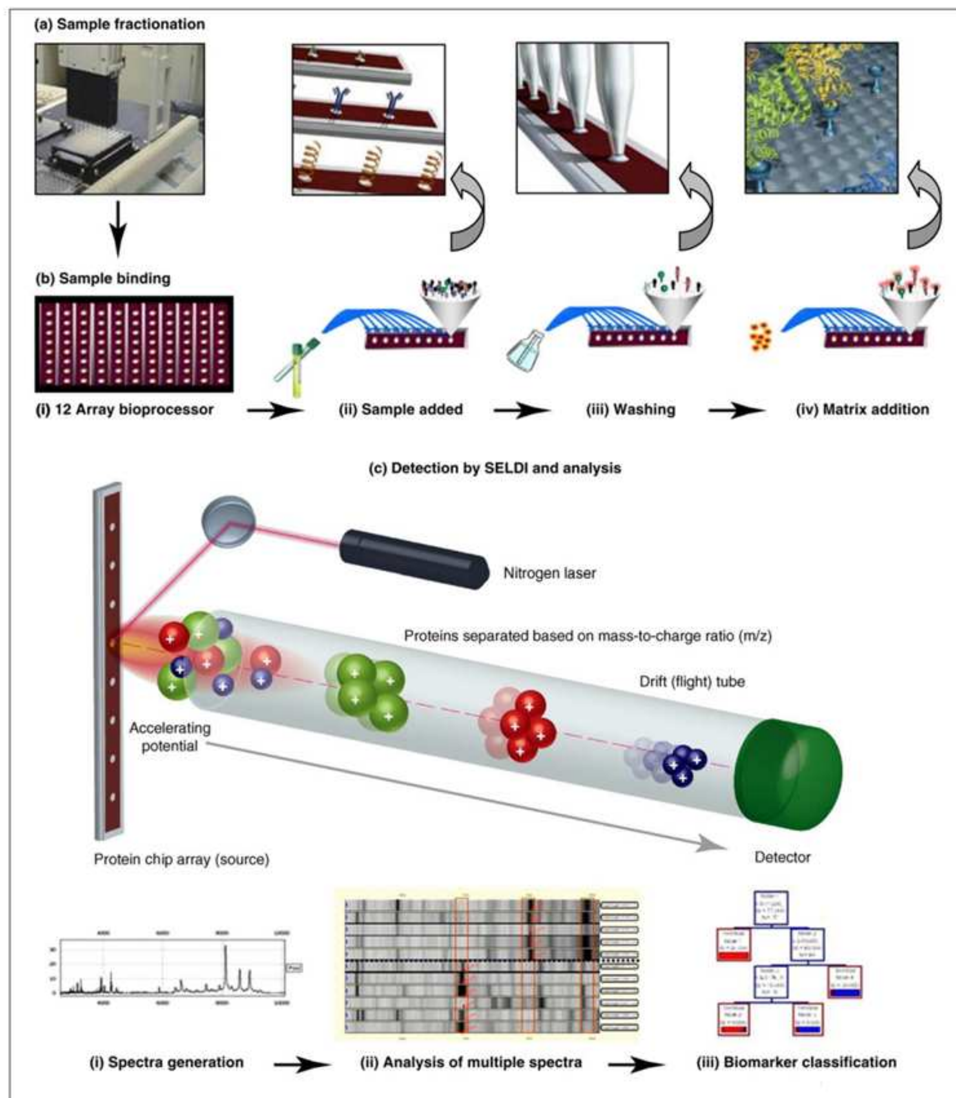


Figure 12: Biomarker discovery procedure using SELDI-TOF-MS

In practice, few µl of samples are first dispensed onto the spot under specific conditions that determine which proteins will be retained by the surface. Then, proteins without affinity for the chromatographic surface as well as interfering substances such as salts, detergent, lipids are removed after washing with an appropriate solvent or buffer. After surface drying, an acid solution of sinapinic acid (SPA) or cyano-4-hydroxycinnamic acid (CHCA) (namely matrix) is loaded onto the spot in order to incorporate the bound proteins into a co-crystallized analytes-matrix complex that will promote the generation of intact gas-phase ions by vaporization and

transfer of positive charge under the laser energy (Figure 12B (a) (b)). After insertion of ProteinChip in the instrument, intact proteins are then desorbed and ionized by the pulsed nitrogen laser source, briefly accelerated in an electric field and separated according to their velocity through the vacuum tube (TOF) (Figure 12B (c)). The time it takes for the ion to reach the detector ("flight time") is recorded. Thanks to a mass calibration equation previously generated externally by acquiring spectrum of a standard peptides or protein mix of known molecular weight, the time information can be converted into a ratio mass/charge for each entity. To assure the best mass accuracy, two calibration equations are defined: one for lower mass range between 1500 and 10000 m/z (mass accuracy of 0.1%) and one for medium mass range from 10000 to 30000 m/z (mass accuracy of 0.2%). Finally, the software generates for each sample a spectrum or a "profile" constituted of peaks characterised by a mass-to-charge-ratio (m/z) and a signal intensity. The intensity of the peak correlates with the abundance of the corresponding entity in the sample.

Data processing: After acquisition of protein profiles, spectra are processed using the manufacturer's software following multiple steps including: noise filtering, baseline substraction, spectrum alignment, total ion current normalisation, peak detection and clustering peaks. In the case of a biomarker discovery study, abundance of protein peaks from the different sample groups (e.g. diseased versus non diseased) are compared using statistical analysis to detect candidate biomarkers. A first list of potential peaks of interest can be generated using the non parametric Mann-Whitney test based on the cluster median intensity of each spectra group. Secondly, peak information can be exported and analysed by bioinformatics software in order to provide a list or pattern of the most discriminative peaks. In collaboration with biostatisticians, a multivariate analysis based on decision trees analysis was developed for analysis of SELDI-TOF-MS data (Geurts et al., 2005). It allows the ranking of peaks according to their relative contribution in the classification of the groups, associated with a percentage of importance. The sensibility and specificity of discriminative models were evaluated using a leave-one-out cross-validation.

Protein identification: As SELDI-TOF-MS can not provide readily protein identification, two strategies can be considered to determine the identity of differentially expressed proteins. The first method is based on the comparison of the m/z ratio of the peaks of interest against a

protein database (UniProtKB/Swiss-Protein database) using an algorithm (Tagident). Among the inferred proteins, those for which identities are the most likely according to the type of sample used and the studied disease can be tested by immunoassay. Immunodepletion process consists in the incubation of sample with the antibody targeted against the candidate protein coupled to agarose beads. Then, bound and unbound fractions are analysed by SELDI-TOF-MS. The identification of protein is considered correct when the peak of interest is present in the bound fraction and undetectable or at low peak intensity in the unbound fraction. An alternative of immunodepletion is the direct analysis of sample on preactived ProteinChip conjugated with the antibody (Jr et al., 1999).
Second approach provides protein identification by high resolution MS (as described in the 2D-DIGE section) after protein purification and enrichment using chromatographic or electrophoretic methods (e.g 1D-SDS-PAGE followed by protein staining). In order to facilitate protein identification procedure, an interface combining ProteinChip to a tandem MS was developed (Caputo et al., 2003) and allows the direct sequencing of peptides <6000 Da (Peng et al., 2009). However, given the complexity of body fluid samples and the lower sensitivity of MS/MS device (Q-TOF) compared to SELDI, enrichment of peptides of interest is necessary and may be a labour intensive and time consuming procedure.

Advantages and limitations: The main feature of SELDI-TOF-MS is its ability to provide a rapid expression profile of intact polypeptides/proteins between 1.5 to 30kDa from a variety of complex biological samples. In addition, the combination of different chromatographic surfaces and binding conditions increases the proteome coverage based on the physico-chemical properties of proteins. Standard quantitation curves obtained by SELDI-TOF-MS in a complex samples have been shown to be linear over 2-3 orders of magnitude (Vorderwulbecke et al., 2005). However, the main disadvantage of this technology is its inability to directly identify ion species due to a limited mass accuracy, low peak resolution and no coupling in tandem.

Applications: The most popular application of SELDI-TOF-MS reported in the literature is body fluid profiling for biomarker discovery (Wright, 2002; Wei et al., 2010). Indeed, early biomarker studies used SELDI spectra as “proteomic signature” that, in conjunction with class prediction algorithms, distinguishes samples from diseased or non-diseased patients. However, SELDI-TOF-MS is also used as a tool for the characterization of biomolecular interactions and protein

posttranslational modifications (Fung et al., 2005), specific assay of protein/peptide abundance (Lomas et al., 2012) as well as for the monitoring of in-process samples during bioprocess development (Berrill et al., 2011).

Reproducibility of results obtained by SELDI-TOF-MS in the case of biomarker discovery studies has been criticized (Hu et al., 2005; McLerran et al., 2008). Besides instrument limitations, poor study design and quality of sample processing were also accountable for biased results (Baggerly et al., 2004; Kiehntopf et al., 2007; McLerran et al., 2008). For this reason, a lot of practical considerations have to be taken into account when using SELDI-TOF-MS (Poon, 2007; De Bock et al., 2010) as a tool for clinical biomarker discovery. Challenges and pitfalls of this technique were the object of our review, presented in appendix.

2D-UPLC/MS^E

This recently introduced proteomic technique, combines 2D or orthogonal ultra performance liquid chromatography with a mass spectrometer constituted of an electrospray as source and a tandem quadrupole - time of flight analyzer (ESI-Q-TOF). After sample fractionation, proteins are digested by specific enzyme (generally trypsin) before injection in the analytical system. The two-dimensional separation device consists of the coupling of two chromatographic columns separating peptides according 2 different properties allowing analyte separation with high resolution, sensitivity and speed (UPLC system: Waters) (Swarts, 2005). In the quadrupole analyzer, ions are filtered by passing through an electromagnetic field generated by 4 parallel rods. Voltages of the same polarity are applied to opposing pole sets and variation of these voltages allows to select or not the ion. Selected ions are subsequently fragmented and product ions are analysed by the TOF analyser.

Traditional MS/MS analysis (or data-dependent analysis) consists of a first acquisition of a MS survey scan followed by selection of precursor ions by the first analyser for MS/MS fragmentation and analysis by the second (Figure 13A). In data-independent analysis (or MS^E), the first analyser (quadrupole) is used as a guide to transfer all ions into the collision cell, in which the collision energy is alternated from low to high at a high rate throughout the run time. Thus, intact peptides are measures in the low energy scans, whereas fragment ions are measured in the high energy scans (Silva et al., 2006; Li et al., 2009; Levin et al., 2011) (Figure 13 B).

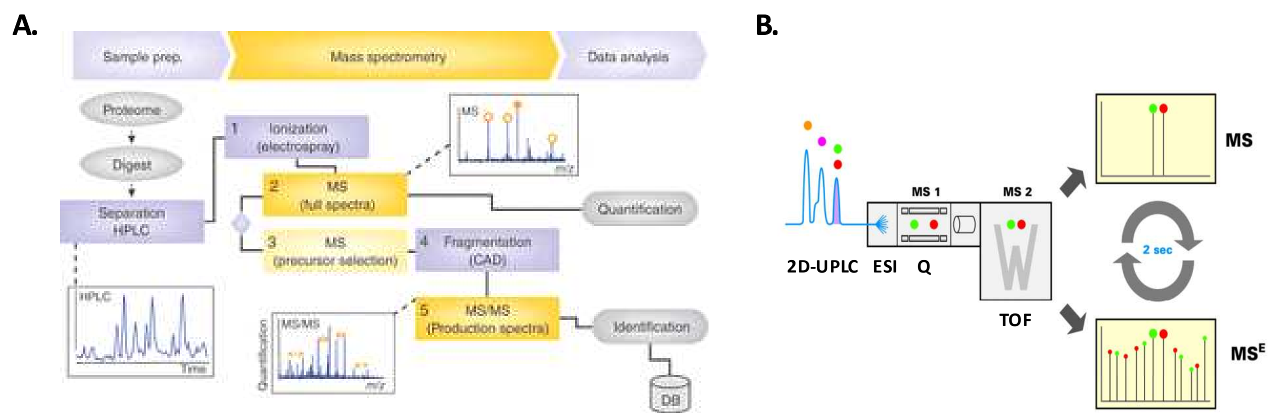


Figure 13:
A. Workflow of a typical proteomic experiment using shotgun MS-based technology
B. Schematic representation of 2D-UPLC-ESI-Q-TOF and MS^E mode.

Data processing: After spectra acquisition, a bioinformatics software (ProteinLynx global server in our study) is used to detect peaks, align spectra and match the product ions (high collision energy) to their corresponding precursor ion (low collision energy) based on retention time, ion intensities, mass accuracy and charge state (Geromanos et al., 2009). Protein identification is obtained by peptide sequencing (Li et al., 2009) (see the section 2D-DIGE) while quantification is based on the sum MS signal response of the three most intense tryptic peptides of a protein. A limitation of this quantification method is that the magnitude of the error is dependent on the size of the protein. Indeed, smaller proteins have fewer tryptic peptides that may have a wide range from the most intense to the next most intense (Silva et al., 2006). Complex computational algorithms based on probabilistic framework are used to determine abundance means, ratios, standard deviation and significance (Richardson et al., 2012). Quantification has been estimated to be linear over a dynamic range of two to three orders of magnitude using 1D LC separation (Levin et al., 2011). Compared to classical MS/MS, MS^E acquisition mode presents the advantage of more reliable and reproducible identifications (Levin et al., 2011).

Advantages and limitations: This method has the advantage to obtain simultaneous sensitive quantification and reliable identification of protein in a single run, and thus is less time consuming. However, this is not a high throughput technique due to the important time run duration and high cost, forcing to work with pool samples. Morever, protein digestion necessary for the analysis leads to the lost of protein information such as specific proteolytic products. Given the large amount of data obtained by run, such technique requires powerful

and automated software for data processing followed by manual checking. Thus, high expertise of the technology is thus necessary.

3.3.2. Gel-based analysis

2D-DIGE

Gel-based method consists of 1) the combination of a bidimensional polyacrylamide gel electrophoresis fractionation (SDS-PAGE) and, 2) subsequent identification of differentially expressed proteins by mass spectrometry. Denatured intact proteins are separated first according to their isoelectric point by isoelectric focusing (IEF) followed by gel separation according to their molecular size.

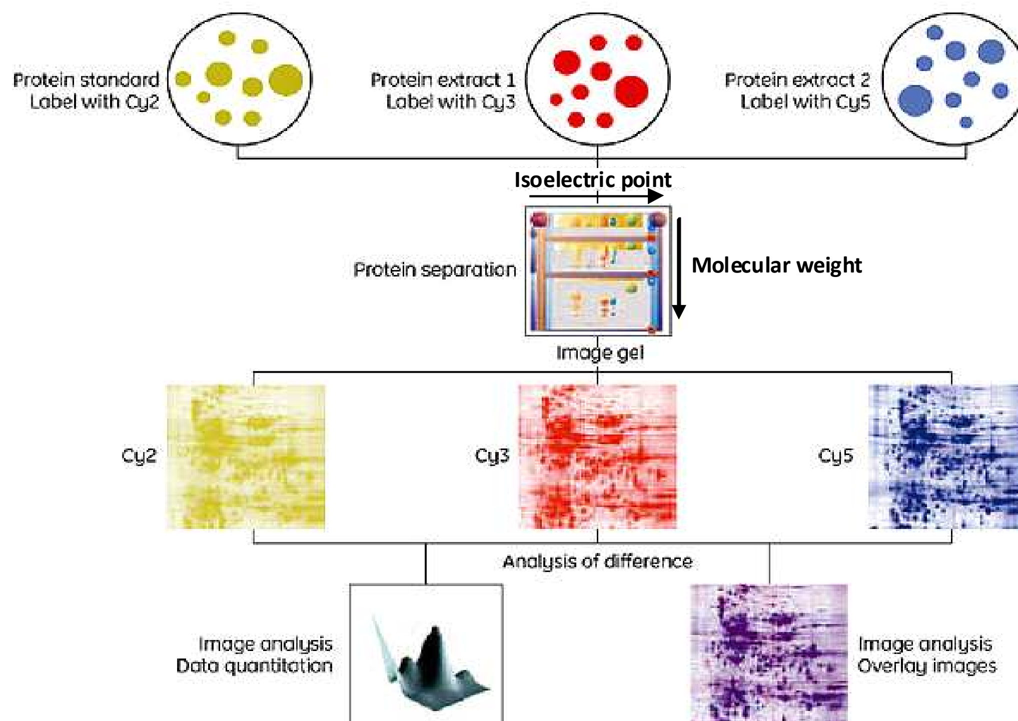


Figure 14: Principle of 2D-DIGE technology
A defined quantity of protein for each sample is labeled with the three distinct Cydyes and samples are mixed together. The proteins are first separated according to their isoelectric point by isoelectric focusing (IEF) in denaturant solution. Then, proteins are separated according to their molecular weight by SDS-PAGE. Acquisition of image is made using a laser scanner using fluorescent scanning mode. Image analysis and differential protein abundance analysis are performed using specific software.

Traditional 2D-gel electrophoresis with the post-migration staining (coomassie blue or silver) is time-consuming and poorly reproducible, making difficult the distinction between technical variations and induced biological changes (Marouga et al., 2005). Development of CyDye staining and 2D-DIGE technology allowed the improvement of reproducibility and quantitative

capabilities, and reduced the number of gels to be run (Unlu et al., 1997). The pre-electrophoresis protein labeling with three spectrally distinct but size and charge-matched fluorescent cyanine dyes (known as Cy3, Cy5 and Cy2) enables the concomitant analysis of two different samples (usually labelled by Cy3 and Cy5) as well as the internal standard (usually labelled by Cy2) on a single gel (Figure 14). The internal standard, consisted of an equal amount of each sample included in the experimental workflow, serves as the reference sample and is run on each gel. The Cydyes have a good sensitivity (detection limit of 25-75pg of protein) higher than silver staining and provide a linear signal over a dynamic range of 4-5 orders of magnitude (Lilley et al., 2002; Viswanathan et al., 2006). Minimal labelling, where <5% of each protein species is labelled (Tonge et al., 2001), is preferable to saturation labelling as it causes no problem of protein solubility and is less likely to interfere with subsequent MS analysis for protein identification (Timms et al., 2008). After bidimensional separation, three fluorescent gel images corresponding to each sample loaded in the gel are acquired using a laser scanner with three distinct excitation and emission wavelengths.

Data processing: Image analysis is subsequently performed by software enabling spot detection, quantification, matching and differential analysis (e.g. DeCyder 2D™ – GE Healthcare). As internal standard sample contains every protein from all samples, it allows the spot matching across gels and then each spot volume of both samples in a gel can be normalised to the corresponding spot volume from the internal standard present in the same gel. Thus, the abundance of each protein is expressed as a ratio, reducing the gel to gel variations. After log transformation of the spot normalized abundance, paired or unpaired T-test is applied to determine discriminative spots between studied groups. Spots with significant differential abundance are excised from the gel manually or automatically using a robotic spot picker.

Protein identification: After the protein separation process and data analysis, proteins of interest are identified by mass spectrometry (MS) or tandem MS either by generating peptide mass fingerprint (PMF) or sequencing by MS/MS, respectively. The main MS techniques used for protein identification are MALDI-TOF, TOF/TOF or nanoLC-MS/MS (i.e. ESI-ion trap or ESI-Q-TOF). Before MS analysis, the proteins are treated with enzyme such as trypsin allowing cleavage of peptide chains mainly at the carboxyl side of the amino acids lysine or arginine

(trypsinisation) in order to generate peptides that are small enough to be efficiently ionised and extracted out of the gel as a mixture of peptides.

In the present work, protein identification after 2D-DIGE analysis was performed by MALDI-TOF-TOF. Proteins were identified using the peptide mass fingerprint (PMF) method, which can be followed by peptide sequencing. Concerning instrumentation, MALDI-TOF is based on the same principle than SELDI-TOF-MS (described in the previous section) except that there is no fractionation of the sample on a chip and entire peptide sample is spotted on the MALDI plate. PMF is generated in MS-mode acquisition and consisted of a unique "fingerprint" of a particular protein, determined by accurate mass measurement of unique protein fragments produced by highly specific enzymatic digestion (Figure 12A). By using a specific enzyme (e.g. trypsin) and software (e.g. MASCOT), the masses of the individual peptides can be predicted by making a theoretical digest of all the entries in large protein databases (e.g. Swissprot database). The measured set of m/z values (with a certain mass tolerance compared to exact mass) from the sample is then compared to the predicted mass values for theoretical digestion of proteins in the sequence database, and the protein is identified by a statistically significant number of matches (Cottrell, 1994; Vertes, 2008). A score based on the probability P that the observed match between the experimental data and the database sequence is a random event is calculated. Morever, the percentage of coverage between the entire identified protein sequence and the observed peptide sequence is also given. Correct identification requires that the database contains the specific protein sequences (or the corresponding DNA sequences) limiting its application to well-known organism species.

As different peptides with different compositions can have the same m/z ratio, peptides providing the most abundant signals can be fragmented and analysed in a second TOF analyser (MS/MS mode) to determine peptide sequence by recording the fragment ion spectrum of the peptide and confirm the protein identity (Figure 12B). This second method using tandem-MS provides the primary structure information of peptides by fragmenting specific ions and analysis of the resulting fragment ions. Three different types of amino acid bonds can be fragmented in the collision chamber: the NH-CH, CH-CO, and CO-NH bonds. This results in six possible fragment ions for each amino acid residue called a, b and c" ions (charge on the N-terminal fragment) and x, y" and z ions (charge on the C-terminal fragment). The mass difference between two fragment ions (more often b and y) resulting of the same bond

cleavage observed on a spectrum corresponds to a particular amino acid residue (Figure 15). Thus, a small amino acid sequence of a specific peptide can be determined.

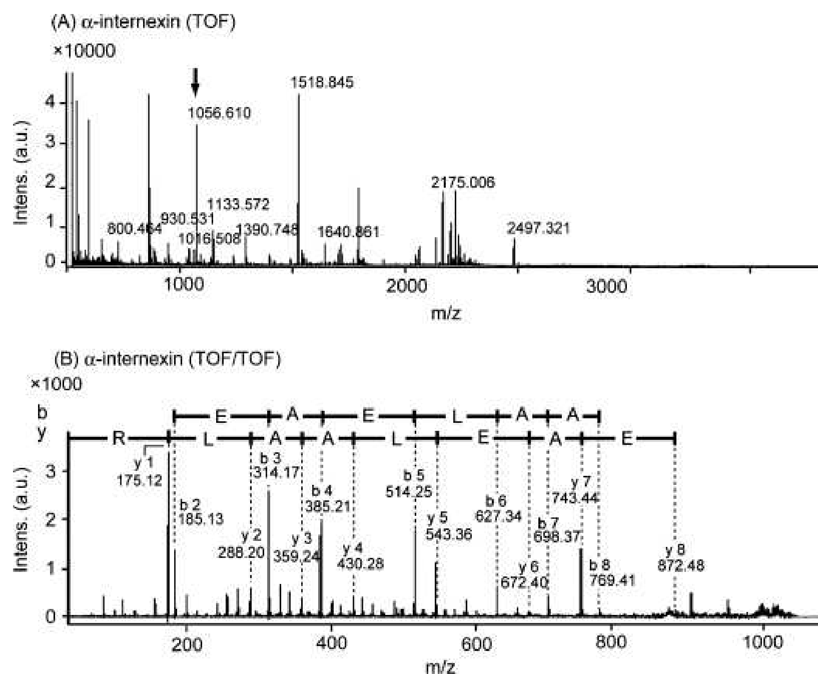


Figure 15: Example of spectra obtained by MALDI-TOF-TOF-MS for protein identification based on (A) PMF and (B) sequencing

Advantages and limitations: Compared to MS-based methods, 2D-DIGE has the advantage to resolve several thousands of intact proteins with high resolution allowing visualization of different protein isoforms, truncated proteins as well as posttranslational modifications. Indeed, most MS-based technology requires protein digestion (except SELDI-TOF-MS) into small peptides before differential analysis, leading to the lost of such information. However, 2D-DIGE analysis does not enable the resolution of proteins below 15 kDa and above 250kDa as well as very hydrophobic or basic proteins. In addition, 2D-DIGE remains a hand-operated and labour intensive technique which necessitates manual checking of image analysis and thus requires qualified personal.

Table 3 presents the technical characteristics of the proteomic technologies used in this word. However, it has to be taken into account that the limit of detection and quantification values as well as the linear dynamic range of the instruments are determined by the nature and the complexity of the analysed sample (Domon et al., 2010).

<u>Table 3</u>: Characteristics of the three proteomic technologies used in this work

	2D-DIGE	SELDI-TOF-MS	LC-MS^E
Sample Throughput	Low (<10 samples per day)	High (300 samples per day)	Low (<10 samples per day)
Mass range	15 – 250kDa	1.5 – 200 kDa	0.05 – 2 kDa
Proteome Coverage	High (~ 2000 proteins)	Low (100-300)	medium (~500 proteins)
Limit of detection	25 – 75 pg	10 fmol	Less than 25 fmol
Linear dynamic range	4-5 orders	2-3 orders	3 orders
Advantages	- Visualization of PTM and fragments - Mature technology - High separation capabilities	- Visualization of specific fragment, dimers, isoform or modified form of peptides/proteins - Rapid and easy to perform - HTP: samples are analysed individually	- High proteome coverage - Simultaneous quantification and identification - Automated
Disadvantages	- Limitation for proteins of extreme pI or hydrophobic - Hand-operated technique and time consuming data analysis - Require protein identification by MS -Low TP: pools of samples	- No readily protein identification due to low mass accuracy and resolution	- Lost of structural information due to protein digestion - Low TP: pools of samples
Cost/sample	Medium	Medium	High

HTP: High-troughput
TP: Throughput

3.4. <u>Confirmation of protein difference and validation</u>

“Qualification” step serves to differentiate true differential protein abundance related to the specific biological condition and false positive induced by the technical process. Ideally, the alternative method used to test candidate biomarkers, particularly in the “verification” step, has to be quantitatively accurate, reproducible, high-throughput and sensitive. Thus, as only several proteins have to be tested, targeted technologies are used, usually antibody-based methods such as the sandwich enzyme-linked immunosorbent assay (ELISA) and Western Blot. Western blot has the advantage to separate different fragments of a protein according to their molecular weight but this method is only semi-quantitative and cannot be applied on hundreds of samples. In the other hand, ELISA quantifies the different forms of a same protein regardless alternative splicing or protein modifications, but it is easier to perform and high-throughput. For the validation step, the most applicable approach for the quantitation of individual proteins remains ELISA. Multiplex immunoassays are also available. However, both techniques are limited to the quality and availability of the antibodies. Indeed, the validation of biomarker candidates may be biaised in favor of proteins, for which an ELISA was already developed,

impeding the validation of potential new biomarkers (Surinova et al., 2011). Moreover, cross-reactivity, particularly with complex samples such as serum or plasma containing large protein species and potential auto-antibody (in the case of autoimmune disease) could bias the result (Tate et al., 2004).

More recently, targeted quantitative MS has been considered for qualification, verification and validation steps with the emergence of single- and multiple- reaction monitoring (MRM). The analytical system constituted of a liquid chromatography coupled to an ESI-triple quadrupole mass spectrometer allows the absolute quantification of one or several proteins in hundred complex samples. In a single reaction monitoring (SRM), the first and the third analysers act as filters to specifically select predefined m/z values corresponding to the peptide ion and a specific fragment ion of this peptide respectively, whereas the second quadrupole serves as a collision cell (Lange et al., 2008). Compared to ELISA, this approach has the advantage to offer the possibility of multiplexing without affecting the sensitivity and selectivity. In addition, the monitoring of particular transitions (precursor/ fragment ion pairs) in combination with the retention time of the targeted peptide allows the quantification of specific protein isoforms or post-translation modifications. However, limit of detection of this technique is 1 µg/ml in complex samples without sample pretreatment and can be improved to 5-25 ng/mL after selective enrichment (Stahl-Zeng et al., 2007) or depletion of abundant proteins (Keshishian et al., 2007; Surinova et al., 2011) which is less sensitive than ELISA.

Although triple quadrupole is the “gold standard” instrumentation for absolute quantification by MS, others mass spectrometers can also be used for protein quantification (e.g. ESI- ion trap).

3.5. GVHD, biomarkers and proteomics

The diagnosis and prognosis of aGVHD rely almost entirely on clinical symptoms, which can be confirmed by biopsy. Currently, no validated laboratory tests exist to predict the risk of developing aGVHD, responsiveness in treatment, or patient survival (Paczesny, 2012). However, early detection of the disease using specific, rapid and noninvasive method would allow preemptive interventions and therapy dose optimisation reducing disease- and treatment-related toxicities. A large panel of potential aGVHD biomarkers has already been proposed by some research groups, comprising genetic disparities and polymorphisms (Hansen et al., 2010), microRNAs (Ranganathan et al., 2012), cellular markers (Magenau et al., 2010; Rubio et al.,

2012) or serum proteins (Paczesny et al., 2009; Chen et al., 2012; Levine et al., 2012; Paczesny, 2012). This section will only focus on circulating proteins/polypeptides as candidate biomarkers.

Proteins that have been proposed as potential biomarkers of aGVHD are derived from hypothesis-driven analysis of proteins involved in immune and inflammatory response linked to aGVHD pathophysiology such as chemokines and cytokines or from non targeted analysis using proteomics approaches like mass spectrometry (Chen et al., 2012). Two categories of aGVHD biomarkers have been described: markers associated with systemic alteration and immune reactivity and those related to GVHD tissue damages. The most reported measurements of protein levels concerns interleukins, chemokines (particularly Th1 response) or other inflammatory proteins and have been mainly performed by single (e.g. ELISA) or sometimes multiplexed immunoassays (e.g. cytometric-bead array) for aGVHD diagnosis. Table 4 shows a non-exhaustive list of hypothesis-driven candidate biomarkers. Increased levels of cytokines such as IL-2, IL-6, IL-7, IL-10, IL-15, and TNF-α and its receptors at day of aGVHD symptoms onset or in the first weeks after HSCT have been associated with occurrence of aGVHD. Level of chemokines such as CXCL10 involved in T-cell trafficking, process in which T-cells migrate towards target organs, was also significantly elevated in serum at the time of aGVHD. However, some of these results have not been validated and these biomarkers are not solely associated with aGVHD but also with other complications involving systemic immune reaction. Other candidate biomarkers, which are not cytokines, have also been studied and include hepatocyte growth factor (HGF), fragments of cytokeratin 18, syndecan-1, C-reactive protein (CRP) and granzymes A and B. Recently, the decrease of the level of albumin, a non specific serum protein, from pretransplantation baseline to the onset of aGVHD treatment has been reported as a predictor of severe (grade III-IV) aGVHD onset (Rezvani et al., 2011).

In order to discover new biomarkers of aGVHD by screening sample protein content, different biological fluids have been investigated by several groups using large-scale proteomic approaches (Table 5). Srinivasan et al. determined, by SELDI-TOF-MS on a small cohort of samples, a serum proteomic signature composed of 8 distinct m/z ratios distinguishing GVHD samples from post-transplant non-GVHD samples with 100% of sensitivity and specificity. No peptide/protein was identified (Srinivasan et al., 2006). In order to observe protein changes after HSCT, Imanguli et al. performed a study using SELDI-TOF-MS complemented by a 2D-DIGE analysis on saliva samples taken at pre-HSCT, 1 month and 2 months post HSCT time points

(Imanguli et al., 2007). Comparison of 1 month post HSCT SELDI-TOF-MS profiles from patients with or without aGVHD showed intensity changes of 30 peaks. No one was identified. Serum samples from a mouse model were also investigated by SELDI-TOF-MS. One peak, corresponding to CCL8 exhibited a significant increase in GVHD samples compared to control mice samples (syngeneic transplant) and changed at different time point after HSCT in an individual mouse. This result was confirmed by ELISA on few human serum samples and CCL8 level was shown to decrease during methylprednisolone treatment (Hori et al., 2008).

The most relevant studies for aGVHD biomarker discovery were performed recently by the groups of Weissinger et al. (Kaiser et al., 2004; Weissinger et al., 2007) and Paczesny, Ferrara et al (Paczesny et al., 2009; Paczesny et al., 2010; Ferrara et al., 2011). Weissinger et al. analysed urine samples using a capillary electrophoresis coupled to a CE-ESI-TOF-MS and identified biomarkers by LC-MS/MS. A pattern of 31 polypeptides discriminating GVHD from no GVHD samples was determined and tested on a large sample cohort allowing a classification with good sensitivity and specificity. Three polypeptides of the pattern were identified as fragments from collagen α-1 (I) and collagen α-1 (III). In a recent conference abstract, Weissinger et al. reported that the screening of a polypeptide pattern including collagen, albumin, beta-2 microglobulin and CD99 allows prediction of aGVHD development at least 10 days before clinical diagnosis in blinded, prospectively collected urine samples (Weissinger et al., 2012). Paczesny et al. first used a target proteomic approach on a small discovery set of plasma samples, allowing the simultaneous evaluation of 120 different proteins comprising acute-phase reactants, cytokines, angiogenic factors, tumor markers, leucocyte-adhesion molecules and metalloproteinases or their inhibitors, by antibody microarray. After confirmation of significant protein level difference by ELISA in a training set, they identified a panel of four proteins: IL-2Rα, TNFR-I, IL-8 and HGF that could optimally discriminate patients with and without aGVHD in a validation set of samples (Paczesny et al., 2009). In addition, to identify specific biomarkers of target organ, they first compared isolated skin aGVHD samples to samples from patients without aGVHD using Intact Protein Analysis System (IPAS). This technique combines a sample pretreatment by immunoaffinity followed by an offline orthogonal chromatographic separation of proteins previously labelled with isotopic acrylamide. The collected fractions are subsequently analysed by 2D nanoLC LTQ-FT. Among the 66 candidate biomarker proteins identified, elafin, a protein primarily expressed in the skin, emerged as the lead biomarker candidate. In a validation study, elafin was found to specifically

increase in isolated skin GVHD compared to patients with gastrointestinal GVHD or non-GVHD rash. It was validated on a larger cohort of samples. Afterwards, Ferrara's group tested prospectively the predictive value of a three-biomarker panel consisting of IL2-Rα, TNFR1 and elafin by measuring levels at pre-HSCT, day 7 and day 14 post HSCT. The prediction model allows predicting grade II-IV GVHD with good specificity (75%) but fair sensitivity (57%) (Paczesny et al., 2011). Following the same experimental design and large-scale proteomics technology, IPAS, Ferrara et al. discovered the regenerating islet-derived 3-alpha (REG3α) protein as a biomarker of gastrointestinal aGVHD. REG3α levels were not elevated in isolated skin GVHD or non-GVHD enteritis showing the specificity of this marker. In addition to these studies, Levine et al. demonstrated that the measurement of a panel of six biomarker levels (IL2-Rα, TNFR1, HGF, IL-8, REG3α and elafin at day of aGVHD treatment initiation and 14 days after) could predict for treatment failure at day 28 and death by day 180 (Levine et al., 2012).

Table 4: Hypothesis-driven candidate biomarkers

Protein	Function	Sample type	Methods	Day post-HSCT tested	Increase or decrease with GVHD	Organ specificity	Specific to aGVHD	Reference
Albumin	Regulation of osmotic pressure – Ion and protein transporter	Serum	Chemical assay	From 30 days before HSCT to 45 days after GVHD treatment initiation	Decrease	Systemic/ GI tract	ND Limited to RIC	(Rezvani et al. 2011)
CRP	Acute phase reactant of inflammation. Promotion of agglutination, bacterial capsular swelling, phagocytosis and complement fixation	Serum	Nephelo-metry	Before conditioning and different time points after HSCT	Increase	Systemic	ND	(Fuji et al. 2008)
Cytokeratin-18 fragments	Intermediate filament in epithelial and parenchymal cells. Marker of apoptosis induction.	Serum	ELISA	Pre-HSCT and routinely collected	Increase	GI tract and liver	No, compared to other GI and liver complications.	(Luft et al. 2007)
Granzymes A et B	Enzymes Involved in target cell lysis in cell-mediated immune responses	Serum	ELISA	From day-6 to day 90 post HSCT	Increase	Systemic	No, compared to CMV infection (only for granzyme A)	(Kircher et al. 2009)
Hepatocyte growth factor (HGF)	Act as a growth factor for many tissues and cell types.	Serum	ELISA	Different time points after HSCT	Increase	Systemic	ND	(Okamoto et al. 2001)
IL-6	Potent inducer of the acute phase response. Lymphocytes and monocytes differentiation	Plasma	ELISA	Before conditioning Once a week	Increase	Systemic	No, compared to other TRC	(Pihusch et al. 2006)
		Serum	ELISA	From the day of BMT until stable neutrophil recovery	Increase	Systemic	No, compared to other TRC	(Schots et al. 2003)
		Plasma	ELISA	From week -1 to week 26	Increase	Systemic	No, compared to other TRC	(Abdallah et al. 1997)
IL-7	T-cell proliferation and differentiation	Serum	ELISA	Before conditioning Different time points until 12 months post HSCT	Increase	Systemic	ND	(Dean et al. 2008)

Table 4 (2): Hypothesis-driven candidate biomarkers

Protein	Function	Sample type	Methods	Day post-HSCT tested	Increase or decrease with GVHD	Organ specificity	Specific to aGVHD	Reference
IL-7/ IL-15	T-cell proliferation and differentiation/ T-cell and NK cells proliferation	Plasma (myeloablative BMT)	ELISA	Before conditioning Day of infusion	Increase	Systemic	IL-15, marker of relapse	(Thiant et al., 2010)
		Plasma (RIC HSCT)	ELISA	Between day 7 and day 90 post-HSCT	Increase	Systemic	ND	(Thiant et al., 2010)
IL-10	Downregulation of Th1 cytokines expression	Serum	ELISA	Day-7 to day 100 post HSCT	Increase	Systemic	ND	(Resende et al., 2010)
		Plasma	ELISA	At time of the lowest leukocyte count and at time of the most rapid recovery leukocyte count	Increase	Systemic	ND	(Takatsuka et al., 1999)
IL-12	T-cell growth factor, stimulates production of TNF-α and IFN-γ	Plasma	ELISA	Day-7 and days 28, 59 and 91 post-HSCT	Increase	Systemic	ND	(Mohty et al., 2005)
IL-18	Activation of NK cells and induction of IFN-γ production	Serum	ELISA	Different time points after HSCT	Increase	Systemic	ND	(Shaiegan et al., 2006)
sCD8	Marker of cytotoxic/suppressor T-cells. May play a role in the process of T-cell mediated killing.	Plasma	ELISA	Before conditioning, day 0, day5, 10 and 15 post-HSCT	Increase	Systemic	ND	(August et al., 2011)
sIL-2R	Soluble portion of the IL-2 receptor, a marker of T-cell activation	Serum	ELISA	Different time points after HSCT	Increase	Systemic	ND	(Shaiegan et al., 2006)
		Serum	ELISA	Before HSCT Once a week for 4weeks	Increase	Systemic	No, compared to other critical TRC	(Mathias et al., 2000)
		Plasma	ELISA	Before conditioning, day 0, day5, 10 and 15 post-HSCT	Increase	Systemic	ND	(August et al., 2011)
Syndecan-1	Heparan surface proteoglycan present on the epithelial cell surface. Links the cytoskeleton to the interstitial matrix.	Serum	ELISA	Routinely collected after HSCT	Increased	Systemic	Yes compared to sepsis	(Seidel et al., 2003)

<u>Table 4 (3)</u>: Hypothesis-driven candidate biomarkers

Protein	Function	Sample type	Methods	Day post-HSCT tested	Increase or decrease with GVHD	Organ specificity	Specific to aGVHD	Reference
TGF-β	Inhibition of T-cell proliferation. Induction of iTreg.	Serum	ELISA	Between 2-4 weeks post-HSCT	Decrease	Systemic	Also increase in cGVHD	(Li et al., 2010)
TNF-α	Marker of activated macrophages. plays a central role in inflammation, immune system development, apoptosis, and lipid metabolism.	Serum	ELISA	From the day of BMT until stable neutrophil recovery	Increase	Systemic	No, compared to other TRC	(Schots et al., 2003)
TNF-RI	Receptor of TNF-α. Measured as a surrogate marker for TNF-α.	Serum	ELISA	Before conditioning Between day 7 to day 180 post HSCT	Increase	Systemic	ND	(Willems et al., 2010)
		Plasma (children)	ELISA	Prior to and at day 7 post HSCT	Increase	Systemic	ND	(Kitko et al., 2008)
		Plasma	ELISA	Prior to and at day 7 post HSCT	Increase	Systemic	No, also in TRM	(Choi et al., 2008)
		Plasma	ELISA	Before conditioning, day 0, day5, 10 and 15 post-HSCT	Increase	Systemic	ND	(August et al., 2011)

Table 5 (1): Biomarkers identified by proteomic approaches

Reference	Protein	Function	Sample type	Methods	Day post-HSCT tested	Increase or decrease with GVHD	Organ specificity	Validated	Specific to aGVHD
(Paczesny et al., 2009)	HGF	Act as a growth factor for many tissues and cell types.	Plasma	Antibody array	At the time of clinical diagnosis	Increase	Systemic	Yes (in a panel)	ND
	sIL-2R	Receptor of IL-2, a marker of T-cell activation				Increase	Systemic		
	IL-8	Chemotactic factor of neutrophils and T-cells. Neutrophil activation				Increase	Systemic		
	TNF-RI	Receptor of TNF, a marker of macrophage activation				Increase	Systemic		
(Ferrara et al., 2011)	REG3α	Protect the epithelial barrier function of the intestinal mucosa	Plasma	IPAS	At the time of clinical diagnosis	Increase	GI tract	Yes	Yes compared to non GVHD enteritis and skin GVHD
(Paczesny et al., 2010)	Elafin	Prevent elastase-mediated tissue proteolysis	Plasma	IPAS	At the time of clinical diagnosis	Increase	Skin	Yes	Yes compared to non GVHD rash and GI GVHD
(Hori et al., 2008)	CCL8	Chemokine attracting leucocytes to sites of inflammation	Plasma (mouse and human)	SELDI-TOF-MS ELISA	Day of HSCT and days 7, 14, 21, 28 post-HSCT	Increase	Systemic	No	ND
(Kaiser et al., 2004)	Leukotriène A4 hydrolase	biosynthesis of the proinflammatory mediator leukotriene B4	Urine	CE-MS	At the time of clinical diagnosis and at different time points after HSCT	Increase	Systemic	No	Yes (in a panel), compared to sepsis
	Albumin (fragment)	Regulation of osmotic pressure – Ion and protein transporter				Increase	Systemic	No	Yes (in a panel), compared to sepsis

Table 5 (2): Biomarkers identified by proteomic approaches

Reference	Protein	Function	Sample type	Methods	Day post-HSCT tested	Increase or decrease with GVHD	Organ specificity	Validated	Specific to aGVHD
(Weissinger et al., 2007)	Collagen α-1 chain (I) fragment	Constituent of connective tissues	Urine	CE-MS	At the time of clinical diagnosis and at different time points after HSCT	Decrease	Systemic	Yes (in a panel)	Yes, compared to chronic renal diseases and CMV/ EBV reactivation
	Collagen α-1 chain (III) fragment	Constituent of connective tissues				Increase	Systemic		
(Weissinger et al., 2012)	Beta-2-microglobulin	Involved in the presentation of peptide antigens to the immune system.	Urine	CE-MS				Yes	ND
	CD99	Involved in T-cell adhesion processes and leucocyte extravasation							

AIMS OF THE WORK

In 2009, more than 26000 allogeneic HCT were carried out worldwide as a therapy for diverse hematologic malignancies including leukemia, lymphoma, myeloproliferative disorders, myelodysplasia as well as congenital immunodeficiency or defective hematopoietic states. However, acute graft-versus-host disease remains a life-threatening complication despite many advances in preventing its occurrence with introduction of new prophylactic strategies and changes in the transplantation procedure. Current diagnostic method based on histological examination is time-consuming, invasive and cannot provide specific diagnosis as aGVHD shares similar features with confounding complications. Therefore, there is a need for a specific, rapid and non invasive assay for the early and accurate aGVHD diagnosis to reduce morbidity and mortality.

The main aim of this work is the investigation of plasma proteome of patients undergoing HSCT in order to characterize early aGVHD biomarkers.

In the 90's, emergence of proteomics provides attractive tools for the rapid evaluation of protein content in complex human samples without a priori. However, current proteomic approaches have a linear dynamic range of detection and quantification limited to 10^4-10^5 orders of magnitude, not sufficient to cover entire plasma proteome. As low abundance proteins deriving from tissue leakage could be considered as more specific disease biomarkers, fractionation of samples in order to facilitate detection of the deep proteome is highly recommended before sample analysis. Thus, the first part of our work consists in the comparison of three different methods for sample fractionation. The three approaches (precipitation by an organic solvent, "equalization" of protein concentration and metal affinity coupled to restricted access materials) were evaluated in terms of reproducibility and their ability to increase the number of detectable peptides/proteins.

Then samples from patients developing aGVHD and patients undergoing HSCT without evidence of aGVHD (control samples) were compared to detect peptides/proteins differentially expressed with significance. In addition, samples taken 15 days before aGVHD symptom appearance were also tested to determine potential early biomarkers. To extent the protein coverage and increase success to find candidate plasma markers, we combined three proteomics approaches: 2D-DIGE, SELDI-TOF-MS and high resolution 2D-LC/MS. Candidate

biomarkers were then evaluated by quantitative targeted methods (ELISA, Western Blot and nanoLC-MS/MS) to confirm the differential protein abundance between groups. Then, a composite biomarker panel with the best discriminative ability to distinguish control from aGVHD samples was build using multivariate model.

Acute GVHD is a typical exacerbated immune-mediated disease resulting from a complex interaction between immune cells and the secretion of proinflammatory cytokines. In parallel to our proteomic approach, we measured by immunoassays the levels of several cytokines (IL-2, IL-6, IL-7, IL-8, IL-10, and IFN-γ) in our sample groups to determine their discriminative potential. Levels of these cytokines in samples taken 15 days before aGVHD were also assessed to investigate the potential properties of these cytokines to predict subsequent acute GVHD.

Finally, as aGVHD is a T-cell mediated complication, a relationship between IL-7 and IL-15 levels and subsequent aGVHD occurrence can be easily suggested. Therefore, we evaluated the association of IL-7 and IL-15 levels at different time points after HSCT with the development of subsequent aGVHD in 70 patients subjected to nonmyeloablative conditioning. In addition, kinetics of cell population reconstitution as well as factors affecting immune recovery and cytokine levels were studied.

RESULTS

Comparison of three methods for fractionation and enrichment of low molecular weight proteins for SELDI-TOF-MS differential analysis

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Article published in Talanta. 2010 Jun 30;82(1):245-54.

Keywords: protein prefractionation, major protein depletion, plasma, serum, SELDI-TOF-MS

Abbreviations:

CM10: weak cationic exchanger arrays

HAP: highly abundant proteins

HMW: high molecular weight

IMAC-RAM: restricted access materials (RAM) combined with IMAC chromatography

LMW: low molecular weight

PF4: Platelet factor 4

PRM-30: Proteomics-30 resin for molecular mass < 30 kDa

RSD: relative standard deviation

RT: room temperature

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SELDI-TOF-MS: Surface-Enhanced Laser Desorption/Ionisation – Time-Of-Flight - Mass Spectrometry

Abstract

In most diseases, the clinical need for serum/plasma markers has never been so crucial, not only for diagnosis, but also for the selection of the most efficient therapies, as well as exclusion of ineffective or toxic treatment. Due to the high sample complexity, prefractionation is essential for exploring the deep proteome and finding specific markers.

In this study, three different sample preparation methods (i.e. highly abundant protein precipitation, restricted access materials (RAM) combined with IMAC chromatography and peptide ligand affinity beads) were investigated in order to select the best fractionation step for further differential proteomic experiments focusing on the LMW proteome (MW inferior to 40000 Da). Indeed, the aim was not to cover the entire plasma/serum proteome, but to enrich for potentially interesting tissue leakage proteins. These three methods were evaluated on their reproducibility, on the SELDI-TOF-MS peptide/protein peaks generated after fractionation and on the information supplied. The studied methods appeared to give complementary information and presented good reproducibility (below 20%). Peptide ligand affinity beads were found to provide efficient depletion of HMW proteins and peak enrichment in protein/peptide profiles.

1. INTRODUCTION

New biomarkers are expected to improve diagnosis, to guide molecularly targeted therapy and to monitor activity and therapeutic response across a wide spectrum of diseases. From a clinical point of view, it is easy to understand why blood biomarker discovery is very attractive. Its sampling is minimally invasive and can be performed repeatedly. To analyse circulating proteins and peptides, cellular components of blood can be removed, either in the presence of anticoagulants or after blood coagulation, yielding to plasma and serum, respectively.

Proteomic profiling of biological fluids for disease biomarker discovery has already improved drastically and is still in constant evolution. Indeed, potentially interesting biomarkers have emerged in literature for several diseases, including cancers and chronic inflammatory diseases [1-4]. Nevertheless, only a few of these have been validated. Much criticism has been made on the poor specificity of some of the discovered biomarkers [5,6]. Actually, most of them are abundant proteins or truncated forms, such as acute phase reactant proteins or proteins linked to clotting or platelet activation during blood sample preparation. However, even if one single marker shows poor specificity, the combination of several candidates could provide a powerful diagnostic tool, as demonstrated by the recently FDA approved OVA1 test combining 5 markers for ovarian cancer diagnostic. However, sample prefractionation appears essential for exploring the deep proteome and highlighting early disease stage biomarkers rather than host response biomarkers.

Analysis of plasma or serum is challenging because of its huge protein abundance dynamic range. It is well known that blood protein concentration covers 10 orders of magnitude, ranging from albumin (35-50 mg/ml in serum) to IL6 (0-5 pg/ml in serum)[7]. The 20 most abundant proteins, including albumin, immunoglobulin, fibrinogen, alpha 1-antitrypsin, alpha 2-macroglobulin, transferrin and lipoproteins, represent approximately 97% of the total protein mass [8-10]. The remaining 3% belong to a complex mixture of middle and low abundance proteins, including proteins of the complement family, hormones or proteins originating from normal tissue secretion or leakage upon cell death or damages. As the dynamic range of the protein amount that can be detected in a single mass spectrum is typically around 2 to 3 orders of magnitude, it is thus not possible to cover the entire range present in blood samples within one experiment [11]. To overcome this, several fractionation procedures have been developed and are now available to narrow the sample protein concentration dynamic range [12-14]. The

most commonly used methods based on physico-chemical peptide/protein properties are centrifugal ultrafiltration, precipitation by organic solvents, electrophoresis and chromatography (on-column or on- magnetic beads) [15-18]. However, these fractionation methods have not yet been evaluated in terms of high throughput capacity and reproducibility in proteomics [19]. Additionally, some proteins can be distributed over several fractions challenging the comparison of their abundance between samples.

Another widely used approach for HAP removal in serum and plasma is their depletion using specific antibodies [20]. But it is worth mentioning that some of the HAP act as carriers for minor abundance proteins, explaining the co-depletion of almost 3000 species as observed by several groups, both fractions being thus interesting to investigate [21,22]. Moreover, this kind of affinity depletion shows also a degree of unspecific binding with non-targeted proteins due to cross reactivity of the antibodies used [23,24].

SELDI-TOF-MS is an instrument used for disease biomarker discovery over a large and fully automated scale. It provides biomarker patterns for a high number of individuals aiming at overcoming the limitation of single markers (i.e., lack of sensitivity and specificity) and may lead to consistent statistical data for a large population [25]. Using SELDI-TOF-MS, many key LMW proteins/peptides with molecular masses below 40 kDa were highlighted [26-28]. Some of these could be used to determine the onset of a given disease [29]. Indeed, LMW proteins/peptides in the serum/plasma include members of several physiologically important classes, such as cytokines, chemokines, and peptide hormones, along with proteolytic fragments of larger proteins, including those generated by disease-specific exopeptidases [30]. SELDI-TOF-MS combines the pre-selection of proteins and peptides on a specific chromatographic surface with a linear time of flight mass spectrometer. Different types of surface are available (hydrophobic, ion exchanger…) and determine the proteins that will be analyzed. Nevertheless, this pre-selection step is limited by the small number of activated groups available on this small surface promoting fixation of the most abundant and sometimes less informative proteins. Therefore, the reduction of sample complexity is essential to ensure the detection of proteins that are present at low concentrations.

In this study, we evaluated three different sample preparation methods (i.e. HAP precipitation, restricted access materials (RAM) combined with IMAC chromatography and equalization) to select the best fractionation step for further differential proteomic experiments focusing on the LMW proteome (MW inferior to 40 kDa). The aim was not to cover the entire plasma/serum

proteome, but to enrich for potentially interesting small MW tissue leakage proteins. The evaluation was based on the number and/or redundant information and on the reproducibility of the tested methods. Those three methods were chosen for their relatively high throughput capacity compared to HPLC, IEF or differential centrifugation. Precipitation is of course very rapid. Proteomics-30® and ProteoMiner® are now being developed in mini-spin columns and 96 well-plates, respectively. Indeed, we intended to deal with clinical material presenting a large biological heterogeneity that requires the comparative analysis of a large number of samples.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Acetonitrile, trifluoroacetic acid, CHAPS, sodium chloride, Trizma base, Trizma hydrochloride, Na2HPO4, imidazole, thiourea were supplied by Sigma-Aldrich (St. Louis, MO, USA), whereas urea was from Amersham and acetic acid from Vel. Sodium acetate, ammonia solution 25% and ammonium chloride were from Merck. All reagents were of analytical grade. RC-DC protein assay kit, weak cationic exchanger arrays (CM10) and sinapinic acid (SPA) were provided by Bio-Rad (Hercules CA, USA).

2.2. Human samples

EDTA plasma and serum were provided from healthy donors. Serum, after 30 min of clotting, and plasma were centrifuged at 800g for 10 min at room temperature prior to being aliquoted and stored at -80°C. Before each sample treatment, thawed serum and plasma were centrifuged at 16100g for 15 min to remove most of the lipids and insoluble materials.

2.3. Peptide ligand affinity beads

Peptide ligand affinity beads, also called ProteoMiner®, were provided by Bio-Rad. Each column contains 500 µl of beads (20% beads, 20% ethanol, 60% water). One milliliter of crude serum or plasma was directly loaded on column without previous dilution. Loading such an important sample volume should ensure the concentration of low and medium abundance proteins [31].
Plasma and serum samples were analyzed in six independent experiments. Briefly, beads were washed successively by the addition of 1 ml of deionized water and 1 ml of wash buffer (150 mM NaCl, 10 mM NaH2PO4 pH 7.4). Then, 1 ml of sample was loaded on columns and incubated with beads for a period of 2 hours at RT. Columns were centrifuged twice for periods of 2 mins and 1 min and all column flowthroughs were collected for further analysis (called FT). Columns were then washed 3 times for 5 min. Proteins and peptides retained on beads were eluted by 300 µl of a solution made of 8 M urea, 2% CHAPS in 5% acetic acid buffer and then directly stored at -80°C.

2.4. Precipitation

First, serum and plasma were denatured with 1.5 volume of a solution made of 7 M urea, 2 M thiourea, 2% CHAPS in a 50mM TRIS pH 9 buffer for 30 min at room temperature. Then, 1.25

vol. of an acetonitrile / 0.1% trifluoroacetic acid solution was progressively added to the sample and incubated for 30 min at RT. Next, samples were centrifuged at 16100g for 20 min and supernatants were collected and adjusted with HPLC water to obtain a final dilution of 1/6.

2.5. IMAC-RAM

These resin column materials, also called Proteomics-30®, were provided by Affiland (Belgium) in a context of scientific collaboration. Each kit is composed of Proteomics-30® resin columns, washing buffer and elution buffer. 100µl of crude serum was loaded into the resin after equilibration with 4ml of washing buffer. After incubation, 900µl of washing was added to obtain a final volume of 1 ml. The column was then washed twice with 1 ml and once with 7ml of the equilibration buffer. Elution was performed adding 3x1 ml of elution buffer. 500µl of the second elution fraction was finally dialysed against acetate buffer pH 4 or Tris buffer pH 9 before for further steps.

2.6. One dimension (1D-) gel electrophoresis

The concentration of each sample, including crude samples used as the reference, was measured using the RC-DC protein assay kit (Bio-Rad). SDS-PAGE analysis was carried out loading 5µg of proteins on NuPAGE 12% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA). The gel was further stained using a SilverQuest silver staining kit (Invitrogen). All samples were processed according to the manufacturer's instructions.

2.7. ProteinChip Array Preparation and Analysis

Samples were analyzed on anionic (CM10) ProteinChip arrays (Bio-Rad, Belgium), as previously described [28]. The supernatant obtained after the precipitation process and ProteoMiner® samples (eluate and flowthrough) were respectively diluted 6 and 10 fold in binding buffer: 100 mM acetate, pH 4 or Tris buffer pH9. ProteoMiner eluates were equilibrated by the addition of ammonium buffer pH 10.5. CM10 arrays were equilibrated 3 times with pH 4 or pH 9 binding buffer. 10µl of sample were applied on ProteinChip arrays and incubated for 1h, at RT, in a humidity chamber. The spots were washed with binding buffer (10 ul) followed by a quick rinse with Milli-Q water (10 ul). After 20 min of air drying, 1 µl of saturated sinapinic acid (SPA) solution (prepared following the manufacturer's recommendations) was applied to each spot. CM10 arrays were then analyzed in a PCS4000 SELDI-TOF-MS reader (Bio-Rad). Spectra were calibrated using external calibration against peptides and proteins from an All-in-one Peptide

kit (1.5 – 8 kDa mass range) and an All-in-one Protein kit (8 – 40 kDa mass range). Laser intensity was optimized for ion detection in these two mass ranges averaging 1560 shots per spot and avoiding signal saturation. Autodetection of peaks was performed for m/z ranging from 1500 to 80000. A minimal signal-to-noise ratio threshold of 3 and a valley depth between 0.68 and 1.9 were the two criteria used for peak cluster formation. Baseline subtraction and normalization on total ion current were performed for all spectra using Protein Chip data manager software (Bio-Rad).

3. RESULTS AND DISCUSSION

The presence of HAP in serum and plasma such as albumin and IgG is detrimental to the detection of low abundant biomarkers. To address the complexity of these samples, it is essential to remove HAP and to concentrate proteins of low abundance before proteome analysis. Due to the high number of samples required for clinical proteomics (ideally between 100 and 1000 samples), the high throughput capacity of the whole procedure is also an important aspect. The present study consists in the comparison of three depletion methods of abundant proteins in serum and plasma samples, namely protein precipitation, IMAC-RAM (or Proteomics-30®) and peptide ligand affinity beads for equalization (or ProteoMiner®). Crude serum and plasma were used as reference samples. The experiment layout is presented in Figure 1. After sample pretreatment, protein content was quantified and analyzed by SDS-PAGE and Surface-Enhanced Laser Desorption/Ionisation - Time-Of-Flight - Mass Spectrometry (SELDI-TOF-MS).

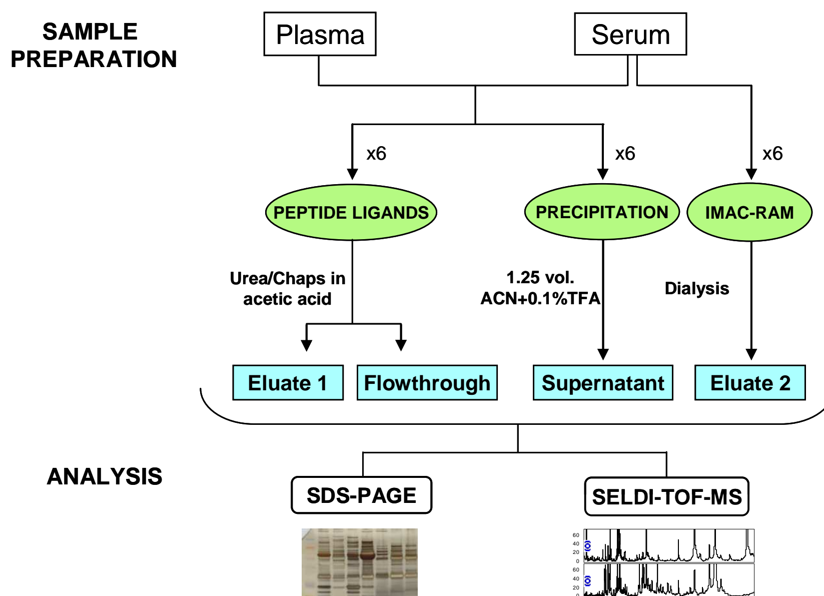


Figure 1: Experimental layout

SELDI-TOF-MS profiling based on weak cationic exchanger arrays (CM10) was used to evaluate the gain of information (profile enrichment), the reproducibility (n=6) and complementarities between profiles. To broaden the field of investigation, samples were analyzed on CM10 at two pH binding conditions (pH 4 and pH 9). Peak detection was performed within two mass ranges (1.8-8 kDa and 8-80 kDa) with properly mass calibration using two different and adequate calibration curves.

3.1. Sample pre-treatments and 1D-gel

3.1.1. Precipitation

Major protein depletion from plasma and serum was investigated using precipitation with various organic solvents (acetonitrile, isopropanol and methanol) at different percentages. After centrifugation and pellet removal, determination of the remaining protein content was performed. Plasma and serum supernatants were then analyzed by 1D-gel and SELDI-TOF-MS. As described by other authors [21,32], acetonitrile added to 0.1% TFA was found to give the best results in terms of number of protein peaks detected below 40 kDa and resolution. Denaturation of sample before organic solvent addition also improves the protein profile (data not shown).

As can be seen in Figures 2A (column 2) and 2B (column 2), the majority of the high molecular weight proteins (HMW) (> 40 kDa) are depleted after ACN/TFA protein precipitation, compared to crude serum (Figure 2A, lane 1) and plasma (Figure 2B, lane 1). Most of the high abundant proteins in blood are larger than 40 kDa. The addition of 1.25 vol. of acetonitrile (ACN) containing 0.1% TFA leads them to precipitate. Subsequent centrifugation removes 97-98% of the proteins, as determined after total protein content determination.

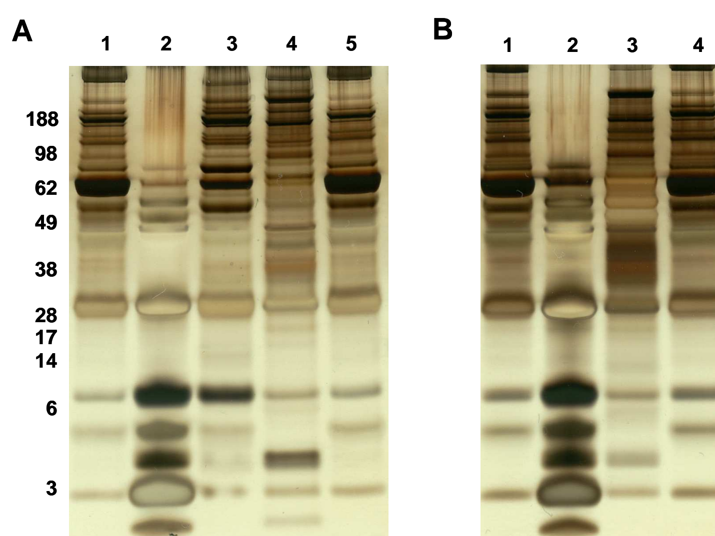


Figure 2: Representative silver stained SDS-PAGE for each prefractionation method. Protein profiles observed in 2,000 to 200,000 m/z range.

A. Serum: (1) crude serum, (2) organic precipitation, (3) Proteomics-30®, (4) ProteoMiner® eluate, (5) ProteoMiner® flowthrough

B. Plasma: (1) crude plasma, (2) organic precipitation, (3) ProteoMiner® eluate, (4) ProteoMiner® flowthrough

Protein precipitation is not a specific method like immunodepletion [33]. Indeed, non-targeted proteins, including potential valuable biomarkers, may be lost during precipitation. Some

proteins may also remain partly soluble and are present in both fractions, compromising differential analysis. Another disadvantage could also be the important dilution of the sample by the addition of solvent. However, the solvent-precipitation method is rapid, simple and cheap. Moreover, the presence of an organic solvent dissociates protein complexes which may facilitate the detection of potential biomarkers associated to HAP.

3.1.2. IMAC-RAM

Proteomics-30® resin combines two principles, IMAC-Cu chromatography and size fractionation with a cut-off of 30 kDa. It is an Affiland patented Metal pentadentate chelator (PDC) resin (EP0972566 B1) which recognizes principally all proteins and/or peptides with MW<30 kDa. PDC coupled to a resin is able to form complexes with all polyvalent metal ions and to give an octahedral Metal ion-Chelator complex with five coordination sites occupied by the chelator. It provides a high stability of the Metal ion-Chelator complex. It also results in one free site for interaction and selective binding of accessible cysteine/histidine residue and chiefly histidine containing biomolecules. PDC-Cu chromatography is used to bind mostly peptides/proteins with MW below 30 kDa and get rid of HMW proteins, salts and lipids. Because of the presence of EDTA in the plasma, this sample pretreatment method was only investigated on serum samples (Figure 2A, lane 3).

The major difference between crude serum and Proteomics-30® pre-treated samples visible on 1D gel is a strong decrease in albumin content, also observed by SELDI-TOF-MS profiling (cf. Paragraph 3.2.). However HMW protein depletion is less efficient compared to the two other fractionation approaches. The total protein content was decreased by 76% after Proteomics-30® procedure.

3.1.3. Peptide ligand affinity beads

This new fractionation approach, recently developed by Righetti and Boschetti, implies the use of a combinatorial library of hexapeptides grafted on micro beads on which, in theory, only one copy of a unique ligand binds. This approach, named ProteoMiner®, simultaneously dilutes HAP and concentrates low and medium abundant proteins [34,35]. The main interest of this equalization method is the dynamic range reduction between high and low abundant proteins and peptides. However, it was shown that, despite the decrease in dynamic range, this technology used for differential studies was only applicable for proteins or peptides which do not reach saturation, i.e. the range of low and medium abundance proteins [36]. ProteoMiner®

was also found to reduce the risk of codepletion that may occur with immunoaffinity methods and presents a much higher loading capacity. Simo et al showed that interactions between protein and amino acid baits are mainly due to hydrophobic interactions, especially with aromatic moieties in priority, followed by hydrogen bonding, and finally ionic interactions [37].
As shown in Figures 2A (lane 4) and 2B (lane 3), equalization of the protein concentration range promotes the detection of new protein peaks, as compared to crude serum (column 1). Interestingly, 1D-gel profiles from eluates (Figure 2A, lane 4 and 2B lane 3) and flowthroughs (Figure 2A, lane 5 and 2B lane 4) obtained after ProteoMiner® sample pre-treatment gave complementary information. Concerning the total protein content, a decrease of 96 and 98% was measured for plasma and serum, respectively.

3.2. Abundant protein depletion

To have an idea of the fractionation method efficiency for abundant protein depletion, m/z values of peaks detected in our spectra were correlated to those described and identified in the literature using the same binding conditions on chip arrays [38]. Moreover, we consider that if a prefractionation method is efficient, proteins abundantly present and detected in crude sample should be significantly depleted in fractionated samples. For example, ProteoMiner® fractionation was found to present a good efficiency for abundant protein depletion after comparison between ProteoMiner® eluate, ProteoMiner® flowthrough and crude sample profiles (cf. Figure 3).
As can be seen in the Figure 3A, several peaks in the 2500 – 7000 m/z range nearly disappeared in serum ProteoMiner® eluate profile compared to crude serum profile. These should correspond to fibrinogen α-chain fragments (2932, 3191, 3240, 3262, 5902 Da) and inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) (3157 Da) [39,40]. Apo-C1 variants (6431, 6629 Da) were found to be decreased in ProteoMiner® eluate and precipitation profiles; while it is completely depleted in Proteomics-30® profiles [41]. As expected, these proteins were largely present in the flowthrough profile (cf. Figure 3A). Moreover, using ProteoMiner®, Figures 3B and C showed the efficient depletion of transthyretin (13765/13886 Da), hemoglobin alpha and beta chains (15121 Da and 15863 Da), B2-microglobulin (11728 Da) nd albumin (~66000 Da) proteins [42-44]. These proteins are among the most abundant ones in serum/plasma [38]. Similar observations could be made with precipitation and Proteomics-30® prefractionation methods (cf. Figures 3A-C). After Proteomics-30® treatment, Apo C-I, lysozyme (14685 Da), Apo

A1 (28084 Da) and albumin were depleted [45,46]. Precipitation seemed to be less efficient for depletion of abundant proteins below 40 kDa. However, B2-microglobulin, lysozyme and albumin were depleted while signals of other abundant proteins were increased (transthyretin, hemoglobin chains).

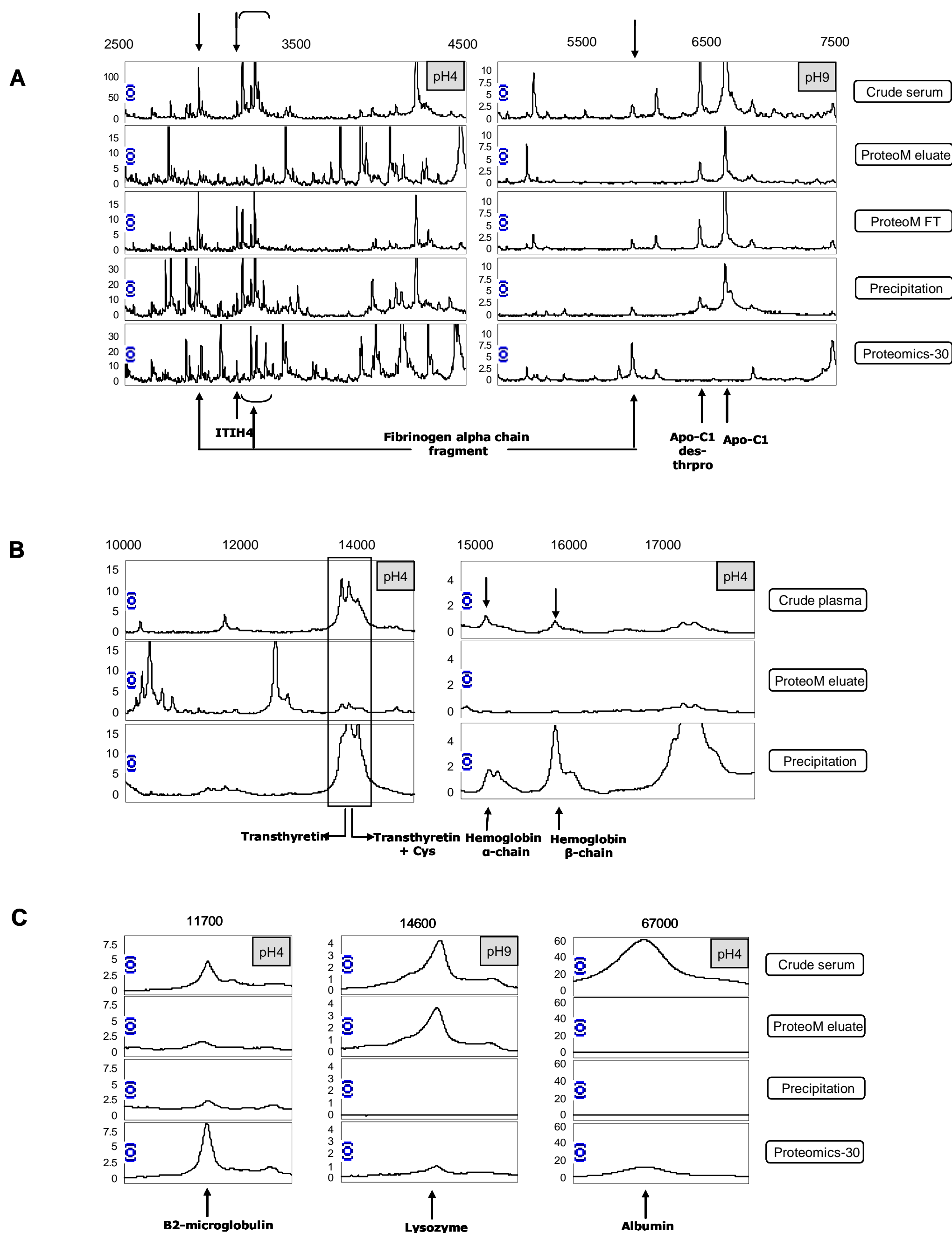


Figure 3: Spectra examples of HAP depletion

(A) 2500-7000 m/z range ; (B) 10000-18000 m/z range ; (C) B2-microglobulin, lysosyme and albumin depletion

As protein binding onto chromatographic surface depends on its affinity, its concentration, but also on chip surface binding capacity, one can imagine that competition between different

proteins for binding sites is rather complex. One can also assume that when high abundant proteins are depleted, low or medium abundant proteins should bind to the available free activated groups of the protein chip surface. This was supported by new peaks appearance in SELDI profiles after sample prefractionation (cf. Figures 3A-C).

3.3. SELDI-TOF-MS protein profiles obtained after fractionation

SELDI-TOF-MS profiles were studied more particularly within the 1.8-40 kDa mass range. It is noteworthy that these profiles were extended to 80 kDa in order to detect the presence/absence of albumin (m/z: 66,000). Using CM10 array at pH 4, 104 peaks were detected in the crude serum sample (cf. Table 1). Fewer peaks (73) were observed with crude plasma, probably due to the presence of a high amount of fibrinogen or coagulation related proteins, which might saturate protein arrays [47].

As can be seen in Table 1, precipitation of serum and plasma with ACN/TFA showed nearly the same number of peaks (91 and 73, respectively) at pH 4 compared to the crude sample and only few proteins were observed above 30 kDa (Figures 2, 4B and Supplementary data 1B).

Using Proteomics-30® material, 110 peaks were detected for serum. Despite the less efficient albumin depletion compared with precipitation and peptide ligand affinity beads (cf. Figure 4B), the SELDI-TOF-MS profile obtained at pH 4 was significantly enriched compared to crude serum (cf. Figure 4A).

Eluate and flowthrough obtained after ProteoMiner® treated serum samples were also studied by SELDI-TOF-MS (cf. Figure 4). ProteoMiner® eluate showed a gain of peaks compared to crude serum (115 vs 104). In addition, the treatment of plasma using ProteoMiner® was rather efficient since the number of peaks almost doubled: 122 versus 73 peaks at pH 4. It is worth noting that rather efficient albumin depletion was also observed (Supplementary data 1B).

In this pH4 condition, serum treated by Proteomics-30® and ProteoMiner® profiles showed a similar number of peaks (~110) while ACN/TFA treatment gave less information (~90 peaks) despite efficient albumin and IgG removal (cf. Figure 4B). For plasma samples, the ProteoMiner® approach showed profile enrichment compared to precipitation (cf. Table 1). Experiments were also carried out at pH 9 as albumin does not bind on cationic exchange surface at this pH (albumin pI : 4.7). In theory, proteins with a pI>9 should bind to the chip.

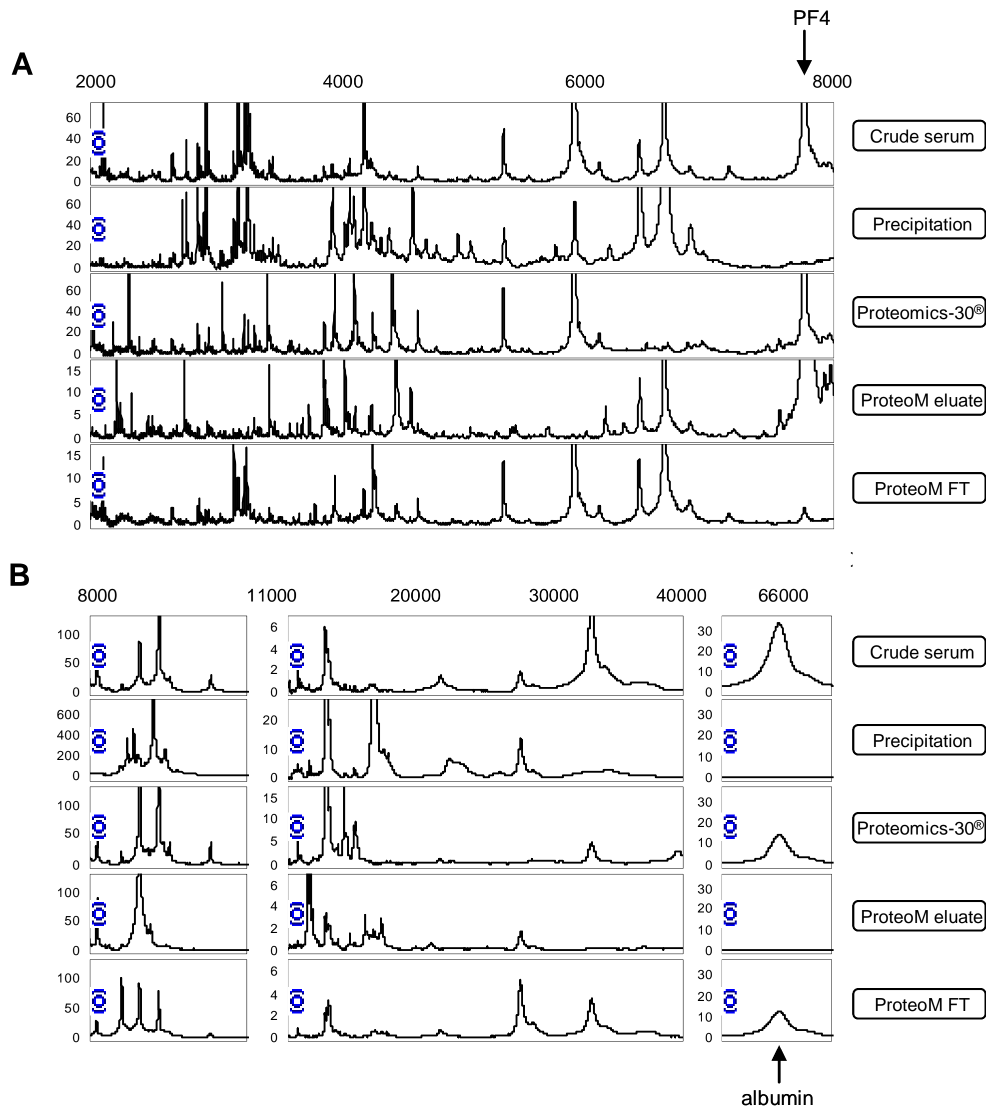


Figure 4: Comparison of serum prefractionation methods by SELDI-TOF-MS using CM10 at pH 4.

A. Protein profiles obtained in 2,000 to 8,000 m/z range.

B. Protein profiles obtained in 8,000 to 70,000 m/z range.

Table 1: Number of detected peaks and RSD (%; n=6) for each prefractionation method.

	SERUM				PLASMA			
	pH4		pH9		pH4		pH9	
	Peaks	RSD	Peaks	RSD	Peaks	RSD	Peaks	RSD
Crude	104	12	98	10	73	12	86	14
Precipitation	91	8	27	18	73	10	48	15
Proteomics-30® eluate	110	12	78	17	ND	ND	ND	ND
ProteoMiner® eluate	115	10	92	13	122	13	136	16
ProteoMiner® FT	90	14	82	14	91	14	69	15

Studied mass range: 1.8 – 40 kDa.

RSD: Relative standard deviation

As can be seen in Table 1, the spectra of serum and plasma pretreated by ProteoMiner® presented almost the same number and more peaks than the crude samples respectively (92 peaks for serum and 136 for plasma eluates compared to 98 and 86 peaks for crude serum/plasma sample profiles). Precipitation of serum and plasma at pH 9 revealed a very poor profile (only 27 and 48 peaks, respectively). Proteomics-30® serum eluate profile gave 78 peaks. Finally, ProteoMiner® eluate profiles revealed more peaks (136 peaks) than the two other approaches.

From these experiments, it can be concluded that SELDI-TOF-MS profiles obtained after ProteoMiner® pre-treatment showed enrichment on cationic chip arrays at both pH conditions, especially for plasma. Information gain was mostly observed in the 2,000 – 10,000 m/z range.

3.4. Sample pretreatment reproducibility

The reproducibility is a prerequisite for accurate differential proteome analysis of clinical samples process as well as for biomarker quantification.

Reproducibility was evaluated on six independent experiments for all sample preparations (cf. Table 1). Relative standard deviations (RSDs) were calculated on the intensity of all SELDI-TOF-MS peaks detected within the 1.8 - 40 kDa range, after replicates clustering. The reproducibility of the experiments performed with crude sample was also evaluated for comparison with treated samples. The amount of peaks detected for a specific prefractionation protocol was found to be the same. However, as mentioned in Table 1, the final number of peaks depended on the prefractionation method considered (i.e. 104 peaks detected in crude serum compared to 115 peaks detected in ProteoMiner® serum eluate) and on the sample type (serum vs plasma).

All the conditions tested on CM10 showed satisfactory RSD values (below 20%, this is the maximal tolerance of the FDA for bioanalysis (cf. FDA guidelines)). RSD values obtained for crude serum and plasma, used as reference samples, were both 12% at pH 4 and, at pH 9, 10% and 14%, respectively. The lowest variability was observed with acetonitrile precipitation at pH 4 (8% and 10%). For Proteomics-30® method, RSD values were also satisfactory (12% and 17% at pH 4 and pH 9, respectively).

As shown in Table 1, ProteoMiner® eluate and flowthrough gave similar RSD values than the reference sample at pH 4, while at pH 9, they were slightly higher.

In semi-quantitative and quantitative proteomic studies, it is important to keep in mind the risk of unselective loss and the functional changes of prefractionation material adsorption ability [48,49]. To our point of view, it is therefore critical, for proteomic analysis, to implement single-use devices which avoid carryover between samples. Indeed, incomplete elution with multiple-used devices can lead to a decrease of binding capacity and to some carryover on subsequent samples, compromising reproducibility and then efforts to find proteins and peptides in relation with disease process. Furthermore, during sample handling, protein degradation might occur. Duration of sample pretreatment processing is therefore an important point to take into account. This step is critical in preserving proteins/peptides integrity.

3.5. Sample pretreatment recovery

To evaluate the overlap between the 3 methods, Venn diagrams, based on the comparison of m/z values of the detected peaks across the different samples, were constructed (Figure 5, Figure 6C, Figure 7 and Supplementary data 4C and 5).

Figure 5 provides Venn diagrams showing information overlaps obtained by SELDI-TOF-MS between serum and plasma ProteoMiner® eluates bound at pH 4 (A) and pH 9 (B).

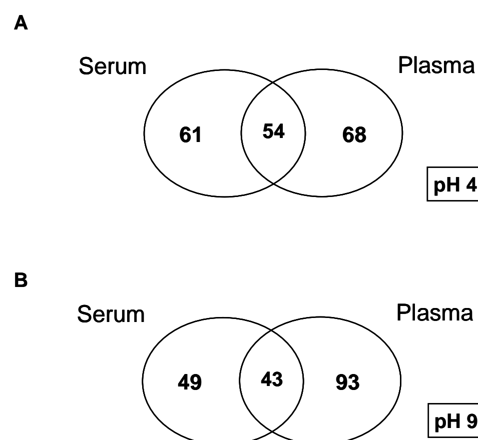


Figure 5: Venn diagrams showing information overlaps obtained by SELDI-TOF-MS between ProteoMiner® eluate of serum and plasma analyzed at pH 4 (A) and pH 9 (B).

As can be seen in this Figure, at both pH conditions, plasma generated more information than serum. The comparison of plasma profiles obtained with ProteoMiner® eluates at pH 4 and 9 showed 50 common peaks (Figure 6C), pH 9 condition being more informative (86 non common peaks versus 72 at pH 4). Profiles obtained at pH 4 and pH 9 bring complementary information

(cf. Figure 6C). In a biomarker discovery trial, it is interesting to combine information from different conditions.

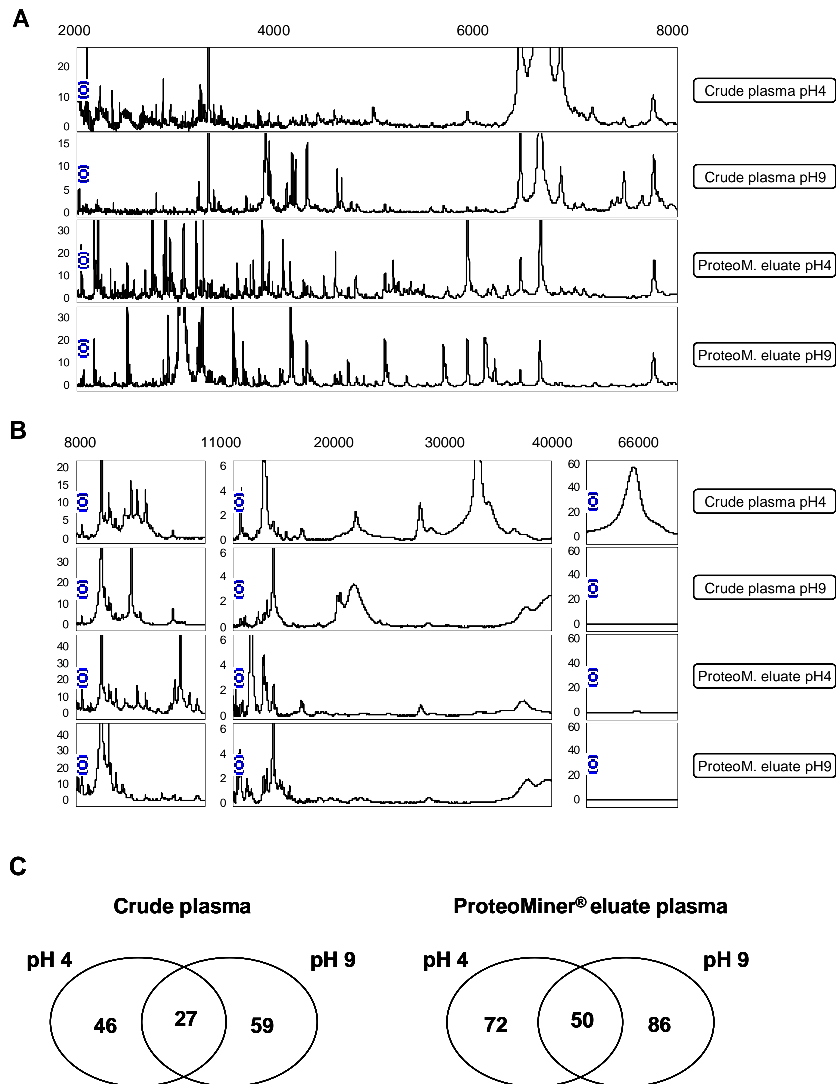


Figure 6: Representative SELDI-TOF-MS spectra and Venn diagrams from plasma prefractionation with ProteoMiner® at pH 4 and pH 9.

A. Protein profiles obtained in 2,000 to 8,000 m/z range.

B. Protein profiles obtained in 8,000 to 70,000 m/z range.

C. Venn diagrams

Figure 7 showed the prefractionation method information overlaps for serum and plasma at both pH conditions. These Venn diagrams clearly indicate that the three fractionation methods are complementary as the information overlap is poor. Indeed, only 9 and 7 peaks were in common when comparing SELDI spectra obtained after serum prefractionation at pH 4 and pH 9, respectively. For plasma samples, almost 30-40% of the peaks detected after precipitation

were also present in ProteoMiner® eluate profiles, while these common peaks represented only 15% of total peaks obtained from ProteoMiner® eluate profiles.

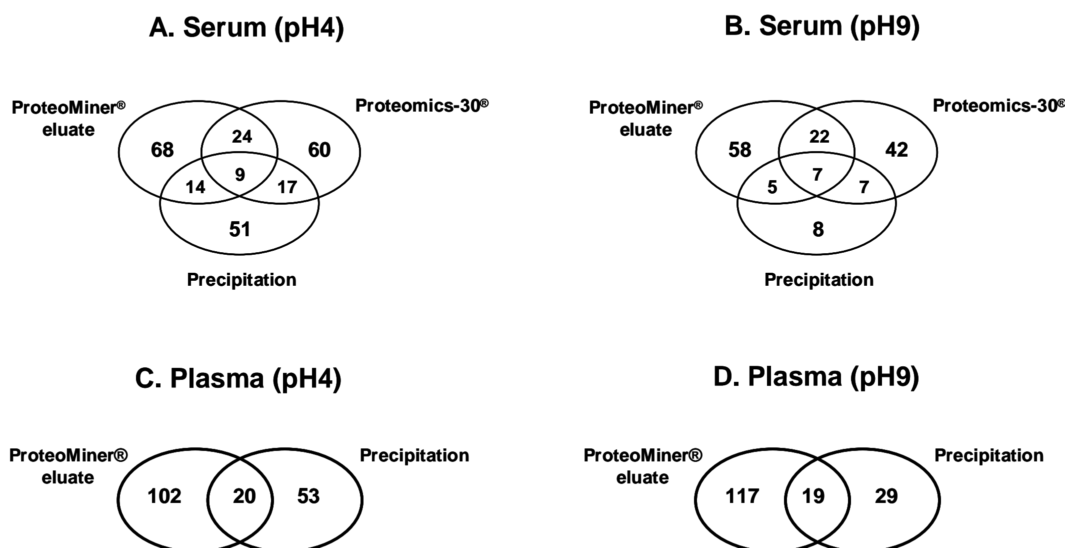


Figure 7: Prefractionation method information overlaps on SELDI spectra

A. Serum (bound at pH4)
B. Serum (bound at pH9)
C. Plasma (bound at pH4)
D. Plasma (bound at pH9)

In Supplementary data 5, Venn diagrams comparing SELDI profiles obtained at pH 4 with crude samples and ProteoMiner® serum eluate, showed 65 new peaks that emerged after ProteoMiner® compared to the 32 new ones in crude serum. Proteomics-30® showed the same gain of information compared to crude sample (65 peaks), while precipitation is less informative (46 peaks vs 59 peaks in crude serum). The same comparison performed with plasma also showed a gain in information compared to crude plasma (75 vs 16 peaks). As expected, there was an important overlap between flowthrough and crude samples (~ 30 peaks for serum and ~25 peaks for plasma, cf. Supplementary data 5).

Finally, the comparison of crude serum, Proteomics-30® chromatography and ProteoMiner® eluates showed a more important information gain at both pH conditions after ProteoMiner® treatment (Supplementary data 5). However, we observed low information redundancy between the three approaches at both pH conditions (Figure 7). Then, we can consider that these three prefractionation methods are complementary.

4. CONCLUSIONS

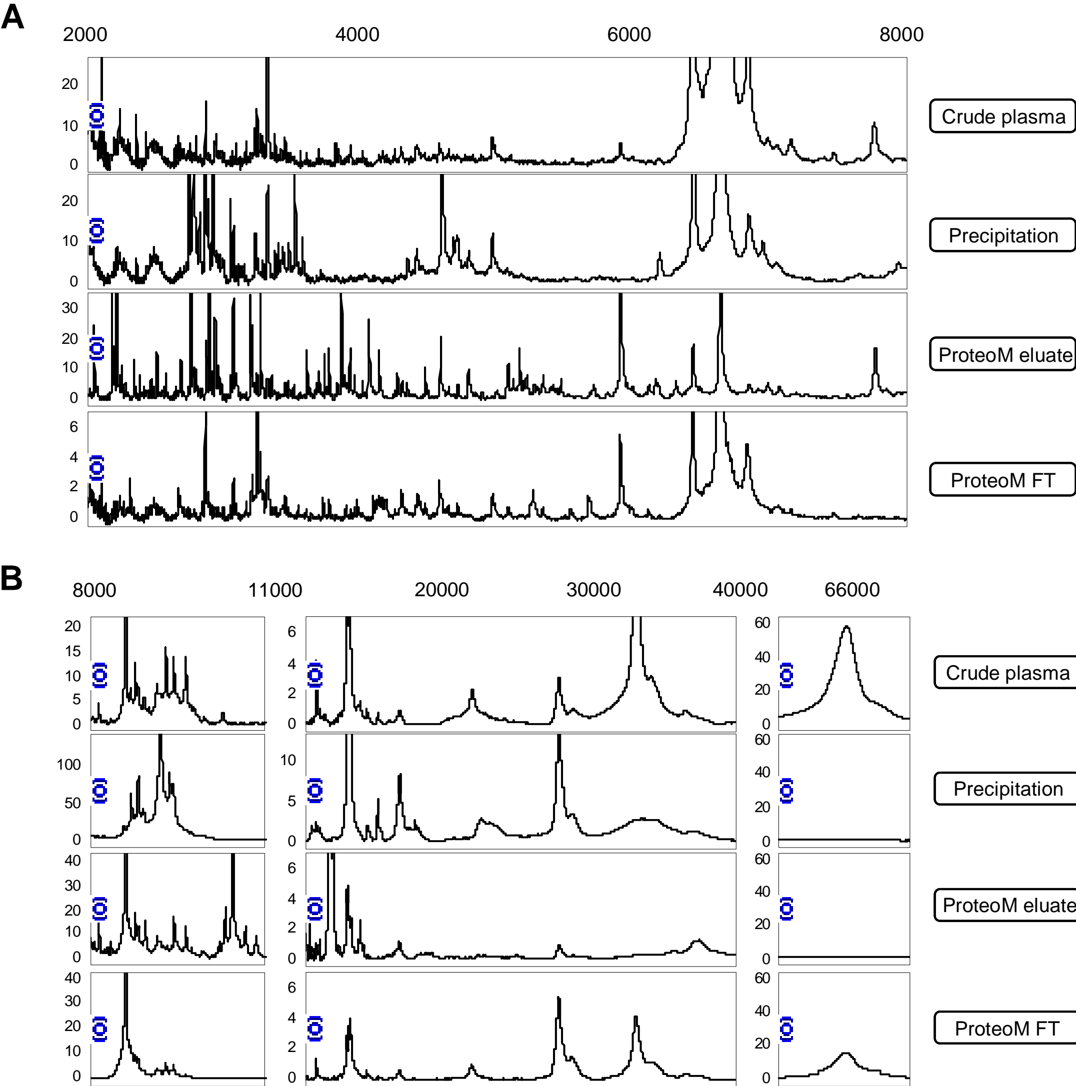
In this paper, three methods of serum and plasma preparation were evaluated according to their capacity of high molecular weight protein depletion and gain of new potential biomarkers. The methods are based on three different approaches: proteins precipitation, metal affinity coupled to restricted-access-material and equalization by peptide ligand affinity. All three appeared to give complementary information and presented good reproducibility (< 20%). The organic solvent precipitation did not supply a real gain in new peptide/protein peaks when studied by SELDI-TOF-MS but the depletion of the abundant proteins with a MW > 40kDa was very efficient. On the contrary, despite of the less efficient depletion of HMW proteins, IMAC-RAM treatment led to additional peaks with low MW. Finally, peptide ligand affinity beads were found to provide efficient depletion of HMW proteins and peak enrichment in protein/peptides profiles.

Acknowledgements

M. F. is Research Associate, M.P.M. Senior Research Associate and A.-C. S. Postdoctoral Researcher at F.R.S.-FNRS (National Fund for Scientific Research). We thank the “Centre Anti-Cancéreux” (Liège, Belgium), the FNRS, the “Fonds Léon Frédéric” (Liège, Belgium), the “Télévie” for their financial support.

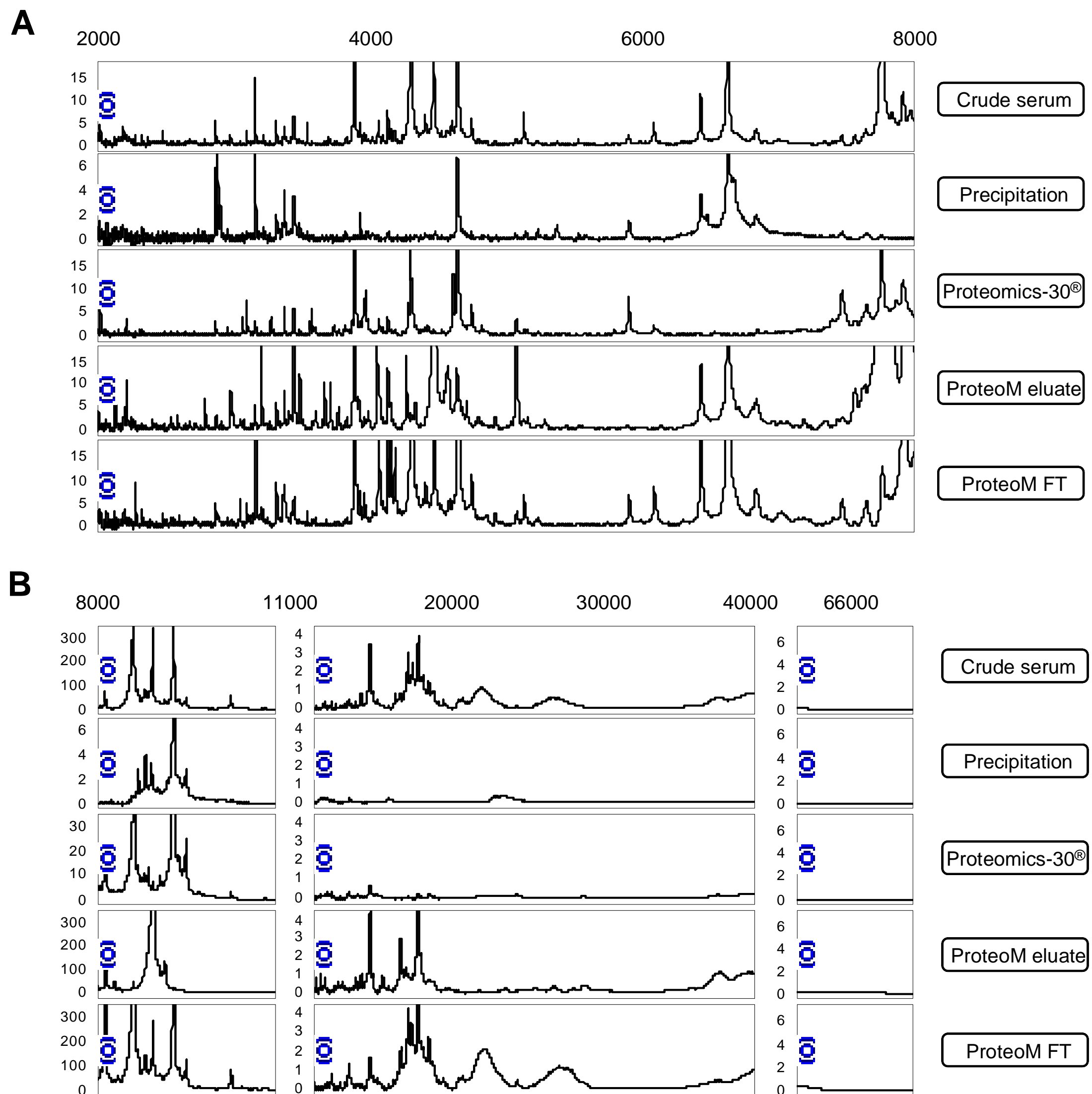
5. SUPPLEMENTARY DATA

Supplementary data 1:
SELDI-TOF-MS profiles of plasma prefractionation methods at pH 4



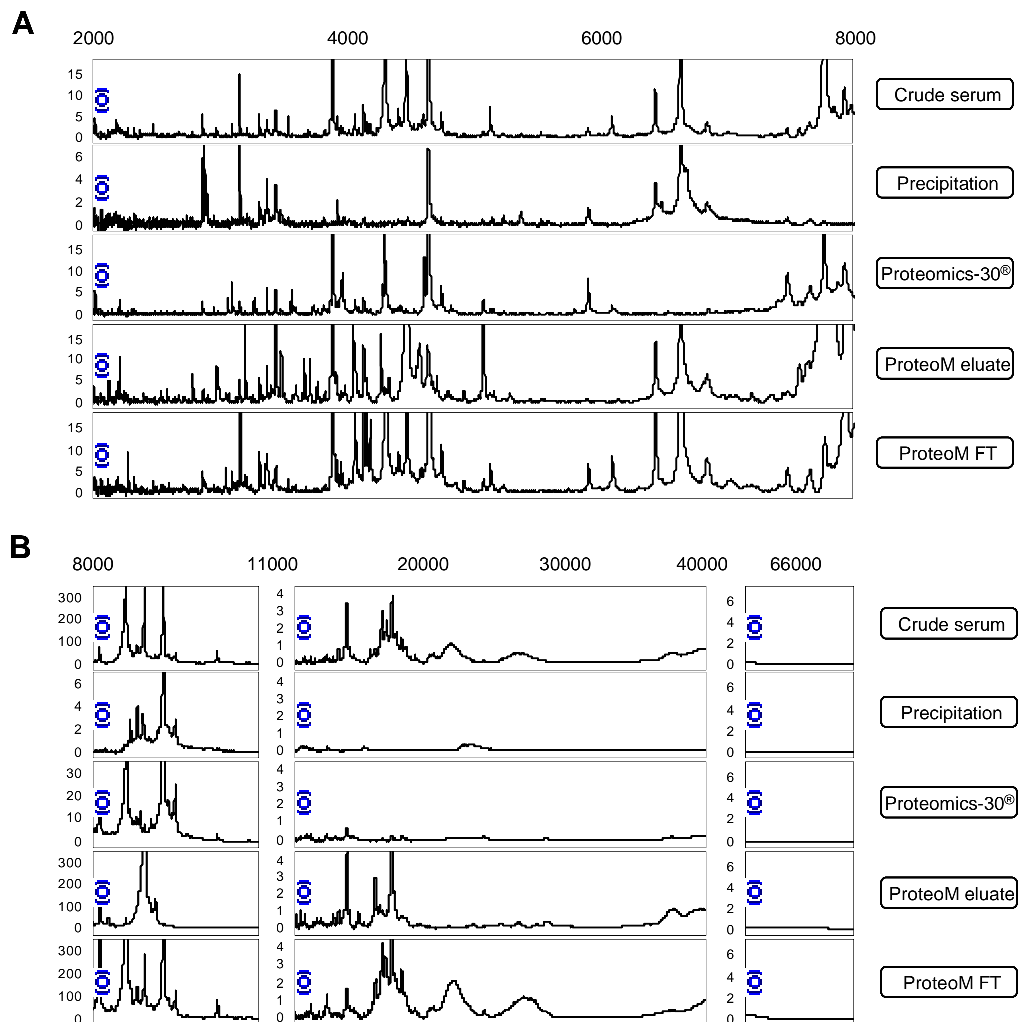
Supplementary data 2:

SELDI-TOF-MS profiles of serum prefractionation methods at pH9

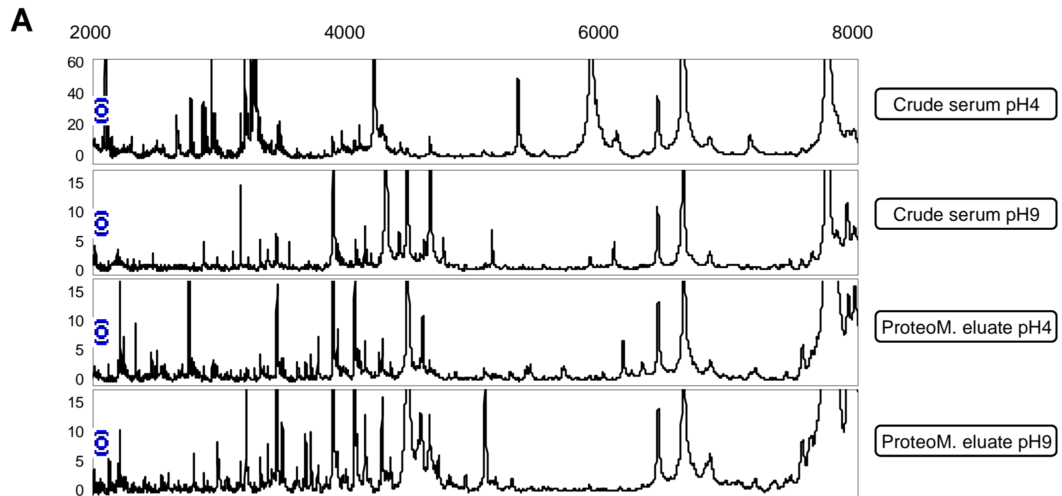


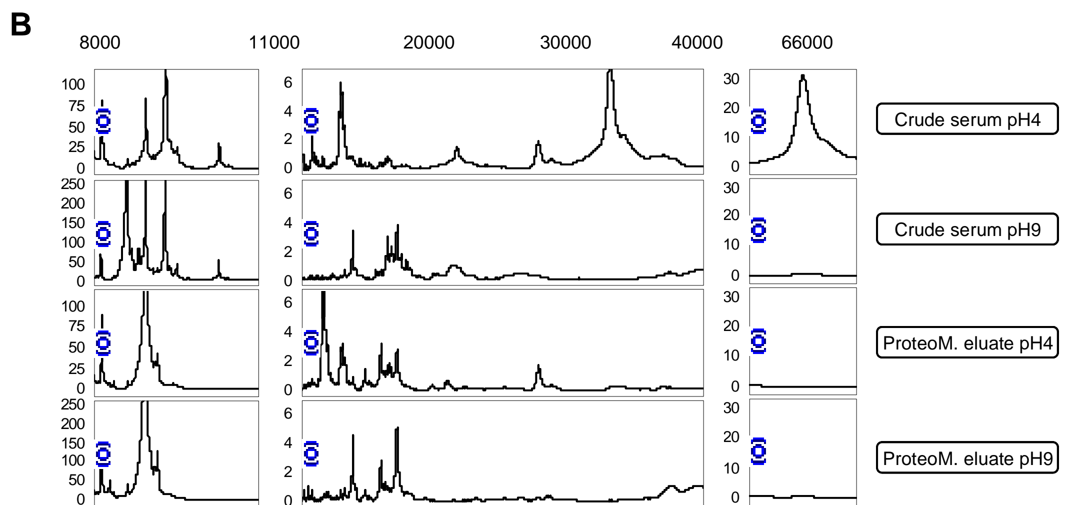
Supplementary data 3:

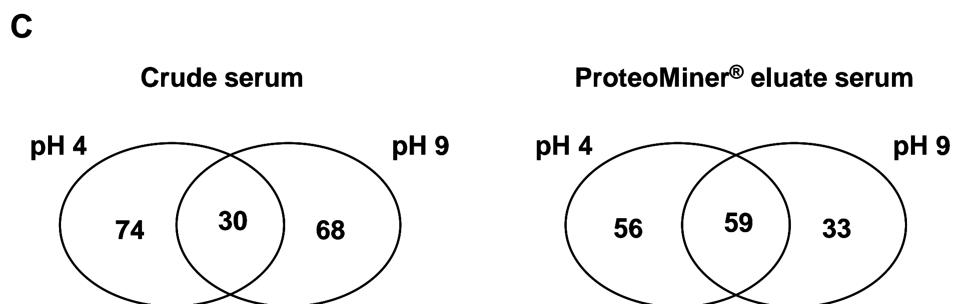
SELDI-TOF-MS profiles of plasma prefractionation methods at pH9



Supplementary data 4:
SELDI-TOF-MS profiles at pH 4 and pH 9 of serum prefractionation using ProteoMiner®

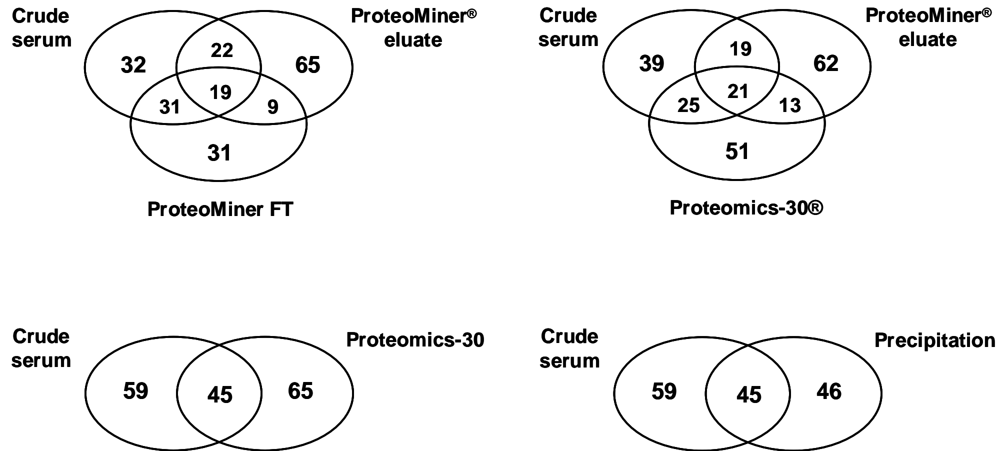


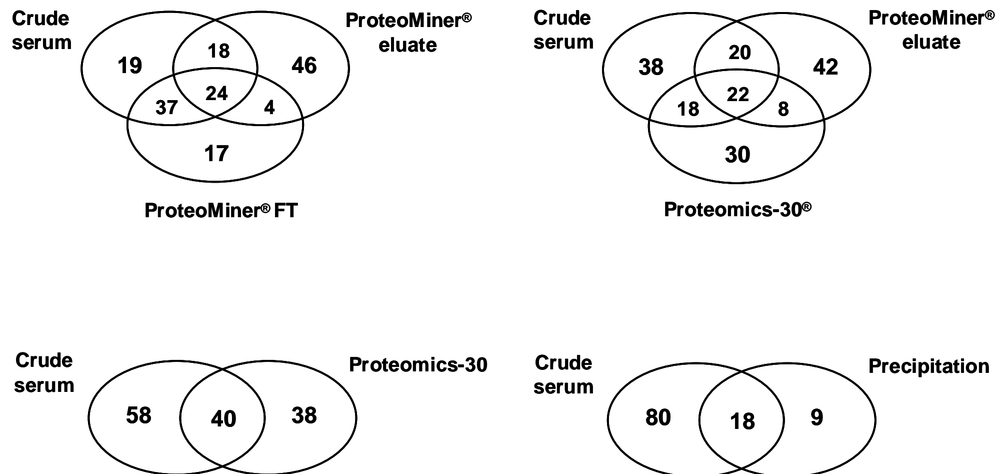




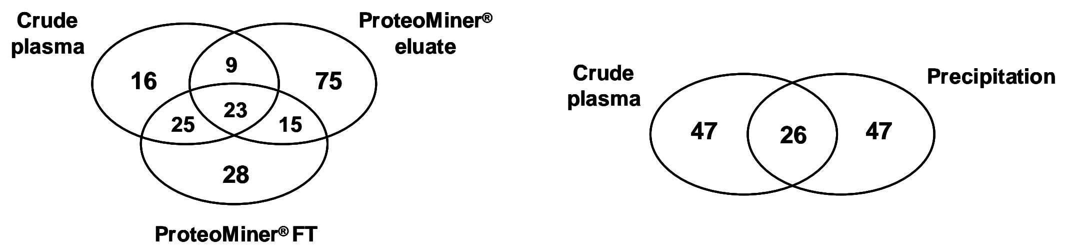
<u>Supplementary data 5</u>:

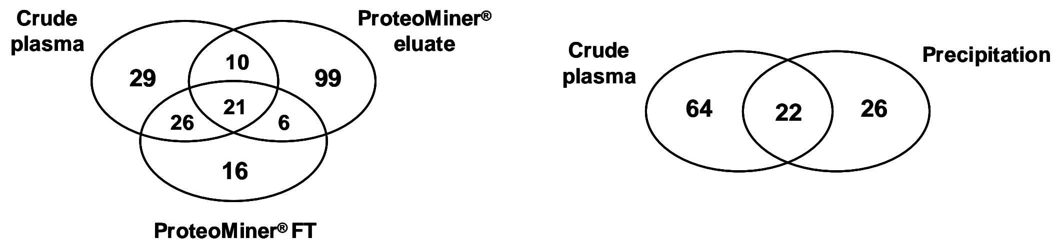
Serum prefractionation information overlaps of at pH 4 (A) and pH 9 (B)





Supplementary data 5:
Plasma prefractionation information overlaps of at pH4 (C) and pH9 (D)





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Comprehensive plasma profiling for the characterization of graft-versus-host disease biomarkers.

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Manuscript in preparation for submission

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Keywords:

Graft-versus-host disease, biomarkers, hematopoietic stem cell transplantation, clinical proteomics

Abbreviations:

aGVHD: Acute graft-versus-host disease

ASB-14: Amidosulfobetaine-14

CRP: C - reactive protein

HRG: Histidine-rich glycoprotein

HSCT: Hematopoietic stem cell transplantation

SAA: Serum amyloid A

UPLC: Ultra Performance Liquid Chromatography

Abstract

Acute graft-versus-host disease (aGVHD) remains a life-threatening complication of hematopoietic stem cell transplantation (HSCT), limiting its application. To optimize management of aGVHD and reduced therapy-related toxicity, early specific markers are needed. The main objective of this study was thus to uncover diagnostic biomarkers comparing plasma protein profiles of patients at the onset of acute GVHD diagnosis and of patients undergoing HSCT without aGVHD. Additional analysis of samples taken 15 days before aGVHD diagnosis was also performed to evaluate the potential of the newly discovered biomarkers for early diagnosis. To extract a maximum of information from plasma samples, we used three complementary proteomic approaches, namely 2D-DIGE, SELDI-TOF-MS and 2D-LC-MS^E.

We identified and confirmed by means of independent techniques, the differential expression of several proteins indicating significant increased inflammation response and disturbance in the coagulation cascade. The variation of these proteins was already observed 15 days before GVHD diagnosis, suggesting the potential early detection of the disease before symptoms appearance.

Logistic regression analysis determines a composite biomarker panel comprising fibrinogen, fragment of fibrinogen beta chain, SAA, prothrombin fragments, apolipoprotein A1 and hepcidin that optimally discriminated patients with and without GVHD. The area under the receiver operating characteristic curve distinguishing these 2 groups was 94.7.

1. INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) has been included in the therapeutic arsenal of hematological malignancies and genetic disorders for many years. Although this therapeutic approach has demonstrated good rates of success for disease eradication, life-threatening complications such as severe infections or graft-versus-host disease (GVHD) remain a major problem after HSCT.

GVHD can be defined as an exacerbated immune reaction mediated by the infused donor immunocompetent cells present in a genetically different and immunosuppressed host. Damaged host cells and bacterial products such as bacterial lipopolysaccharides induce the secretion of proinflammatory chemokines and cytokines, such as TNF-alpha, IL-1 and IL-6 that activate antigen-presenting cells. Presentation of host alloantigens to donor T cells leads to their proliferation and differentiation, thereby inducing a “cytokine storm” leading finally to the activation of cellular effectors amplifying host tissue injury [1-3]. Skin, liver and gastrointestinal tract are the main organs affected by acute GVHD. The staging of this pathology is based on the localization and the severity of injury.

Although improvements have been achieved in the prevention of GVHD through introduction of new immunosuppressive drugs, changes in the source of cells and graft manipulation [4-6], prophylactic approaches appear to be insufficient to avoid such complications. Moreover, these improvements are hampered by an increased rate of relapse, because of the close correlation between GVHD and graft-versus-tumor effects [7, 8], and thereby compromise the efficacy of HSCT. Concerning GVHD treatment, although consensus has emerged supporting the use of high-dose (methyl)prednisolone or prednisone for initial treatment of acute GVHD, practices differ among centers with respect to the initial glucocorticoid dose to be applied, the use of additional immunosuppressive agents, the management of treatment withdrawal after initial improvement, and the treatment of patients who failed to respond to steroids [9]. Second line of treatment for steroid-refractory aGVHD includes increased dose of immunosuppressive agent (cyclosporine, mycophenolate mofetil or tacrolimus), antithymocyte globulin, monoclonal antobodies as well as extracorporeal photophoresis or mesenchymal stem cell infusion [10]. For all these reasons, GVHD remains a challenge for clinicians in the application of HSCT.

Currently, diagnosis and grading of acute GVHD are based on clinical manifestations and histopathological analysis of involved organ biopsies [11]. Those are time-consuming, invasive and poorly specific practices. Measurement of biomarkers from fluids such as blood or urine could be a useful tool to diagnose and even predict the GVHD onset allowing an earlier initiation of treatment and a better management of this complication. Moreover, the identification of new biomarkers of GVHD could give novel insights on the underlying mechanisms and physiological processes of this pathology.

Although many studies report the monitoring of chemokines and cytokines as potential acute GVHD (aGVHD) biomarkers [12-17], only few investigations based on non-targeted proteomic approaches have been performed [18-25]. Among the studies performed to predict aGVHD after HSCT and to identify new biomarkers, Weissinger et al proposed a capillary electrophoresis-mass spectrometry analysis of urine samples. They identified peptides generated from collagen, albumin, beta2-microglobulin and CD99, indicating significant disturbances in collagen metabolism and T-cell activation [18, 22, 26]. Moreover, recent studies from Ferrara's group by antibody microarrays and Intact Protein Analysis System identified a panel of GVHD plasma biomarkers (namely IL-2 alpha, TNFR1, HGF, IL-8, elafin and reg3alpha) [15, 19, 23]. They validated those markers and showed that they could discriminate between therapy responsive and non-responsive patients and predict survival in patients receiving GVHD therapy [27, 28].

Diagnosis based on cytokine evaluation could suffer from a lack of specificity due to their immunomodulating roles in various diseases. Proteomic approaches present the advantage to examine in a single experiment a large panel of peptides and proteins, providing a fingerprint of a pathophysiological situation at a given time. Therefore, specific differences in the abundance of multiple proteins may be found when comparing samples from diseased and non-diseased patients. As a single biomarker could be the indicator of many unrelated pathological changes, the simultaneous detection of several markers is a key to improve specificity [29]. In this particular disease and considering the diversity of complications after HSCT, combination of biomarkers should assure a more specific diagnosis.

From a clinical point of view, blood biomarker discovery is very attractive because it is less invasive than tissue biopsy. Nevertheless, analysis of plasma or serum is challenging compared

to that of urine because of their complexity and their huge protein abundance dynamic range (blood protein concentrations cover 10 orders of magnitude)[30]. Currently the dynamic range of protein concentrations that can be detected in a single mass spectrum analysis is typically around 4-5 orders of magnitude, which means that it is not possible to cover the entire concentration range present in blood samples within one experiment and that sample pretreatment is required.

In this study we compared plasma protein profiles of patients at acute GVHD diagnosis and of patients undergoing HSCT without developing aGVHD. Additional analysis of samples taken 15 days before aGVHD diagnosis was also performed to evaluate the potential of the newly discovered biomarkers for early diagnosis. To extract a maximum of information from plasma samples, we used three complementary proteomic approaches. A gel-based (2D-DIGE) and two MS-based (SELDI-TOF-MS and 2D-LC-MS^E) proteomic approaches were chosen for aGVHD biomarker discovery. 2D-DIGE is a classical method of differential proteome comparison able to efficiently detect protein post-translational modifications and allowing relatively easy protein identifications [31]. SELDI-TOF-MS generates intact protein profiles of samples in short analysis time, allowing individual patient sample analysis [32, 33]. Finally, a label-free quantitative LC-MS^E approach was used that provides high sensitivity and direct identification of digested proteins within a concentration ranging over 3-4 orders of magnitude [34, 35]. For each approach, particular attention was paid to sample pretreatment.

2. MATERIALS AND METHODS

2.1. Patients and sample collection

All patients were enrolled after giving written informed consent, following approval of the institutional medical ethics committee. They were transplanted at the University Hospital Center of the University of Liège with peripheral blood stem cells following myeloablative or reduced-intensity conditioning regimens for haematological malignancies. Patients with active infection on sampling day were excluded. Diagnosis and grading of acute GVHD were based on clinical symptoms and biopsies according to established criteria [11].

EDTA plasma samples were prospectively collected weekly until day 100 after HSCT and every 2 weeks until day 365. Within 3 hours after collection, samples were aliquoted and stored at -80°C until analysis. aGVHD samples were taken on the day of diagnosis, before corticosteroid administration (GVHD D0). In addition, samples taken 15 days before the acute GVHD diagnosis (GVHD D-15) were collected. Patients considered as controls (patient under HSCT without developing aGVHD) were matched for sex, age, conditioning intensity regimen and time of sample collection after HSCT. Patient characteristics are summarized in Table 1 and supplementary table S1.

2.2. 2D-DIGE proteomic approach

Set of patients and plasma processing

Thirty-two patients including 16 with aGVHD and 16 control patients were enrolled. The median day of the onset of grade II aGVHD was day 36 (range from day 13 to 139). In addition, ten samples taken 15 days before aGVHD diagnosis were analysed. Three sample groups were thus considered: controls, GVHD D0 and GVHD D-15 (cf. Supplementary data S1).

In order to deplete very abundant plasma proteins, which could disturb the electrophoretic process and prevent detection of low abundance proteins, a bead-based combinatorial peptide library technology (ProteoMiner®, Biorad Laboratories Inc., Hercules, CA, USA) was applied. This approach leads to the removal of high abundant proteins while low abundance proteins are concentrated tending to the equalization of protein levels [36]. The protocol followed the

manufacturer's recommendations [37]. Briefly, 1 ml of plasma was incubated during 2 hours after washing the beads with PBS buffer and milli-Q water. After removal of the unbound fraction with PBS buffer (3x5min of incubation), proteins were eluted with 3x100µl of a 2D-DIGE compatible elution solution (25mM Tris, 7M urea, 2M thiourea, 4% CHAPS) to avoid a subsequent desalting step.

Table 1: Patients' characteristics

	2D-DIGE		**SELDI-TOF-MS**		**2D-LC-MS^E**	
Characteristics	***Control (n=16)***	***Grade II aGVHD (n=16)***	***Control (n=16)***	***Grade II aGVHD (n=16)***	***Control (n=23)***	***Grade II aGVHD (n=23)***
Median age, years (range)	58 (16-66)	57 (21-67)	63 (30-66)	61 (23-70)	61 (30-72)	61 (23-70)
Gender						
Male	13	14	11	14	15	16
Female	3	2	5	2	8	7
Diagnosis						
Acute myeloblastic leukemia	6	4	7	4	9	5
Lymphoma	2	5	3	6	3	7
Multiple myeloma	4	5	1	2	4	4
Myelodysplastic syndrome			1	3	2	5
Other malignancies	4	2	4	1	5	2
Donor						
Related	4	7	3	3	4	5
Unrelated	12	9	13	13	19	18
Conditioning regimen intensity						
Myeloablative	4	5	0	0	0	0
Reduced	12	11	16	16	23	23
ATG administration	6	6	0	0	0	0
Acute GVHD						
Skin		15		12		19
Gut		7		7		7
Liver		1		0		0
Combined		7		3		3
Day of onset of acute GVHD, median (range)		34 (13-139)		46 (15-245)		
Post-HSCT day of samples, median (range)	34 (10-139)	36 (14-139)	46 (21-259)	46 (18-248)	47 (21-259)	47 (18–248)

2D-difference Gel Electrophoresis (2D-DIGE)

As described in Supplementary Figure 1A and 1B, pools of control and grade II GVHD D0 and D-15 samples were separated by 2D-DIGE.

Protein content was determined using PlusONE 2-D Quant Kit (GE Healthcare, Uppsala, Sweden). Twenty-five µg of proteins from each pool were labelled separately with 0.2 nmol of Cy3 or Cy5 dyes for an incubation time of 30 min. The reaction was stopped by adding 10 mM lysine. To avoid experimental variation, an equal distribution of Cy3 and Cy5 dyes between control and GVHD samples was realized (Supplemental Figure 1B). An internal standard labelled with Cy2 dye was prepared with equal amounts of proteins from each sample included in the experimental procedure. After combining the internal standard with labelled control and GVHD samples, the volume was adjusted to 450 µl by adding rehydration buffer (7 M urea, 2 M thiourea, 2% (w/v) ASB 14, 1.2% (v/v) Destreak reagent and 0.6% (v/v) pH 3-10 NL IPG buffer). This was added to a 24 cm pH 3-10 NL strip for passive rehydration for 8h at 20°C. Isoelectric focusing (IEF) was conducted at 500 V for 1 h, gradient 1 kV for 3 h, gradient 8 kV for 3h and constant 8 kV for 8 h 45 at 20 °C with a maximum current setting of 50 µA per strip (IPGphor isoelectric focusing unit, GE Healthcare). After the first dimension separation, IPG strips were equilibrated in a solution made up of 6 M urea, 50 mM Tris pH 8.8, 30% glycerol, 1.6% sodium dodecyl sulfate and a trace of bromophenol blue, containing first 1% dithiothreitol (DTT) and then 5% iodoacetamide, each time for 15 min. Second dimension electrophoresis was performed by overnight orthogonal SDS-PAGE of the proteins on 12% (w/v) acrylamide gels at 20 °C in an Ettan Dalt II system (GE Healthcare) at 1 W/gel.

Gel analysis

Spot profile images were obtained with a Typhoon 9400 Laser Scanner (GE Healthcare) by scanning at three different wavelengths corresponding to the emission spectrum of the three CyDyes. Image and data analysis were performed using DeCyder software (GE Healthcare). After spot detection and normalisation of spot volumes with the internal standard, each normalised spot volume was compared between groups and a p-value and ratio were assigned. Spots with a p-value < 0.05 calculated by Student t test and a volume ratio > 1.5 (increase or

decrease) between groups were considered as spots of interest. Statistical significance of GVHD D0 versus GVHD D-15 comparison was evaluated using paired t-test.

Protein identification

Preparative gels containing unlabelled proteins and the internal standard were run in parallel and used for excision of spots of interest with the Ettan Spot Picker robot (GE Healthcare). Trypsin in-gel digestion was performed on the Janus working station (Perkin Elmer, Waltham, MA, USA). Pieces of gel were successively washed with 50 mM NH_4HCO_3 following by 50 mM NH_4HCO_3 / ACN (50/50) solution. After reduction with 50 mM DTT and alkylation with 55 mM iodoacetamide, gels were washed as previously described and dried out with ACN. Then digestion with trypsin (Promega, Madison, WI) was performed and peptides dissolved in a 0.1% TFA solution were recovered and spotted on a MALDI plate prior to the addition of 1 µL of R-cyano-4-hydroxycinnamic acid (CHCA) (7 mg/mL, 50% v/v ACN, 0.1% v/v TFA, Sigma Aldrich, MO).

PMF and MS/MS analysis were performed on an MALDI-TOF-TOF-MS, Ultraflex II (Bruker Daltonics, Billera, MA, USA) operated in positive ion mode. Automatic spectra acquisition was piloted with the Flex control ™ v3.0 software and real time analysis by Flex analysis ™ v3.0 software (Bruker Daltonics). Searches on databases were managed in real time with BioTools ™ v3.1 (Bruker Daltonics) on the Mascot server v2.2.2. Identification searches were performed on the Swissprot database restricted to Human taxonomy with 100 ppm of mass accuracy in MS and 300 ppm in MS/MS. The 4 most intense peaks detected within each PMF were selected for MS/MS.

2.3. SELDI-TOF-MS proteomic approach

Set of patients and plasma processing

Thirty-two patients undergoing HSCT with reduced intensity conditioning were included. Plasma samples were divided in three groups: controls (n=16), GVHD D-15 (n=11) and GVHD D0 (n=16). Given that SELDI-TOF-MS is a high throughput technology, ten patients developing septicaemia were also analysed. Plasma samples were processed individually with

Proteominer® and proteins were eluted with a solution made of 8 M urea, 2% CHAPS and 5% acetic acid.

SELDI-TOF-MS analysis

Each sample was analysed individually (cf. Supplementary data S1C). Peptide and protein profiles were generated using CM10 cation ion exchange array (Biorad Laboratories Inc.) with complementary pH 4 and pH 9 binding conditions. Samples were diluted ten times in either 100 mM sodium acetate binding buffer (pH 4) or in Tris-HCl 100 mM binding buffer (pH 9) and analysed in duplicate. To control technical variations, a plasma sample treated by ProteoMiner® was run on multiple arrays and was used as quality control. Diluted samples were incubated for 1 hour on chromatographic surfaces activated with the corresponding binding buffer. Spots were washed with appropriate binding buffers and milli-Q water and air dried. Finally, satured sinapinic acid matrix solution was applied. Two ranges of mass (low-mass (LM) 2000-8000 Da and medium mass (MM) 8000-30000 Da ranges) were processed separately according to the two calibration equations generated externally using All in one peptide and All in one protein standard (Biorad Laboratories Inc.). Spectra were acquired using PCS4000 SELDI-TOF-MS (Biorad Laboratories Inc.) by averaging 1200 shots at laser intensity of 4400 (LM) and 5000 (MM) for pH 4 condition and 4200 (LM) and 5000 (MM) for pH 9 conditions. Focus mass was set for low and medium mass range at 4500 Da and 10000 Da respectively.

Data processing

Data were processed using Proteinchip Data Manager Software (Biorad Laboratories Inc.). After baseline subtraction, noise calculation, spectra alignment and total ion current normalisation, peak clusters were formed by optimizing peak detection for each condition.

Statistical analysis

P-values were calculated using the non-parametric Mann-Whitney test based on the cluster median intensity of each spectra group.

Protein identification by immunodepletion

Three µg of anti-serum amyloid A1 (Santa Cruz Biotechnology, CA, USA), 20 µg of anti-apolipoprotein AII (Acris Antibodies, Herford, Germany), 20 µg of anti-apolipoprotein AI or 15 µg of anti-hepcidin-25 (Abcam, Cambridge, UK) antibodies were coupled with 50 µl of protein G+ beads overnight at 4°C. ProteoMiner eluate samples diluted 1:10 with Tris-HCl pH 9 buffer were then incubated with beads for 2 hours at 4°C. After washing with PBS containing 0.1% of Tween-20, bound fraction was eluted with 100 mM acetic acid containing 30% acetonitrile. Unbound and bound fractions were concentrated and diluted with 100 mM sodium acetate pH 4 buffer before analysis on CM10 array by SELDI-TOF-MS. Protein G+ beads without antibody or coupled with another antibody served as negative controls.

2.4. 2D-LC-MS^E proteomic approach

Set of patients, plasma processing and data collection

A total of 46 patients, 23 control patients and 23 patients developing grade II aGVHD were included in this analysis. All samples analysed by SELDI-TOF-MS were enrolled in this cohort. Crude plasma samples were equally divided (7-8-8) to constitute three pools for each group (control and GVHD D0) (Supplementary data Figure S1D).

After protein quantification using RCDC kit (Biorad), 1500 µg of total protein per sample were depleted of high abundant proteins using Seppro IgY14 spin column kit (Sigma-Aldrich, St. Louis, MO, USA) applying manufacturer protocol two times. After reduction, alkylation and complete protein trypsin digestion, peptide digests were desalted on C18 Zip Tip (Millipore, Billerica, CA, USA). The eluted peptides were finally diluted in 100 mM ammonium formiate adjusted at pH 10 with ammonia and spiked with a commercial mix of 4 protein digests. To monitor sample processing, 15 µg of Bacillus licheniformis α-amylase and 21 µg of Saccharomyces cerevisiae invertase were added to samples prior depletion and digestion steps respectively.

Analysis were performed using a nanoAcquity system (Waters Corporation, Mildford, MA, USA) coupled with the Q-TOF Synapt HDMS^{TM} G1 system mass spectrometer (Waters Corporation). The configuration of the 2D-nano UPLC system was a reverse phase pH 10 / reverse phase pH 3 based 2D separation. First, samples were loaded at 2µl/min (20 mM ammonium formiate, pH

10) on a X-Bridge BEH C18 5µm column (300µm, 50 mm) followed by five acetonitrile gradient elution steps (10, 14, 16, 20 and 65%). Each fraction was desalted and equilibrated at pH 3 online on a trapping column Symmetry C18 5µm (180 µm, 20 mm) before separation on the second analytical column (BEH C18 1.7µm (75 µm, 250 mm): flow rate 300 nL/min, solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile), gradient 0 min, 97% A; 90 min, 60% A.

Data were acquired by collecting spectra every 1s in a data-independent MS^E positive mode with alternating low and elevated energy (ramping) over a 50-1500 m/z range. The UniProtKB database search for protein identification involves trypsin as protease, with 1 possible misscleavage, with carbamydomethylation (C) as fixed modification and oxidation (M) and phosphor (STY) as variable ones. Raw data were processed (deconvoluted, deisotoped) and the protein identification and relative quantification were performed using ProteinLynx Global SERVER (PLGS) v2.5. The following parameters were used: PLGS differential quantitative relative analysis was performed with the assumption that a protein is identified and quantified and reached significance in the comparison using the 3 biological replicates per sample group. The relative abundance was calculated for each identified protein and expressed as ratio based on the mean ± SD of the expression of each protein from the replicate values. The calculated p-values determined the probability of regulation, a p-value associated to the difference of ratio or LogE ratio < 0.05 or > 0.95 indicated a 95% likelihood of downregulation or a 95% likelihood upregulation, respectively.

Data set of differentially expressed proteins and their respective ratio change were examined by the “Core analysis” function of Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood city CA, USA) in order to group proteins into molecular networks, similar signaling and metabolic pathways as well as biological function classes.

2.5. <u>Protein determination</u>

Western blotting (WB)

Potential biomarkers revealed by 2D-DIGE, SELDI-TOF-MS and 2D-LC-MS^E were tested by WB on 56 individual crude plasma samples; except for Ig lambda and kappa that were only tested on

the 46 samples used for LC-MS^E experiment. This set of samples included 32 plasma analysed by 2D-DIGE and 24 non-redundant samples tested by SELDI-TOF-MS and 2D-LC-MS^E. A set of 10 samples from patients undergoing HSCT and developing a septicaemia was also included to test the specificity of candidate biomarkers. Equal volumes of plasma samples from control and aGVHD patients were loaded on sodium dodecyl sulfate (SDS)-polyacrylamide gel. Proteins were transferred on polyvinylidene difluoride membranes (Millipore) and then blocked in 5% milk solution for 1 hour. Membranes were incubated with primary antibodies: mouse monoclonal anti-plasminogen (1/1000), mouse monoclonal anti-coagulation Factor XIII (1/200), goat polyclonal anti-fibrinogen beta chain (1/200), mouse monoclonal Ig lambda light chain (1/400) (Santa Cruz biotechnology, Santa Cruz, CA, USA), rabbit monoclonal anti-serum amyloid A4 (1/1000) or mouse monoclonal Ig kappa light chain (1/2000) (Abcam). After washing membranes, corresponding horseradish peroxidase-conjugated secondary antibodies were added with a dilution of 1/10000 for mouse and rabbit (GE Healthcare) and 1/2000 for goat (Dako, Glostrup, Denmark) antibodies. Western blot band signals were revealed using enhanced chemiluminescence detection reagent (ECL kit, Thermo Scientific, MA) and were detected by Imaquant LAS 4000 Mini luminescence image analyzer (GE Healthcare). The intensity of each band was measured using Imagequant TL® software (GE Healthcare). To normalize protein levels, the value of the band corresponding to each protein was divided by the band intensity of the intergel controls (i.e. the same sample loaded twice on each gel). Statistical analysis was performed by the Mann-Whitney test using Prism 4.00 Software (Graph pad, San Diego, CA, USA), with statistical significance accepted at $P < 0.05$.

Measurement of hepcidin-25 in plasma samples by LC-MS/MS

Hepcidin-25 levels of 54 plasma samples (28 controls and 26 aGVHD samples) were measured by a LC-chip coupled to a nanoelectrospray/ion trap/MS operating in positive mode. Extraction procedure, calibration standard preparation, chromatographic and MS parameters were previously described by Houbart et al. [38]

Quantification of SAA and Apolipoprotein A1 levels by ELISA

SAA and Apo A1 levels were measured by ELISA in serum samples from 28 controls and 28 aGVHD at the time of aGVHD onset. In addition, these proteins levels were also evaluated in 19 serum samples taken 15 days before aGVHD onset. SAA and Apo A1 levels were measured by ELISAs following the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA / Abnova, Taipei city, Taiwan). Samples were diluted 1:200 and 1:100 for SAA and Apo A1 assays, respectively. Patient samples whose cytokine levels were out of standard curve range, were re-assessed after dilution.

Quantification of fibrinogen, CRP and prothrombin fragments 1+2 in plasma samples

Levels of C-reactive protein (CRP), fibrinogen and prothrombin fragments 1+2 were assessed in samples from 28 controls and 28 aGVHD patients. Samples from aGVHD group were taken at time of GVHD onset and 15 days before aGVHD diagnosis. Heparinized and citrated plasma samples were used for C-reactive protein and fibrinogen measurement respectively. CRP level was evaluated by immunoturbidimetric assay using modular Roche PPE-U and PPE-R (Roche Diagnostics, Indianapolis, IN, USA). Reference range is 0-6mg/L. Citrated plasma fibrinogen level was measured by Clauss method using BCS system (Siemens, Deerfield, IL). Normal level ranges between 2.3 and 4.3 g/L. Additionally, quantification of prothrombin fragments was performed on EDTA plasma using ELISA assay (Enzygnost F1+2 monoclonal, Siemens).

2.6. Statistical analysis

Univariate and multivariate statistical analyses were used to evaluate the ability of the markers to predict the onset of aGVHD based on sample values obtained by Western Blot, ELISA, LC-MS/MS and routine tests (n=56). Biomarkers with skewed distributions were ln transformed and then used as continuous covariates in logistic regression models. Proteins includes in the analysis were plasminogen, coagulation factor XIII, fibrinogen beta chain, SAA4, SAA1, CRP, fibrinogen, prothrombin F1+2, hepcidin and apolipoprotein AI. Multivariate logistic regression with stepwise backward model selection was applied to generate the best composite panel of biomarkers that discriminated between control and aGVHD samples. Area under the curve

(AUC) for individual markers as well as for the composite panel was computed using logistic regression.

3. RESULTS

In the present study, differential protein analysis was performed on plasma samples from patients subjected to HSCT and developing or not aGVHD. Three complementary proteomic approaches (2D-DIGE, SELDI-TOF-MS and 2D-LC-MS^E) were applied to collect a maximum of information from complex samples. The experimental design of the different studies is summarized in supplementary data Figure S1.

2D-DIGE analysis

2D-DIGE was used to provide plasma protein distribution patterns ranging from 15 kDa to 150 kDa. As sample pre-treatment, hexapeptide combinatorial library on beads technology (ProteoMiner®) was applied to deplete highly abundant plasma proteins as described previously [39]. The differentially expressed proteins with significant p-values (<0.05) for grade II GVHD versus controls were identified after in-gel digestion using MALDI-TOF-MS peptide mass fingerprinting and confirmed by MALDI-TOF-MS/MS (Table 2).

Table 2: Summary of proteins differentially expressed between control and grade II aGVHD D0 samples revealed by 2D-DIGE and identified by MALDI-TOF-MS and MS/MS.

2D-DIGE spot number	Protein Name	Uniprot ID[a]	Theor. MW (Da)	Theor. pI	p-value	GVHD / Control Ratio	PMF Mascot score	PMF seq. cov. (%)	PMF Matched queries	MS/MS Mascot score	MS/MS Matched queries
372	**Plasminogen**	P00747	93247	7.3	0.025	-1.55	232	36	33	172	4
564	**Coagulation factor XIII B chain**	P05160	77742	6.0	0.044	-1.71	123	33	16	111	4
581	**Coagulation factor XIII B chain**	P05160	77742	6.0	0.0027	-1.62	180	46	26	287	4
2384	**Fibrinogen beta chain**	P02675	56577	8.5	0.026	1.54	49	12	7	59	2
3712	**Serum Amyloid A4**	P35542	14851	9.2	0.0059	1.54	/	/	/	50	2

[a] http://www.uniprot.org/

In the comparison between grade II aGVHD D0 and control patients, four differentially expressed proteins were identified (plasminogen, coagulation factor XIII beta chain, fibrinogen beta chain fragment and serum amyloid A4, see Figure 1). While the first two proteins were found at a lower level in grade II GVHD plasma samples, the two others showed more elevated levels (Figure 2A, 2B, 2C, 2D respectively).

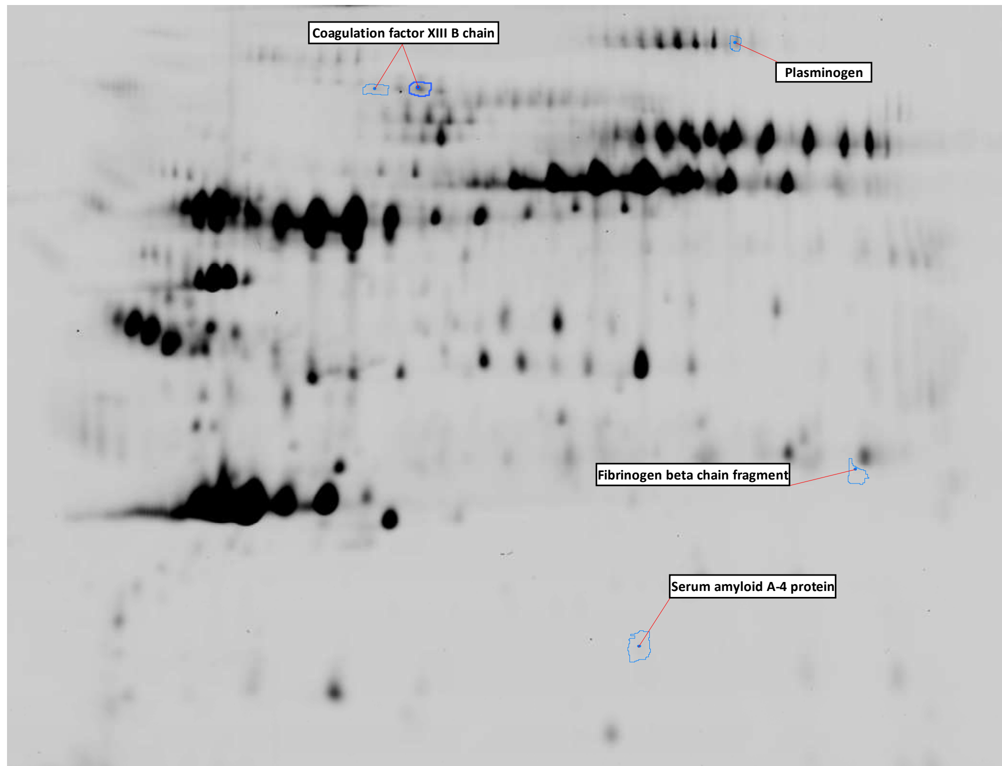


Figure 1: Representative 2D-DIGE gel of plasma pretreated with ProteoMiner®: comparison of controls and grade II aGVHD plasma samples showing proteins differentially expressed (p-value <0.05 and fold change >1.5).

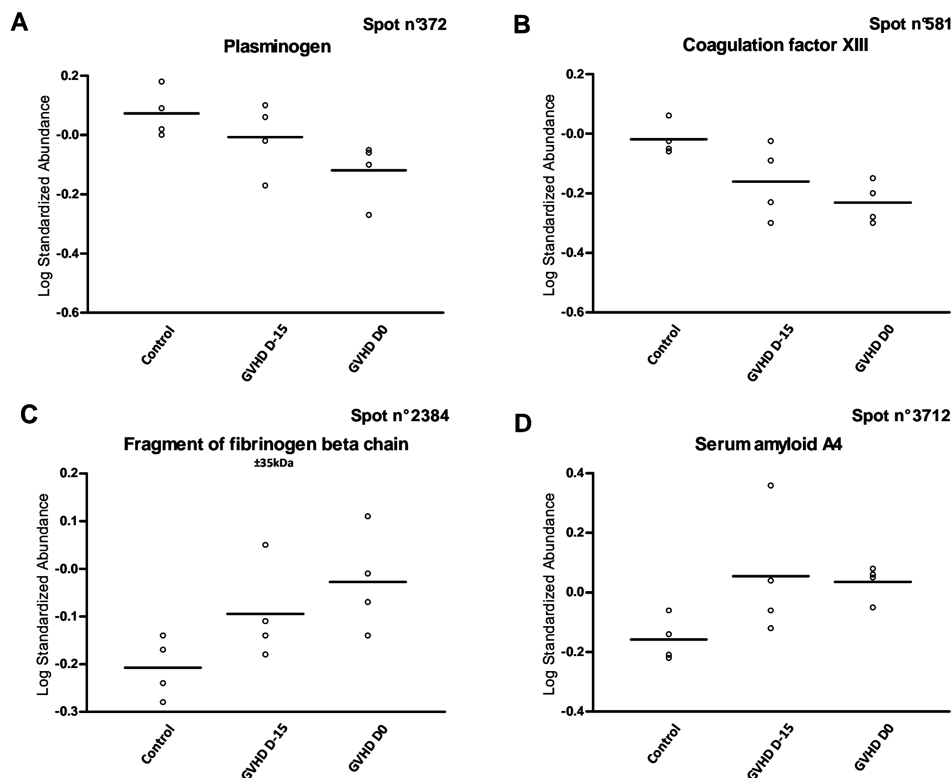


Figure 2: Relative abundance of differentially expressed proteins observed by 2D-DIGE for controls and grade II patient samples. The graph shows the logarithm of protein abundance standardized with the internal standard of each biological replicates (o) and the mean value (—). GVHD D0 and GVHD D-15 are respectively the day of the onset of aGVHD and 15 days before.

Two different forms of coagulation factor XIII beta chain were found to be expressed at a significantly lower level in aGVHD patients compared to controls. Those two forms are supposed to correspond to post-translationally modified proteins (ie. phosphorylated or acetylated proteins) explaining a shift in their isoelectric point. No proteins were found to be significantly differentially expressed between 15 days before and the day of aGVHD diagnosis. However, as can be seen in Figure 2A, 2B, 2C, 2D, the identified proteins showed, in GVHD D-15 samples, an intermediate level between controls and aGVHD D0. This could suggest that the abundance of those proteins already varied before the aGVHD diagnosis, although levels were not statistically different than in controls.

SELDI-TOF-MS analysis

After Proteominer® pre-treatment, the plasma samples from 16 control patients and 16 patients developing grade II aGVHD (on the day of aGVHD diagnosis and taken 15 days before) were analysed on cation exchange arrays under pH 4 and pH 9 conditions. The eluted plasma proteins were analysed in duplicate, generating 172 spectra (86 spectra per condition). Each spectrum provided 220 and 209 detected peaks ranging between 1500 and 30000 m/z for pH4 and pH9 experimental conditions, respectively. After cluster generation, the mean of peak intensity for each duplicate was considered before applying a Mann-Whitney statistical test. Peak intensities of 27 and 9 different m/z values (at pH 4 and pH 9 respectively) were found to discriminate significantly the control and aGVHD D0 samples (Table 3). Protein identities could be assumed based on m/z values, literature reports [40] and the TagIdent tool (http://web.expasy.org/tagident/). Thereby, peaks at m/z of 2792, 11687, 17271 and 28120 were hypothesized to be hepcidin-25, serum amyloid A1, a truncated form of Apolipoprotein AII and Apolipoprotein AI, respectively. As shown in Supplementary data S2, immunodepletions confirmed the identification of these proteins. Identification of hepcidin-25 was additionally confirmed after correlation of its abundance evaluated by LC-MS/MS and by SELDI-TOF-MS in 26 plasma samples. Indeed, a strong positive correlation between hepcidin-25 concentration provided by LC-MS/MS analysis (expressed in ng/mL) and peak intensities obtained by SELDI-TOF-MS was calculated by the Spearman’s correlation test (P< 0.0001, Rs 0.87, Supplementary data S3).

<u>Table 3</u>: Differentially expressed proteins between controls and GVHD D0 samples using SELDI-TOF-MS. Protein identities were confirmed by immunoprecipitation or MS/MS. NI are proteins not yet identified.

	pH4				pH9			
Rank	Median m/z value	p-value	Protein name	Expression GVHD D0 / control	Median m/z value	p-value	Protein name	Expression GVHD D0 / control
1	**11687**	0.0020	Serum amyloid A1 (SAA1)	increased	**2345**	0.0023	NI	increased
2	**21737**	0.0059	NI	decreased	**12658**	0.0047	NI	increased
3	**28120**	0.0067	Apolipoprotein A1	decreased	**5302**	0.0116	NI	decreased
4	**11527**	0.0093	N-arginine truncated SAA1	increased	**11687**	0.0143	Serum amyloid A1	increased
5	**9308**	0.0104	NI	increased	**2792**	0.0195	Hepcidin-25	increased
6	**3020**	0.0129	NI	increased	**9304**	0.0215	NI	decreased
7	**22636**	0.0143	NI	decreased	**4590**	0.0262	NI	increased
8	**11446**	0.0159	Serum amyloid A1 (3-104)	increased	**2998**	0.0317	NI	increased
9	**4433**	0.0176	NI	increased	**2678**	0.0458	NI	increased
10	**2792**	0.0215	Hepcidin-25	increased				
11	**6189**	0.0215	NI	decreased				
12	**10072**	0.0215	NI	decreased				
13	**2815**	0.0262	NI	increased				
14	**2678**	0.0288	NI	increased				
15	**5846**	0.0288	Serum amyloid A1 (2H+)	increased				
16	**5992**	0.0288	NI	decreased				
17	**17271**	0.0317	C-term-Glu-missing homodimer apo AII	decreased				
18	**8348**	0.0348	NI	decreased				
19	**21277**	0.0348	NI	decreased				
20	**3883**	0.0382	NI	decreased				
21	**7977**	0.0382	NI	decreased				
22	**3045**	0.0418	NI	increased				
23	**2440**	0.0458	NI	increased				
24	**5767**	0.0458	N-arginine truncated SAA1 (2H+)	increased				
25	**6436**	0.0458	Truncated Apolipoprotein C-I	decreased				
26	**14049**	0.0458	Apolipoprotein A1 (2H+)	decreased				
27	**2858**	0.0500	NI	increased				

As depicted in Figure 3A and 3B, the peak intensities of hepcidin and SAA1 were found significantly increased on the day of aGVHD occurrence. In addition, their peak intensities were already found higher in GVHD D-15 samples compared to controls (Figure 3A). Peak intensities of apolipoprotein AI and AII were found decreased in GVHD D0 and also in GVHD D-15 samples suggesting an early change in protein levels (Figure 3A).

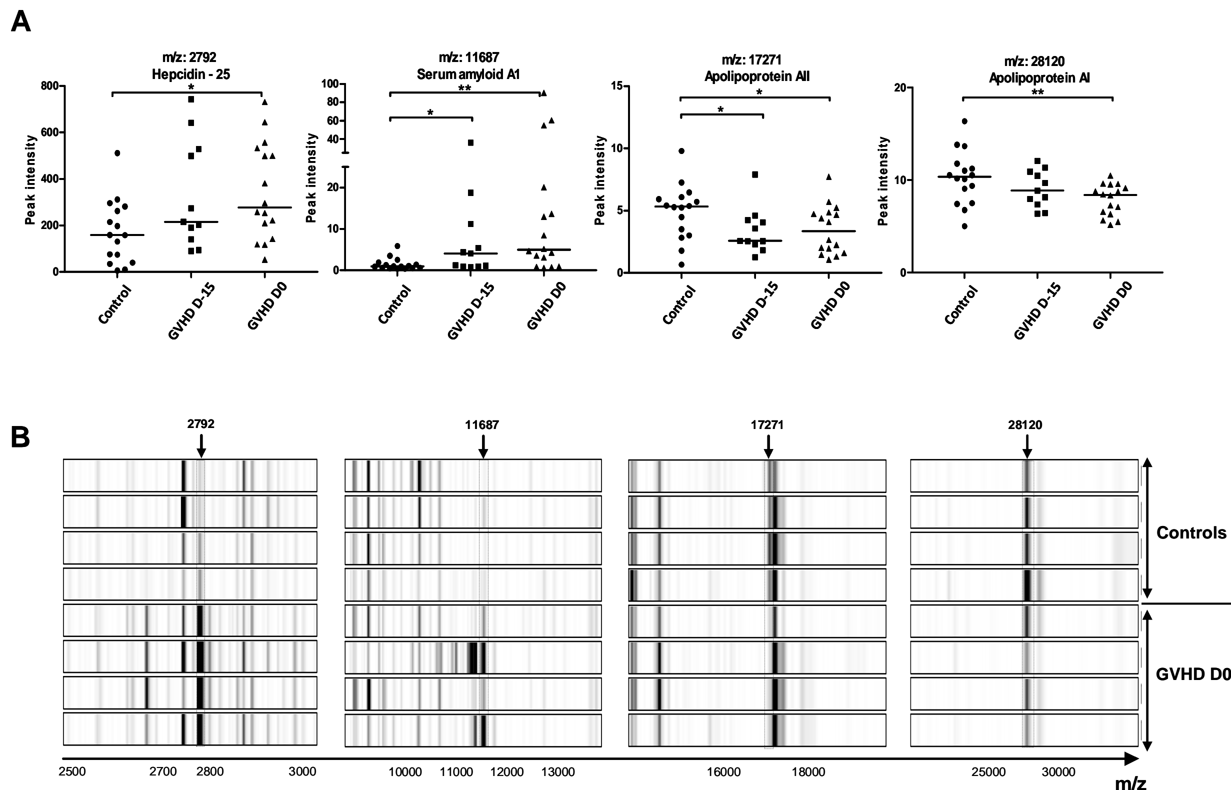


Figure 3: Results of SELDI-TOF-MS analysis

A. Peak intensity distribution of potential biomarkers for aGVHD diagnosis. Median of m/z 2792, 11687, 17271 and 28120 peak intensities are depicted for controls, D-15 and D0 aGVHD samples.
B. Gel view spectra representing peak intensities of m/z 2792, 11687, 17271 and 28120 for 4 controls and 4 GVHD D0 samples.

2D-LC-MS^E analysis

Six pools of samples, 3 grade II aGVHD D0 and 3 controls (Supplementary data Fig. S1D) were compared using a label-free quantitative LC-MS^E approach. Prior to analysis, plasma pools were immunodepleted against fourteen highly abundant plasma proteins. Ninety differentially expressed proteins were identified as shown in supplementary data Table S2. Statistical significance was reached for confidently identified protein with a p-value < 0.05 (downregulation) or > 0.95 (upregulation). A ratio (GVHD/control) and the logarithm of this ratio were assigned to each protein, showing 56 downregulated and 34 upregulated proteins in the aGVHD group. Proteins were classified by Ingenuity Pathway analysis software according to their canonical pathways as presented in Table S2.

Many identified proteins are involved in inflammation, the immune system, blood coagulation and lipid metabolism (see Table S2). Indeed, the six significant canonical pathways altered identified to be involved in aGVHD are acute phase response signalling, LXR/RXR activation,

coagulation system, intrinsic and extrinsic prothrombin pathway activation as well as primary immunodeficiency signalling. Levels of some negative acute phase proteins (APP), such as transthyretin and retinol-binding protein 4, were found to be lower in GVHD D0 samples, while levels of positive APP such as α1-antitrypsin, haptoglobin or complement components were found increased. C-reactive protein was the most increased protein in aGVHD samples (ratio: 3.39), while the most decreased proteins were related to immunoglobulin chain structure, especially kappa light chain. In addition, some proteins involved in coagulation were altered in aGVHD (see Table S2). Interestingly, plasminogen and histidine-rich glycoprotein levels were found to be decreased while levels of various fibrinogen chains increased in GVHD D0 samples, which is in accordance with 2D-DIGE results.

Validation of potential biomarkers obtained by proteomic approaches

In order to validate the potential biomarkers revealed by the proteomic approaches, five proteins of interest were quantified by Western Blot using 56 non-redundant individual crude plasma samples included in the different proteomic experiments (28 controls and 28 grade II aGVHD). An equal volume of undepleted plasma samples was loaded in each well. Figure 4 shows quantification results for plasminogen, coagulation factor XIII, the beta-chain of fibrinogen and serum amyloid A4 (SAA4), which confirmed the differential plasma levels observed by 2D-DIGE for these proteins. Indeed, as shown in Figure 4, levels of plasminogen and coagulation factor XIII were significantly decreased in patients with aGVHD compared to controls ($0.01<P<0.05$) (Figure 4A and 4B). An up-regulation of fragment of fibrinogen beta-chain (~ 35kDa) (NS) and SAA4 (NS) in patients with aGVHD versus control patients was also confirmed (Figure 4C and 4D). Moreover, abundance of a fragment of histidine-rich glycoprotein (~ 50kDa) was found to decrease at aGVHD onset by Western Blot confirming the LC-MS^E experiments (Figure 4E). Furthermore, a significant increase of hepcidin levels in aGVHD samples (median 82.6; interquartile range 50.8 - 117.1 ng/mL) compared to controls (median 57.1; interquartile range 27.1 - 76.3 ng/mL) was observed by LC-MS/MS ($P=0.009$)(Figure 4F). Finally, the SAA1 levels obtained by ELISA were significantly more elevated ($P<0.001$) in aGVHD D0 samples compared to controls while Apo A1 levels was decreased with onset of aGVHD (NS), confirming the results obtained by SELDI-TOF-MS analysis (Figure 4G and 4H).

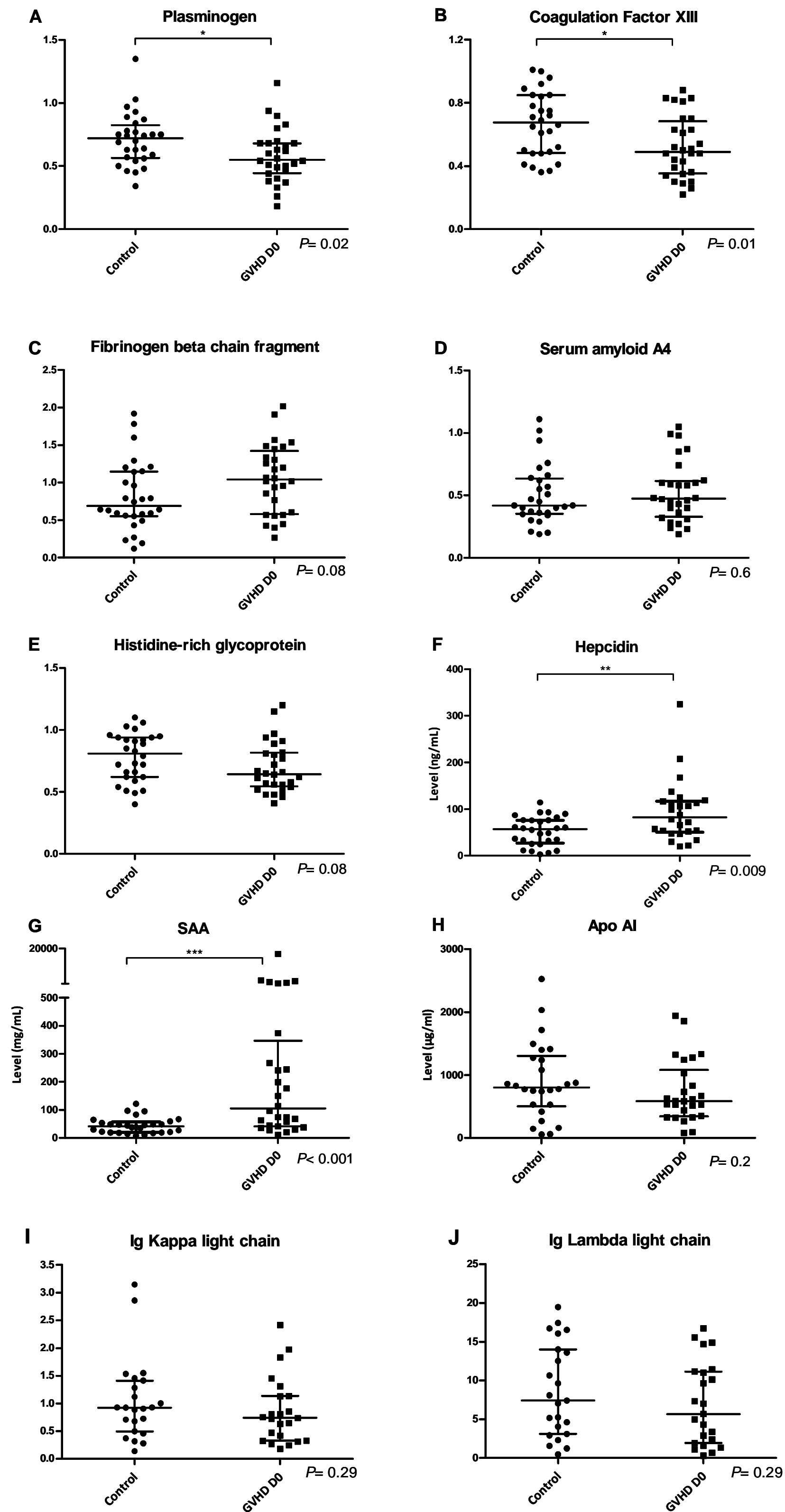


Figure 4: Comparison of plasminogen (A), coagulation factor XIII (B), fibrinogen beta-chain fragment (C), SAA4 (D), histidine-rich glycoprotein (E), hepcidin (F), SAA1 (G) and Apo A1 (H) levels from control and grade II aGVHD patients. Western-Blot was used to evaluate protein amounts denoted as the ratio of band volumes between sample and intergel control sample. LC-MS/MS and ELISA techniques were applied to measure hepcidin and, SAA and Apo A1 levels, respectively. Western blot was used to assess Ig kappa (I) and lambda (J) light chain levels. P-values were calculated by the Mann-Whitney test. * P< 0.05; ** P< 0.01; *** P< 0.001.

Additionally, samples taken 15 days before aGVHD onset exhibit higher SAA1 levels than control samples (P= 0.06) while D-15 aGVHD Apo A1 levels were found lower (NS) than controls as observed by SELDI-TOF-MS (Supplementary Data 3). These results suggest that the levels of these proteins are already altered before aGVHD symptom onset.

Levels of several potential biomarkers were also determined on 10 samples from HSCT patients developing septicaemia (cf. Table S3). Their levels were also found to be modulated, sometimes even more than at GVHD diagnosis.

As Ig lambda and Ig kappa light chain proteins exhibited a significant difference between the two groups when compared by 2D-LC-MS^E, their protein levels were measured by WB on the 46 samples used for this analysis. As presented in Figure 4I and J, Ig kappa (4I) and Ig lambda (4J) light chain showed a slight decrease in the aGVHD group compared to controls.

Level of inflammation- and coagulation-related markers obtained by clinical routine assays

As many aGVHD markers detected by proteomic analysis are involved in inflammatory and coagulation processes, C-reactive protein (CRP) and total fibrinogen levels were also evaluated by clinical routine assays. CRP and fibrinogen levels of control samples (n=28), GVHD D-15 (n=21) and GVHD D0 (n=28) are depicted in Figure 5A and 5B.

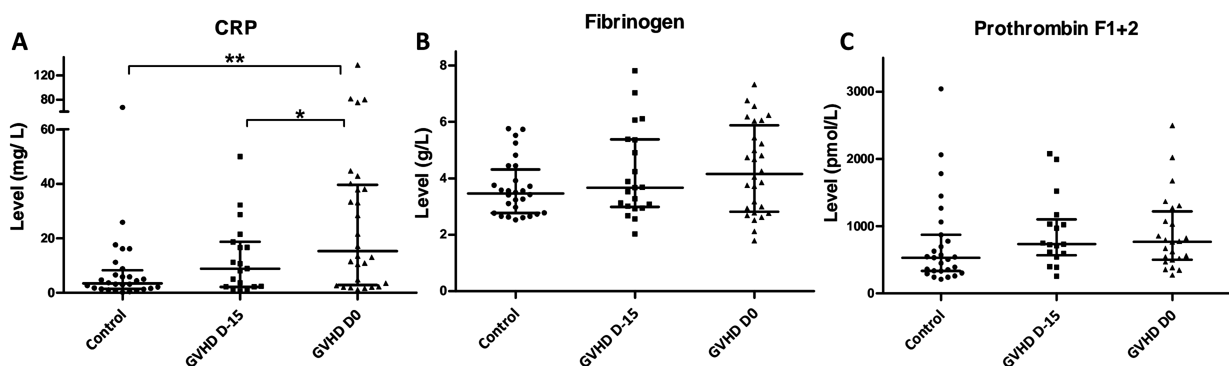


Figure 5: Comparison of CRP (A), fibrinogen (B) and prothrombin fragments F1+2 (C) between control samples and grade II aGVHD samples on the day of diagnosis. Levels of CRP and fibrinogen were measured by routine clinical tests. Prothrombin fragment levels were evaluated by ELISA. Levels of CRP, fibrinogen and prothrombin F1+2 in samples (n=21) taken 15 days before aGVHD onset are also depicted. P-values calculated using the Mann-Whitney test. * P< 0.05; ** P< 0.01; *** P< 0.001.

As expected, these two markers of inflammation increased at the onset of aGVHD compared to controls (CRP and fibrinogen p-values were 0.0017 and NS, respectively). However, it can be noticed that CRP showed only a slight (not significant) increase 15 days before aGVHD onset. Moreover, to assess a potential activation of coagulation at the time of aGVHD occurrence, prothrombin fragments 1+2 levels were measured in controls, GVHD D-15 and GVHD D0 samples (Figure 5C). A non significant increase of these activation peptide fragments was observed at aGVHD onset. Interestingly, the level of these fragments already increased at GVHD D-15, suggesting early coagulation activation.

Correlation

Pairwise associations between the different biomarkers in samples taken on the day of aGVHD diagnosis were evaluated using the Spearman's test and are listed in Table 4. Normalized band volumes obtained by WB of SAA4, fibrinogen beta-chain fragment, histidine-rich-glycoprotein, coagulation factor XIII and plasminogen as well as hepcidin, SAA1 and Apolipoprotein A1 levels from 28 plasma samples (D0 aGVHD samples) were considered for statistical analysis. In addition, levels of CRP, fibrinogen and prothrombin fragments 1+2 measured by routine assays were included.

<u>**Table 4**</u>**: Correlation coefficients** for proteins analysed by clinical assay, WB, ELISA and LC-MS/MS in aGVHD D0 samples * *P*< 0.05; ** *P*< 0.01; *** *P*< 0.001. NS: not significant

GVHD D0										
	CRP [a]	**SAA1[c]**	**Hepcidin [d]**	**Fibrinogen [a]**	**Fibrinogen β chain [b]**	**Plasminogen [b]**	**Factor XIII [b]**	**Prothrombin F1+2 [c]**	**HRG [b]**	**SAA4 [b]**
CRP [a]	/	/	/	/	/	/	/	/	/	/
SAA1[c]	**0.74 *****	/	/	/	/	/	/	/	/	/
Hepcidin [d]	**0.62 *****	NS	/	/	/	/	/	/	/	/
Fibrinogen [a]	**0.58 ****	**0.52 ****	**0.54 ****	/	/	/	/	/	/	/
Fibrinogen β chain [b]	NS	NS	NS	**0.59 *****	/	/	/	/	/	/
Plasminogen [b]	**-0.41 ***	NS	NS	NS	NS	/	/	/	/	/
Factor XIII [b]	**-0.44 ***	NS	NS	NS	**0.41 ***	**0.55 ****	/	/	/	/
Prothrombine F1+2 [c]	NS	**-0.41 ***	NS	NS	NS	NS	NS	/	/	/
HRG [b]	NS	NS	NS	NS	NS	**0.49 ****	NS	NS	/	/
SAA4 [b]	NS	NS	NS	NS	NS	NS	**0.45 ***	NS	NS	/
Apo AI [c]	NS	NS	**-0.42 ***	NS	NS	NS	NS	NS	**0.40 ***	NS

[a]concentration determined by clinical assay
[b]normalised band volume from Western blot analysis
[c]concentration obtained by ELISA
[d]concentration measured by LC-MS/MS

Significant positive correlations were found between CRP, an acute phase reactant, and other inflammation-related proteins such as SAA1, hepcidin and fibrinogen. On the contrary, CRP level correlated negatively with coagulation-related factors such as plasminogen and factor XIII. Although SAA correlated positively with CRP and fibrinogen, and negatively with prothrombin F1+2, another member of the SAA family, SAA4, showed no correlation with inflammatory markers. As expected, plasminogen positively correlated with coagulation factor XIII and histidine-rich glycoprotein, both involved in coagulation. Apolipoprotein AI levels, involved in cholesterol transport, were associated with both hepcidin and HRG.

Logistic regression analysis and ROC Curves with multiple markers

As presented in Table 5, logistic regression was used to calculate the p-values, odds ratios (OR) and AUC of ROC curves for each 11 individual biomarkers confirmed by ELISA, WB and LC-MS/MS in our cohort of 56 samples.

Table 5: P-values, odd ratios (OR) and area under the ROC curve (AUC) of individual candidate biomarkers calculated using logistic regression analysis.

	Control	GVHD D0			
Biomarkers	**n**	**n**	**OR**	**p-value**	**AUC**
Plasminogen	28	28	0.048	**0.036**	68.0
Histidine	28	28	0.115	0.12	63.6
Ln SAA4	28	28	1.32	0.64	54.0
Fib B chain	28	28	3.02	0.073	63.7
Factor XIII	28	28	0.025	**0.0123**	70.2
Fibrinogen	28	28	1.48	0.069	62.0
Ln CRP	28	28	2.03	**0.0023**	74.4
Ln SAA ELISA	28	28	3.34	**0.0019**	78.4
Ln Prothrombin	27	24	2.04	0.12	64.7
Ln APO A1	26	26	0.84	0.58	60.5
Ln Hepcidin	28	26	3.26	**0.012**	70.6

After removal of samples with missing values, univariate and multivariate logistic regression were applied on 47 remaining samples (25 controls and 22 GVHD). Multivariate logistic analysis

determined that a combination of SAA, prothrombin F1+2, fibrinogen beta chain fragment, Apo A1, fibrinogen and hepcidin levels produced the best model to predict the occurrence of aGVHD. Table 6A and 6A present p-values, OR and area under the ROC curve (AUC) for the markers included in the composite panel using univariate and multivariate analysis, respectively. As single biomarker, SAA was found to have the best predictive value (AUC = 73.5) (Table 6A). The generated composite panel markedly increases the AUC to 94.7 (Table 6B).

Table 6: Candidate biomarkers selected by the multivariate model based on values obtained from 47 samples**.**

A) P-value, OR and AUC calculated using univariate logistic regression analysis for each biomarker

B) P-value and OR calculated using multivariate logistic regression analysis as well as the AUC for the composite panel.

A **UNIVARIATE**

Biomarkers	OR	p-value	AUC
Fib B chain	3.01	0.096	63.5
Fibrinogen	1.22	0.39	53.1
Ln SAA ELISA	2.71	**0.0074**	73.5
Ln Prothrombin	1.82	0.20	62.5
Ln APO A1	0.71	0.31	66.2
Ln Hepcidin	2.73	**0.025**	67.1

B **MULTIVARIATE**

Biomarkers	OR	p-value	AUC
Fib B chain	605	**0.0042**	
Fibrinogen	0.13	**0.018**	
Ln SAA ELISA	6.19	**0.015**	
Ln Prothrombin	4.80	0.064	
Ln APO A1	0.15	**0.023**	
Ln Hepcidin	5.93	**0.019**	
COMPOSITE*			**94.7**

*** Predicted probability of developing GVHD= $e^{composite}$/ 1+$e^{composite}$**
Composite= -10.75 + 1.82 *ln*(SAA) + 1.57 *ln* (Prothrombin F1+2) + 6.41 FGB – 1.89 *ln*(ApoA1) – 2.04 fibrinogen + 1.78 *ln*(Hepcidin)

4. DISCUSSION

Although HSCT is the therapy of choice for various malignant or genetic haematological disorders, its application requires a lot of expertise to limit life-threatening complications such as acute graft-versus-host disease. This is why an early and specific diagnosis of aGVHD could help clinicians to better manage and decrease corticosteroid-related toxicity by reducing the dose or administrating other drugs.

The application of proteomic approaches is now commonly recognized as a tool for new biomarker discovery [41, 42]. Proteomic analysis is based on the assumption that the development of pathological states leads to changes in protein expression which should be detected. However, comparative differential analysis of protein patterns has not yet been extensively used for the discovery of biomarkers of acute GVHD [15, 18, 19, 21-23] while a lot of studies have been dedicated to the evaluation of various cytokines levels [12, 13, 43-47]. Although the expression levels of many cytokines were found to be linked to the onset of acute GVHD after HSCT, the use of such proteins as single biomarker suffers from a lack of specificity as they are also involved in many immune processes. Thus, a combination with other biomarkers should be considered to improve specificity.

In the present study, comparison of protein profiles between patients developing aGVHD and patients undergoing HSCT without aGVHD was performed using a combination of three complementary proteomic approaches: 2D-DIGE, SELDI-TOF-MS and 2D-LC-MS^E. We found that the investigation of plasma proteome combining those three different approaches extends the coverage of information. The well-known 2D-DIGE technology based on fluorescent tagging of proteins and bidimensional electrophoresis provides a high resolution map of intact proteins, including their possible post-translationally modified forms that are specific to each sample. Due to the low throughput of this approach, patient samples had to be pooled. As the 2-DIGE technology is not appropriate for the analysis of proteins with extreme pI or molecular weight below 15 kDa, SELDI-TOF-MS was chosen to study peptides and small proteins ranging between 1 to 30 kDa. SELDI-TOF-MS is a high throughput technology generating intact protein profiles, thus allowing rapid analysis of multiple individual samples and thus increasing statistical significance. However, biomarker discovery using SELDI-TOF-MS has to be associated with other

approaches to identify the proteins of interest. Finally, a more recent mass spectrometry-based label-free quantitative proteomics approach was performed on pooled tryptic digested samples.

Our analysis using 2D-DIGE comparing plasma samples from patients undergoing HSCT with or without acute GVHD revealed some potential biomarkers involved in coagulation, inflammation or lipid metabolism-related processes (Fig. 1). In particular, the 2D-DIGE approach allowed the identification of various specifically-generated fragments of several proteins, reflecting the singular ability of this technique to reveal the presence of post-translational modifications of proteins. Significant variations of plasminogen, coagulation factor XIII B chain, fibrinogen beta chain and serum amyloid A4 levels were observed. WB performed on individual samples confirmed the decrease of plasminogen and coagulation factor XIII levels as well as increased fibrinogen beta chain and serum amyloid A4 levels (Fig. 4). Interestingly, the levels of these proteins from samples taken 15 days before aGVHD diagnosis (excepted for SAA4) were found to have an intermediate level between controls and aGVHD D0 samples. These results suggest that pathological changes already occurred before disease diagnosis and thus could be used as early markers.

Except for SAA4, all these proteins are involved in the coagulation cascade, which is a relevant finding since many reports described thrombotic and hemostatic disorders after HSCT [48-52]. Acute GVHD with gastrointestinal damage presents an increased risk of bleeding while inflammation linked to aGVHD could be associated with higher risk of thrombosis [53, 54].

Coagulation factor XIII is a protein stabilizing the fibrin clot. Pihusch reported a decrease of factor XIII in patients presenting gastrointestinal acute GVHD. This difference could be the result of a higher consumption of this protein at the site of damaged epithelium [53]. The decrease of antithrombin III and prothrombin observed by LC-MS^E analysis as well as increase of prothrombin fragments 1+2 measured by ELISA also suggests an activation of coagulation. Moreover, decrease of plasminogen level could also arise from its higher consumption, maybe due to the excessive release of plasminogen activator by injured epithelium resulting in hyperfibrinolysis. Another protein involved in coagulation is fibrinogen which is a dimeric 330 kDa plasma protein composed of two alpha, beta or gamma chains. Under the action of thrombin, fibrinopeptide A and B are cleaved from fibrinogen, releasing soluble fibrin

monomers that form insoluble fibrin polymers stabilized by coagulation factor XIII [55]. In our analysis, fibrinogen beta chain fragment was found to vary between control and aGVHD D0 plasma samples (Fig. 2).

Fibrinogen is not only associated with coagulation cascade but is also, like CRP, a marker of acute phase of inflammation. The measurement of these two protein levels in our cohort showed a concomitant increase at onset of GVHD compared to controls, indicating an inflammatory state (Fig. 5). Elevation of CRP and fibrinogen levels has already been reported as predictive marker of HSCT complications and GVHD [50, 56]. The increase in fibrinogen level was somewhat less significant that of CRP, which suggests that inflammation-dependent stimulation of fibrinogen release is counterbalanced by its consumption through coagulation activation.

Reduced level of histidine-rich glycoprotein (HRG) was noticed in grade II aGVHD in the LC-MS^E experiment. HRG possesses a multifunctional role in different physiological processes, among others coagulation / fibrinolysis and modulation of cell adhesion to T-cells [57]. For example, Tsuchida-Straeten et al demonstrated an enhancement of coagulation and fibrinolysis processes in a HRG-/HRG- mouse model, suggesting anticoagulation and antifibrinolytic properties of HRG in vivo [58]. These properties could be partially explained by the high affinity of HRG for plasminogen and fibrinogen. In addition, HRG was described as a negative acute phase reactant and its level was found to be lowered in acute state of inflammation [59]. Our results showing decreased HRG levels in aGVHD samples are thus in agreement with those described by Mauz-Körholz et al [60]. Depletion of HRG could contribute to the induction of coagulation and fibrinolysis cascades in GVHD.

Inflammatory state at aGVHD diagnosis was also evident following demonstration by SELDI-TOF-MS experiments of changes in abundance of other biomarkers, namely apolipoproteins, SAA1 and hepcidin. Three peaks corresponding to SAA1 (intact SAA1 and its Arg and Arg-ser-truncated forms, Table 3) were found to be overexpressed. The serum amyloid A protein family members are involved in the regulation of inflammatory processes, lipid metabolism and lipid transport, and are mainly associated with plasma high density lipoproteins [61]. SAA1 is an acute phase reactant, thus it is not surprising to observe a positive correlation with CRP and fibrinogen levels (Table 4). As SAA1 release occurs within the first hour after acute injury [62,

63], it is interesting to note that its level already increased 15 days (P= 0.06) before symptom appearance, unlike those of CRP and fibrinogen (Fig. S3 and 5). Urieli-Shoval and al. reported an extrahepatic expression of SAA mRNA and protein in epithelial cells of many histologically normal human tissues, such as small and large intestine and skin epidermis [64]. Thus, early elevation of SAA before GVHD onset might result from early tissue leakage.

Similarly to CRP, hepcidin levels were found to gradually increase with aGVHD development (Fig. 3) and were significantly elevated in D0 aGVHD samples compared to controls (Fig 4F). Elevation of plasma hepcidin concentration might result from the increase of IL-6 levels, a potent inducer of hepcidin, following donor-derived T-lymphocyte activation [65]. Moreover, as a negative regulator of intestinal iron absorption, hepcidin has already been associated with GVHD by several authors [66, 67].

Contrarily to SAA1, levels of apolipoproteins AI and AII were found to decrease at aGVHD onset, and even 15 days before (Fig. 3 and S3). Changes of specific isoforms of Apo A-I levels between pre- and post-GVHD samples have already been observed by Wang et al. using intact protein analysis system [24]. HDL associated with Apo A-I is a negative acute phase reactant which was found to decrease by at least 25% during acute inflammation [68]. It plays an inhibitory role by interacting with activated T cells and interfering with monocyte activation responsible for IL-1 and TNF-alpha release [69]. A lower level of apolipoproteins during GVHD compared to controls might be due to early acute inflammation potentially caused by induced tissue injury, although Apo A1 levels do not correlated with CRP and SAA.

The level of another member of the serum amyloid A protein family (SAA4) was found to be elevated in patients with GVHD compared to controls (Fig. 2D and 4D). Although SAA1 functions are well described as acute phase protein of inflammation, the role of SAA4 remains poorly established. SAA4 has been described as a minor acute phase reactant [70], whereas other authors did not find any relation with inflammatory reaction [71]. In this study, SAA4 was not correlated with CRP and SAA1, suggesting that SAA4 does not play a major role in inflammatory process.

Analysis performed using LC-MS^E detected 90 proteins that are differentially expressed between controls and aGVHD patients. Classification of these proteins according to their involvement in biological processes confirmed that aGVHD onset is related to inflammation, as

well as alteration in the coagulation cascade, immune system and glucose/lipid metabolism. Indeed, significant variations of proteins such as plasminogen, histidine-rich glycoprotein, fibrinogen chain, SAA4 and CRP levels were found when comparing controls and GVHD D0 (Table S2). In addition, a decrease of Ig kappa and lambda light chain levels, with a low GVHD D0 / control ratio, was also observed by WB performed on the 46 samples used for the LC-MS^E analysis (Table S1 and Fig. 4). As the separation power of WB is limited, specific fragments of the Ig light chain revealed by LC-MS^E cannot be quantified separately on 1D gel. Targeted analysis should be performed by tandem mass spectrometry to determine whether only specific immunoglobulin regions are altered or if this reflects the decrease of total immunoglobulins associated with aGVHD as previously described by Perreault et al. and Norlin et al. [72, 73].

As we only focused on some differentially expressed proteins revealed by LC-MS^E experiment, further investigations should be undertaken to study the other potential biomarkers (cf. Supplementary data S2).

One limitation met in this study has to be noticed. Indeed, even if the identified biomarkers are relevant with physiological alteration already described for aGVHD, these markers remains abundant and poorly specific proteins. Indeed, variation of protein levels tested by WB was also observed in septicaemia samples, another frequent complication of HSCT. This suggests that in spite of many advances made in the proteomics field of blood biomarker discovery in the last decennia, detection of very low abundant proteins from blood samples remains limited. Indeed, the best discriminating biomarkers are expected to come from tissue leakage, proteins present in very low abundance. In this study, identified proteins are ranged in high abundant to middle abundant protein levels, even after application of 2 different sample preparation approaches. Moreover, given the low number of discriminating markers in our 2D-DIGE study, it can be supposed that disease-related information have been lost after combinatorial hexapeptide libraries pre-treatment due to the dynamic range compression [74].

In addition, the moderated grade of aGVHD studied and the higher number of isolated skin aGVHD compared to gut and liver aGVHD in the diseased sample set could also explain the low number of candidate biomarkers. Indeed, it can be supposed that isolated skin aGVHD lead to

lower systemic alteration (compared to intestinal aGVHD with bleeding) and thus it might be more difficult to distinguish them from controls.

Finally, we evaluated the capability of the candidate biomarkers to correctly discriminate patients with and without aGVHD. The composite biomarker panel generated by multivariate logistic regression provided a superior probability of correctly classify patients with and without aGVHD (AUC 94.7) compared to the best marker considered individually, SAA (AUC 73.5). This result demonstrates that proteomic approaches are useful tools to rapidly identify a set of biomarkers, which can improve the specificity of the disease diagnosis compared to the use of a single marker.

In conclusion, to improve its management, there is an urgent need to identify early and specific diagnostic tools of aGVHD. Current diagnosis of aGVHD is invasive and time-consuming. Using various proteomic approaches, several plasma proteins were found to be modulated by acute GVHD, making them potential biomarkers. Identified proteins are involved in inflammation, coagulation cascade and lipid metabolism. Although these proteins are involved in diverse and non-specific pathophysiological processes, their early expression before appearance of aGVHD symptoms warrants their further examination. These results reflect endothelium damage and activation of immune cells leading to the cytokine storm induced by the aGVHD. Despite the limited number of patients in our cohort, these findings provide some interesting insights into the pathophysiology and early diagnosis of aGVHD.

Acknowledgments

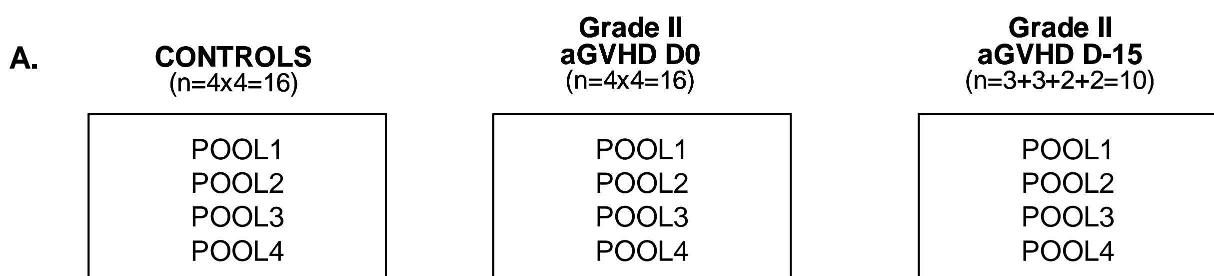
MDB is Télévie Research Fellow. MF, MPM and FB are Senior Research Associate at the National Fund for Scientific Research (FNRS) Belgium. This work was financially supported by the National Fund for Scientific Research (FNRS), the “Télévie” and the “Fonds Leon Frederic”. We thank the proteomic and bioinformatic platforms of the GIGA-Research center.

5. SUPPLEMENTARY DATA

S1 : Experimental design of proteomic analysis

Control and aGVHD groups were compared. For aGVHD group, samples on the day of aGVHD diagnosis and 15 days before aGVHD onset were considered.

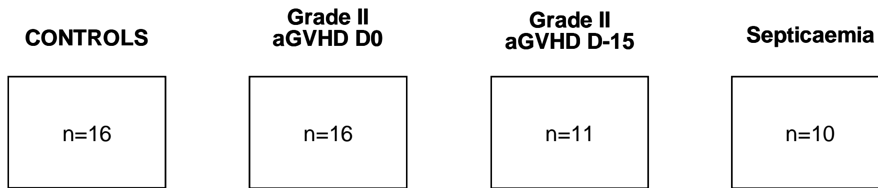
A. Experimental design of 2D-DIGE analysis
B. 2D-DIGE: Randomization of sample labelling and sample distribution on gels. (NB: 3 individual samples of grade IV GVHD were included in the experiment but not discussed in this manuscript because not statistically representative)
C. Experimental design of SELDI-TOF-MS analysis
D. Experimental design of 2D-LC-MSE analysis



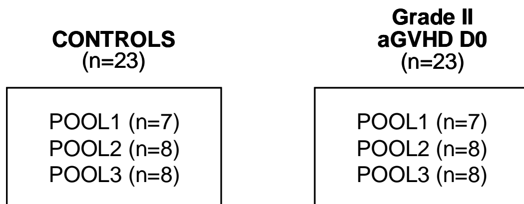
B.

SAMPLES	Gel 1	Gel 2	Gel 3	Gel 4	Gel 5	Gel 6	Gel 7	Gel 8	Gel 9	Gel 10	Gel 11
Grade II GVHD D0 pool 1	Cy3										Cy5
Grade II GVHD D0 pool 2										Cy5	
Grade II GVHD D0 pool 3					Cy5						
Grade II GVHD D0 pool 4								Cy3			
Grade IV GVHD D0 sample 1			Cy3								
Grade IV GVHD D0 sample 2				Cy5							
Grade IV GVHD D0 sample 3						Cy3					
Grade II GVHD D-15 pool 1				Cy3							
Grade II GVHD D-15 pool 2	Cy5										
Grade II GVHD D-15 pool 3							Cy5				
Grade II GVHD D-15 pool 4									Cy3		
Grade IV GVHD D-15 sample 1							Cy3				
Grade IV GVHD D-15 sample 2								Cy5			
Grade IV GVHD D-15 sample 3											Cy3
Grade II control pool 1		Cy3									
Grade II control pool 2									Cy5		
Grade II control pool 3			Cy5								
Grade II control pool 4										Cy3	
Grade IV control pool 1						Cy5					
Grade IV control pool 2		Cy5									
Grade IV control pool 3					Cy3						
Internal standard	Cy2	Cy2	Cy2	Cy2	Cy2	Cy2	Cy2	Cy2	Cy2	Cy2	Cy2

C. SELDI-TOF-MS



D. 2D-LC-MS^E



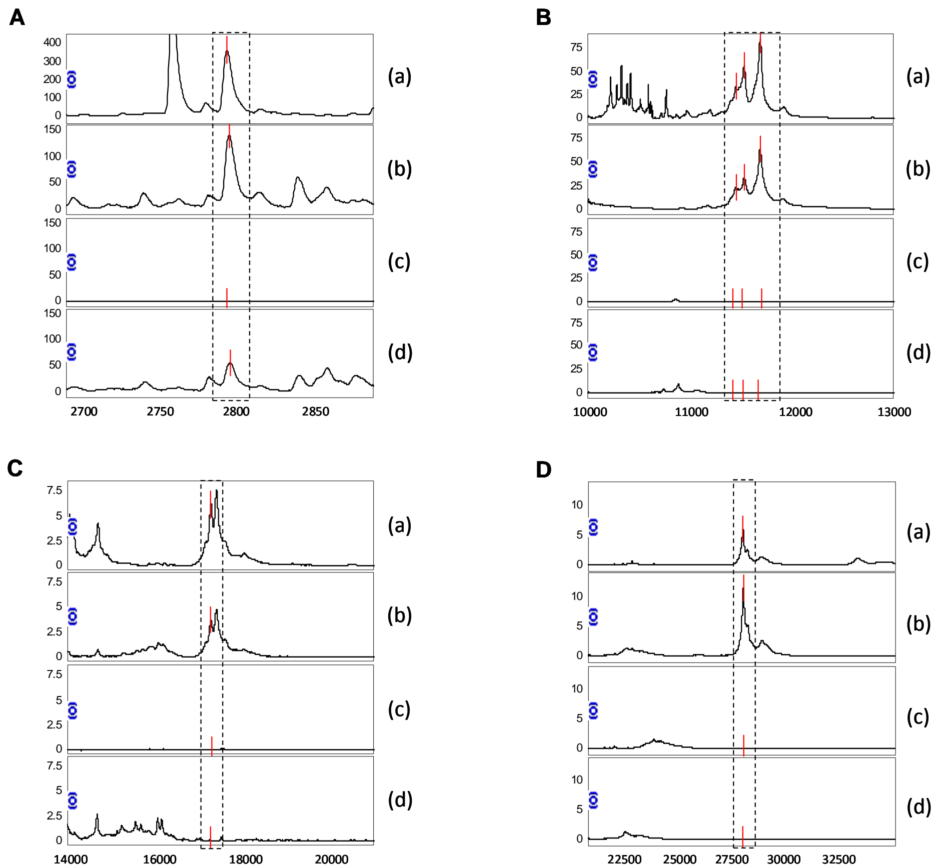
S2: Immunodepletion experiments for identification of peaks at 2792 (A), 11446, 11527, 11687 (B), 17271 (C) and 28120 (D) m/z analysed on CM10 arrays by SELDI-TOF-MS.

plasma sample from aGVHD patient

eluted plasma fraction after incubation with protein G+ coupled with an antibody; namely. hepcidin-25 (A), SAA1 (B), Apo AII (C) or Apo AI (D) antibody

eluted plasma fraction after incubation with protein G+ alone (negative control)

eluted plasma fraction after incubation with protein G+ coupled with another antibody (negative control)



S3: Comparison of SAA1 (A) and Apo A1 (B) levels between control samples and grade II aGVHD samples on the day of diagnosis. Protein levels were evaluated by ELISA. Levels of SAA1 and Apo A1 in samples (n=21) taken 15 days before aGVHD onset are also depicted. P-values calculated using the Mann-Whitney test and Wilcoxon signed-rank test. * P< 0.05; ** P< 0.01; *** P< 0.001.

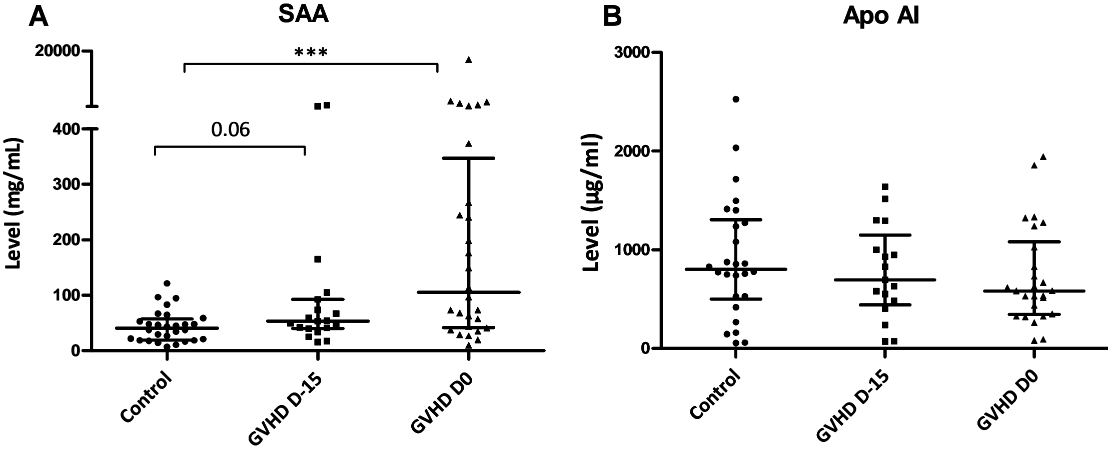


Table S1: Patient characteristics of validation set (n=56)

	Western Blot	
Characteristics	***Control (n=28)***	***Grade II aGVHD (n=28)***
Median patient age, years (range)	59.5 (30 – 72)	59.5 (21 – 70)
Gender		
Male	20	20
Female	8	8
Diagnosis		
Acute myeloblastic leukemia	13	7
Lymphoma	4	9
Multiple myeloma	5	5
Myelodysplastic syndrome	1	4
Other malignancies	5	3
Donor		
Related	6	8
Unrelated	22	20
Conditioning regimen intensity		
Myeloablative	4	5
Reduced	24	23
ATG administration	6	5
Acute GVHD		
Skin		22
Gut		9
Liver		2
Combined		5
Day of onset of acute GVHD, median (range)		44 (13 – 245)
Day after HSCT of samples, median (range)	45 (10 – 259)	44.5 (14 – 248)

Table S2: **Proteins significantly different between control and aGVHD obtained by LC-MS^E classified by Ingenuity Pathway analysis software**

Uniprot ID	Protein name	Score	p-value	Ratio	LogE ratio	LogE ratio SD
Acute phase response signaling						
P02766	Transthyretin	76866	0	0.75	-0.29	0.13
P04196	Histidine rich glycoprotein	6632	0	0.76	-0.27	0.1
P02765	Alpha 2 HS glycoprotein	22652	0	0.78	-0.25	0.06
P02753	Retinol binding protein 4	8741	0	0.79	-0.24	0.11
P02787	Serotransferrin	39719	0	0.79	-0.23	0.04
P02751-6	Isoform Fibronectin V III 15 of Fibronectin	4660	0	0.80	-0.22	0.08
P02751-5	Isoform Fibronectin V I 10 of Fibronectin	4670	0	0.80	-0.22	0.07
P02751-4	Isoform Fibronectin III 15X of Fibronectin	4484	0	0.80	-0.22	0.09
P02751-2	Isoform Migration stimulation factor FN70 of Fibronectin	1415	0.04	0.81	-0.21	0.23
P02751-12	Isoform 12 of Fibronectin	3992	0	0.82	-0.2	0.08
P02751-10	Isoform 10 of Fibronectin	5638	0	0.82	-0.2	0.09
P02768-2	Isoform 2 of Serum albumin	95367	0	0.92	-0.08	0.05
P02768	Serum albumin	136929	0	0.92	-0.08	0.02
P00450	Ceruloplasmin	32216	0	0.92	-0.08	0.02
P02790	Hemopexin	83837	0	0.93	-0.07	0.02
P00751	Complement factor B	16126	0.03	0.95	-0.05	0.05
Q14624-2	Isoform 2 of Inter alpha trypsin inhibitor heavy chain H4	7125	0.97	1.07	0.07	0.07
P01023	Alpha 2 macroglobulin	28934	1	1.07	0.07	0.03
P0C0L5	Complement C4 B	12500	1	1.09	0.09	0.04
P0C0L4	Complement C4 A	15844	1	1.11	0.1	0.03
P02748	Complement component C9	3481	0.96	1.19	0.17	0.19
P01011	Alpha 1 antichymotrypsin	41476	1	1.20	0.18	0.04
P01024	Complement C3	1217	1	1.21	0.19	0.12
Q06033	Inter alpha trypsin inhibitor heavy chain H3	811	0.98	1.22	0.2	0.2
Q06033-2	Isoform 2 of Inter alpha trypsin inhibitor heavy chain H3	685	0.99	1.22	0.2	0.19
P19652	Alpha 1 acid glycoprotein 2	34136	1	1.26	0.23	0.06
P02763	Alpha 1 acid glycoprotein 1	48811	1	1.28	0.25	0.07
P01011-2	Isoform 2 of Alpha 1 antichymotrypsin	8655	1	1.30	0.26	0.08
P01009-3	Isoform 3 of Alpha 1 antitrypsin	17968	1	1.31	0.27	0.09
P00738	Haptoglobin	36658	1	1.32	0.28	0.06
P01009	Alpha 1 antitrypsin	34074	1	1.35	0.3	0.06
P01009-2	Isoform 2 of Alpha 1 antitrypsin	22926	1	1.35	0.3	0.07
P02741	C reactive protein	402	1	3.39		
P02741-2	Isoform 2 of C reactive protein	7100	GVHD **			
Coagulation System						
P01008	Antithrombin III	26662	0	0.88	-0.13	0.06
P00747	Plasminogen	7673	0	0.90	-0.11	0.07
P00734	Prothrombin	13211	0.01	0.91	-0.09	0.06
P01042	Kininogen 1	11458	0	0.92	-0.08	0.07
P02679	Fibrinogen gamma chain	12417	1	1.28	0.25	0.05

Uniprot ID	Protein name	Score	p-value	Ratio	LogE ratio	LogE ratio SD
P02679-2	Isoform Gamma A of Fibrinogen gamma chain	15810	1	1.31	0.27	0.05
P02675	Fibrinogen beta chain	28823	1	1.31	0.27	0.04
P02671	Fibrinogen alpha chain	17124	1	1.36	0.31	0.09
P02671-2	Isoform Alpha of Fibrinogen alpha chain	13256	1	1.36	0.31	0.07
LXR-RXR activation						
P06727	Apolipoprotein A IV	13463	0	0.69	-0.37	0.08
P04217	Alpha 1B glycoprotein	18646	0.02	0.91	-0.09	0.07
P02749	Beta 2 glycoprotein 1	17378	0.01	0.92	-0.08	0.06
P04004	Vitronectin	7645	0.03	0.92	-0.08	0.08
P02647	Apolipoprotein A I	35000	0.98	1.05	0.05	0.05
P00739	Haptoglobin related protein	7277	1	1.39	0.33	0.12
P00739-2	Isoform 2 of Haptoglobin related protein	7193	1	1.42	0.35	0.13
P35542	Serum amyloid A 4 protein	1159	GVHD*			
Primary immunodeficiency signaling						
P01610	Ig kappa chain V I region WEA	22256	0	0.48	-0.73	0.27
P01609	Ig kappa chain V I region Scw	22256	0	0.49	-0.72	0.29
P01594	Ig kappa chain V I region AU	22256	0	0.49	-0.72	0.3
P01608	Ig kappa chain V I region Roy	22256	0.01	0.49	-0.72	0.28
P01607	Ig kappa chain V I region Rei	22256	0.01	0.50	-0.7	0.36
P01599	Ig kappa chain V I region Gal	22309	0	0.49	-0.71	0.3
P80362	Ig kappa chain V I region WAT	22256	0	0.50	-0.7	0.28
P01600	Ig kappa chain V I region Hau	22385	0	0.52	-0.65	0.28
P01593	Ig kappa chain V I region AG	27948	0	0.56	-0.58	0.27
P01877	Ig alpha 2 chain C region OS	10107	0	0.61	-0.5	0.16
P01623	Ig kappa chain V III region WOL	9371	0	0.63	-0.46	0.2
P01622	Ig kappa chain V III region Ti	9371	0	0.64	-0.45	0.24
P04206	Ig kappa chain V III region GOL	9371	0	0.64	-0.45	0.22
P01620	Ig kappa chain V III region SIE	9371	0	0.65	-0.43	0.24
P01861	Ig gamma 4 chain C region	27187	0	0.65	-0.43	0.12
P01876	Ig alpha 1 chain C region	23529	0	0.69	-0.37	0.14
P01842	Ig lambda chain C regions	46555	0	0.70	-0.36	0.14
P01859	Ig gamma 2 chain C region	21558	0	0.75	-0.29	0.08
P01860	Ig gamma 3 chain C region	13856	0	0.78	-0.25	0.13
P01857	Ig gamma 1 chain C region	41191	0	0.79	-0.23	0.07
P04220	Ig mu heavy chain disease protein	5028	1	1.60	0.47	0.19
P01871-2	Isoform membrane bound of Ig mu chain C region	8454	1	1.63	0.49	0.14
P01871	Ig mu chain C region	6222	1	1.67	0.51	0.12
P01764	Ig heavy chain V III region VH26	6695	Control **			
Others						
P06737	Glycogen phosphorylase liver form	701	0	0.73	-0.31	0.18
P51884	Lumican	3073	0.03	0.78	-0.25	0.26
P06396-2	Isoform Cytoplasmic of Gelsolin	2480	0	0.81	-0.21	0.12
P06396	Gelsolin	3477	0.01	0.82	-0.2	0.15
Q03591	Complement factor H related protein 1 OS	2095	0.03	0.83	-0.19	0.2

Uniprot ID	Protein name	Score	p-value	Ratio	LogE ratio	LogE ratio SD
P11217	Glycogen phosphorylase muscle form	2940	0	0.83	-0.19	0.09
P43652	Afamin OS Homo sapiens	7025	0	0.84	-0.17	0.11
P08603-2	Isoform FHL 1 of Complement factor H OS	8240	0	0.88	-0.13	0.07
P01042-2	Isoform LMW of Kininogen 1	9022	0.01	0.91	-0.09	0.07
P08603	Complement factor H	19844	0.01	0.93	-0.07	0.05
P02750	Leucine rich alpha 2 glycoprotein	8140	1	1.26	0.23	0.09
P68871	Hemoglobin subunit beta	21681	1	1.39	0.33	0.14
P02100	Hemoglobin subunit epsilon	1895	0.96	1.49	0.4	0.34
P02747	Complement C1q subcomponent subunit C	1219	Control **			
P61769	Beta 2 microglobulin	1460	GVHD **			

P-value < 0.05 or > 0.95 were considered as significative

* Proteins detected in all GVHD samples and in only one control sample.

** Proteins detected in at least 2 samples out of 3 in the noticed group and not detected in the other

Table S3: Median with 25th-75th percentile of normalised band volumes obtained by WB[a] and peak intensities obtained by SELDI-TOF-MS[b] for controls, GVHD D0 and septicaemia samples. P-values were calculated by the Mann-Whitney test.

Protein name	Controls	GVHD D0	Septicaemia	Control *versus* GVHD D0	Control *versus* Septicaemia
	Median (25th-75th)	Median (25th-75th)	Median (25th-75th)	P-value	P-value
Plasminogen[a]	0,72 (0.56 – 0.83)	0,55 (0.45 – 0.68)	0,49 (0.37 – 0.57)	**0.02**	**0.001**
Coagulation factor XIII[a]	0,68 (0.48 – 0.85)	0,49 (0.35 – 0.68)	0,49 (0.44 – 0.60)	**0.01**	**0.04**
Histidine-rich glycoprotein[a]	0,81 (0.62 -0.94)	0,65 (0.55 – 0.82)	0,60 (0.35 – 0.75)	0.08	**0.02**
Serum Amyloid A4[a]	0.42 (0.35 - 0.64)	0.48 (0.33 – 0.62)	0.64 (0.31 – 0.89)	0.62	0.49
Hepcidin[b] (m/z 2792)	159 (49 – 277)	277 (159 – 525)	322 (174 – 762)	**0.02**	**0.03**
Serum Amyloid A[b] (m/z 11687)	0,93 (0.6 – 1.8)	4,9 (1.5 – 18.5)	38,5 (4.5 – 81)	**0.0021**	**< 0.0001**
Apolipoprotein AII[b] (m/z 17271)	5,3 (3.1 – 6.0)	3,4 (1.7 – 4.8)	2,2 (1.9 – 4.1)	**0.03**	**0.03**
Apolipoprotein AI[b] (m/z 28120)	10,4 (7.9 – 11.7)	8,4 (6.4 – 9.4)	5,2 (3.4 – 6.9)	**0.007**	**0.001**

[a] controls (n=28), GVHD D0 (n=28), septicaemia (n=10)
[b] controls (n=16), GVHD D0 (n=16), septicaemia (n=10)

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Evaluation of circulating cytokine levels as predictive serum markers of acute graft–versus–host disease after hematopoietic stem cell transplantation

Abstract

Acute graft-versus-host disease (aGVHD), a common complication encountered after allo-HSCT, is an exacerbated immune reaction between donor and host cells mediated by a cytokine-dependent mechanism. Early diagnosis and management of this life-threatening complication could significantly improve survival and quality of life in patients undergoing HSCT. In order to assess whether pro-inflammatory and anti-inflammatory cytokines implicated in aGVHD pathophysiology could be early predictor of aGVHD onset, we compared levels of IL-2, IL-6, IL-7, IL-8, IL-10, interferon-γ (IFN- γ) and TNF-R1 by multiplex immunoassay between patients with or without grade II-IV aGVHD. Significant increase of serum IL-6, IL-10 and TNF-R1 levels between samples from patients without aGVHD and samples taken on the day of aGVHD symptom onset was observed. In addition, IL-10 levels measured 15 days before aGVHD onset were found significantly higher than in patients without grade II-IV aGVHD. Thereby, to further investigate whether IL-10 levels in the early post-transplant period could predict subsequent occurrence of mild to severe aGVHD, the pre-conditioning, day 14 and day 25 IL-10 levels were evaluated by ELISA in 65 patients undergoing HSCT following nonmyeloablative conditioning. No difference was observed between IL-10 levels before conditioning from patients with and without experiencing grade II-IV aGVHD while day 14 and day 25 median IL-10 levels were higher in aGVHD group. In addition, a significant difference was observed in the 180-day cumulative incidence of grade II-IV according to day 14 and day 25 median IL-10 levels between patients with and without grade II-IV aGVHD. In a multivariate Cox model, the median level of IL-10 on day 14 and 25 was the only significant predictive factor of subsequent onset of grade II-IV aGVHD. In conclusion, evaluation of the IL-10 level in the first month after HSCT may be useful as a diagnostic marker in predicting the occurrence of moderate to severe acute GVHD after HSCT.

1. INTRODUCTION

Acute GVHD remains a major factor of transplant-related mortality after hematopoietic stem cell transplantation (HSCT). The main organs affected by this complication are skin, gastrointestinal tract and liver, and according to the involvement of each organ, aGVHD severity can be ranged between minor reactions (grade I) to a severe and lethal status (grade IV). Moreover, aGVHD development impairs thymic function that affects immune recovery after HSCT (1), leading to a higher risk of infection and relapse. In addition, toxicities related to high-dose corticosteroid administration as a first line of GVHD treatment increase risk of mortality related to aGVHD. Thus, to reduce the rate of transplant-related mortality, early detection and management of aGVHD is a critical clinical problem.

Acute GVHD is an immune-mediated complication mounting by genetically incompatible donor immunocompetent cells infused in an immunocompromised host. This cascade reaction is mediated by complex cell interactions and dysregulation of cytokine production that may initiate and perpetuate tissue damages. The pro-inflammatory cytokines, IL-1, IL-6 and TNF-α are key mediators of the initiation step of aGVHD physiopathology. Cytokine secretion results from the deleterious effect of the preparative regimen on host tissue cells and subsequent lipopolysaccharide translocation in the gastrointestinal tract. The subsequent APCs and T-cell activation followed by cell differentiation, proliferation and effector recruitment require the release of mediator molecules such as IL-2, interferon-γ and IL-7. Pathophysiology of aGVHD is a complex processus based on the balance production of pro- and anti-inflammatory cytokines derived from Th1 and Th2 cells respectively (2). A variety of cytokines have been demonstrated to be associated with aGVHD, making them potential predictive serum markers of aGVHD onset (3-7). Among them, a higher level of IL-10, a regulatory cytokine secreted by Th2 T-cell, monocytes and macrophages, has been associated with onset of acute GVHD (4, 8, 9) although this conclusion remains controversial (10-12). In addition to the effect of various external stimuli, production of IL-10 is influenced by genetic variations such as haplotype and single nucleotide polymorphism of IL-10 gene. (13, 14).

As the linear dynamic range coverage of current proteomic strategies is limited for the detection of low abundance proteins, the levels of pro-inflammatory (IL-2, IL-6, IL-7, IL-8, IFN- γ and TNF-RI) and immunoregulatory cytokines (IL-10) were evaluated in patients developing or

not aGVHD by multiplex immunoassays in order to complete our biomarker discovery for aGVHD diagnosis. As IL-10 was found the most promising early serum marker, we subsequently evaluated its kinetic course levels during the first month post-HSCT in 65 patients undergoing nonmyeloablative HSCT.

2. MATERIALS AND METHODS

All studied patients were transplanted at the University of Liège. Serum samples were collected prospectively twice a week until day 100 post-HSCT and once a week until day 365. After centrifugation of blood at 3000 rpm for 10 min, serum samples were aliquoted and stored at -80°C until analysis.

2.1. Patients and evaluation of cytokine levels by cytometric bead array

Patients: All 28 patients were given G-CSF-mobilized peripheral blood stem cells (PBSC) after high-dose conditioning (n=7) or nonmyeloablative regimen consisted of low-dose [2 Gy or 4 Gy] total body irradiation (TBI) (n=19) or total lymphoid irradiation/anti-thymocyte globulin (n=2). Seven patients undergoing myeloablative regimen received ATG. Median age of patients with or without grade II-IV aGVHD was 56 (range 44-67) and 51 (range 16-66), respectively. High-dose recipients received cyclosporine with or without methotrexate as preemptive therapy of GVHD while an association of tacrolimus and mycophenolate mofetil was administered to nonmyeloablative recipients. Diagnosis and clinical grading of aGVHD were performed according to established criteria (15). Fourteen patients do not develop grade II or more aGVHD (namely control) while 12 and 2 patients experienced grade II aGVHD and grades III-IV aGVHD, respectively. The median day of aGVHD occurrence was day 41 (range 20-286). Serum samples from 14 control patients and 14 patients developing aGVHD were analysed. Control samples were taken around day 42 (range 39-47). For aGVHD group, samples collected around the day of aGVHD diagnosis before initiation of treatment and 15 days before symptom appearance were evaluated.

Cytokine levels: Multiplex measurement of IL-2, IL-6, IL-7, IL-8, IL-10, IFN and TNF-RI levels were performed using cytometric bead array method (CBA) following manufacturer instructions (Beckton Dickinson Biosciences). TNF-RI was measured as a surrogate marker of TNF-α. Briefly, all beads coated with different antibodies were mixed together, excepted for TNF-R1 which was analysed alone and were subsequently washed. Based on preliminary tests, a 4 fold-dilution of serum was performed for TNF-R1 analysis while no dilution was applied for others 7 cytokines. For each serum sample and cytokine standard mixture, 50 µl of sample was first added to a solution of 50 µl of mixed capture bead followed by 50µl of a secondary antibody conjugated

with detector pycoerythrin (PE) reagent and incubated for 1h and 2h at room temperature, respectively. After washing, beads were resuspended in wash buffer and analysed by flow cytometry.

Flow cytometry analysis: Data acquisition and analysis were performed using a FACSCanto™ II flow cytometer (Beckton Dickinson Biosciences). Levels of cytokines in the same mixture were determined simultaneously, since beads that detect a given cytokine have distinct fluorescence intensity. Particles (7.5 µm in diameter) were internally labelled with different concentrations of a dye that emits fluorescent signals after excitation by the 633nm laser and signals were measured in the APC and APC-Cy7 channel (FL620/20 and 780/60-735LP). Cytokine concentrations were determined by the PE fluorescence intensity, which was measured in the PE channel (585/42-556LP). The standard curves were determined using a four or five-parameter logistics depending of the type of beads. The data were analysed with FCAP Array software provided by the manufacturer.

2.2. Measurement of IL-10 and CRP levels

Sixty-five patients transplanted between March 2007 and April 2011 at the University of Liège were included in the study. All patients were given G-CSF-mobilized peripheral blood stem cells (PBSC) after low-dose [2 Gy (n=60), or 4 Gy (n=10)] total body irradiation (TBI)-based nonmyeloablative regimen. Among them, 18 patients developed grade II-IV aGVHD. IL-10 serum levels were evaluated in serum samples taken before conditioning, 14 and 25 days after HSCT. IL-10 levels were measured by ELISAs following the manufacturer's protocol (High sensitivity IL-10, R&D Systems, Minneapolis, MN, USA). No sample dilution was performed for IL-10 assay. Patient samples whose cytokine levels were out of standard curve range, were reevaluated after dilution. C-reactive protein measurement (CRP) levels were evaluated by immunoturbidimetric assay using modular Roche PPE-U and PPE-R (Roche Diagnostics, Indianapolis, IN, USA). Reference range is 0-6mg/L.

2.3. Statistical analyses

All analyses were conducted using GraphPad Prism 5 (Graphpad Software, San Diego, CA) and SAS version 9.2 for Windows (SAS Institute, Cary, NC, USA). The Mann-Whitney and Wilcoxon signed-rank tests were used to compare different groups. Associations between variables were

evaluated using Spearman test. Cumulative grade II-IV aGVHD incidence curve based on median IL-10 levels on day 14 and 25 were estimated using Kaplan-Meier method. Comparison between survival curves was calculated with the log rank test. Multivariate Cox model was performed to evaluate risk factors of developing aGVHD. Factors introduced in the model included median levels of day 14 and 25, 2 or 4 Gy TBI, donor type, donor and patient age, female donor to male recipient or other gender combination, and number of CD3+ and CD34+ cells infused.

3. RESULTS

3.1. Levels of serum cytokines in patients with and without aGVHD

Levels of 7 cytokines; IL-2, IL-6, IL-7, IL-8, IL-10, IFN-γ and TNF-R1 were measured by cytometric bead assay in 39 serum samples from 28 patients undergoing HSCT after myeloablative or nonmyeloablative conditioning. Cytokine levels in serum samples from patients with (n=14) or without developing grade II-IV aGVHD (n=14) were compared. In the aGVHD group, 14 samples at day of aGVHD symptom onset and 11 serum samples taken 15 days before aGVHD diagnosis were also examined in order to evaluate potential early level change.

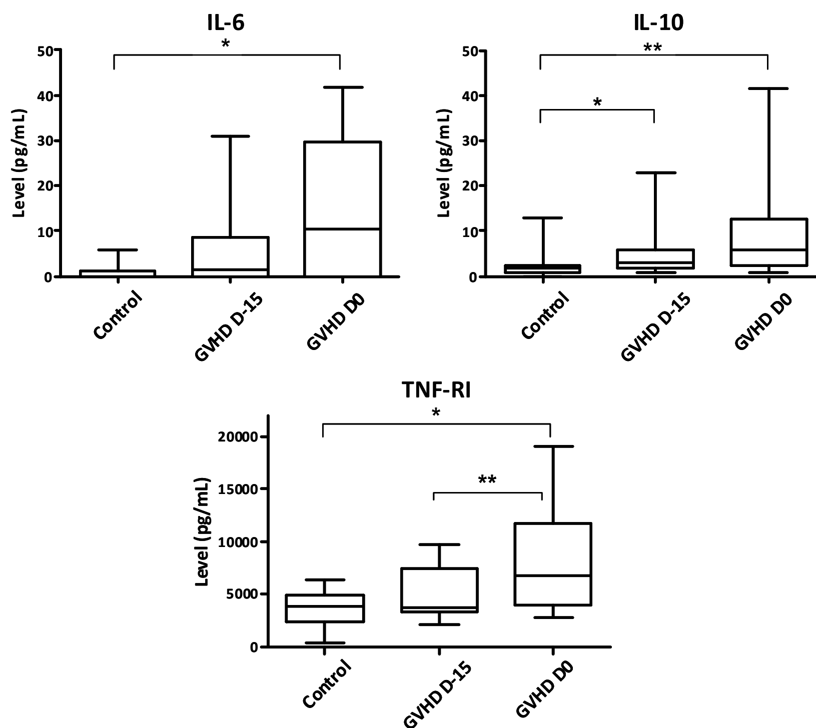


Figure 1: Comparison of serum IL-6, IL-10 and TNF-R1 levels (median with upper and lower quartiles as well as the 5-95 percentiles) from patients without GVHD and samples from patients with grade II-IV GVHD taken on the day of onset and 15 days before. P-values were calculated using the Mann-Whitney test. *, P <0.05; **, P <0.01 ; ***, P <0.001.

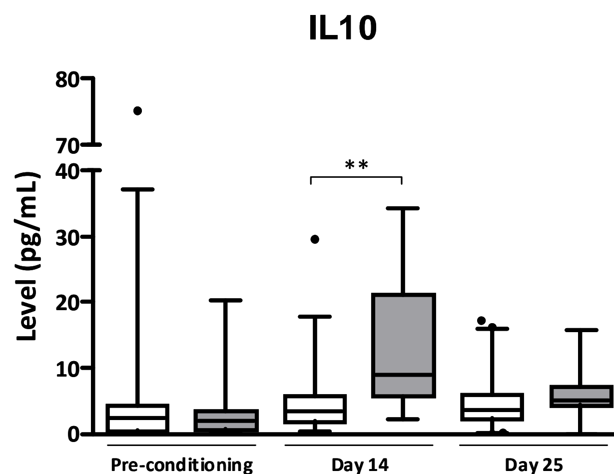
As presented in Figure 1, among the 7 cytokines studied, patients with aGVHD exhibited significant higher serum concentrations of IL-6 (p-value 0.021), IL-10 (p-value 0.0072) and TNF-R1 (p-value 0.036) compared to controls. Interestingly, IL-10 level in samples taken 15 days before aGVHD onset was found significantly elevated compared to control (p-value 0.046) suggesting IL-10 secretion before symptom appearance. However, no significant difference of

IL-8 and IFN- γ levels between groups with and without aGVHD was observed. Levels of IL-2 and IL-7 were not detectable.

3.2. Association between IL-10 levels in the first month after nonmyeloablative transplantation and aGVHD onset

In the preliminary study, IL-10 levels were found significantly increased at the time of GVHD diagnosis compared to controls. In addition, the significant difference of IL-10 levels between samples taken 15 days prior aGVHD symptoms onset and controls suggests that IL-10 could be an interesting predictive serum marker. Thus, we measured the IL-10 concentrations at three different time points in the serum of 65 patients undergoing nonmyeloablative conditioning HSCT in order to evaluate if IL-10 level after HSCT could predict subsequent aGVHD onset. Grade II-IV aGVHD was observed in 18 patients and median time for diagnosis of aGVHD was day 43.

Figure 2 shows difference in IL-10 levels between patients who do not experienced mild or severe aGVHD (including grade I aGVHD) and those who developed grade II-IV aGVHD before conditioning, on day 14 and 25 post-HSCT.



<u>Figure 2</u>: Kinetics of IL-10 levels before conditioning and the first month after HSCT in patients experiencing or not aGVHD. Box plots represent median values, lower and upper quartiles as well as the 5-95 percentiles. White and grey boxes correspond to grade 0-I group and grade II-IV group, respectively. P-values were calculated using the Mann-Whitney test. *, P <0.05; **, P <0.01 ; ***, P <0.001.

Before conditioning, median IL-10 levels were low and there was no significant difference between both groups (median 2.33 pg/mL versus 2.16 pg/mL). On the day 14 post-HSCT, there was a slight IL-10 level elevation compared to pre-HSCT levels in grade 0-I aGVHD patients while

increase is more pronounced in patients developing grade II-IV aGVHD (Wilcoxon paired test, p-value 0.02). In addition, median IL-10 level in patients with subsequent grade II-IV aGVHD was significantly higher compared to those without developing aGVHD (p-value 0.0023). On the day 25 post-HSCT, the grade II-IV aGVHD patients exhibit an increased median IL-10 concentration compared to patients without aGVHD (5.14 pg/mL versus 3.7 pg/mL), although the difference was not significant (p-value 0.14).

To further evaluate whether IL-10 levels could predict subsequent onset of grade II-IV aGVHD, cumulative incidence analysis were used to estimate grade II-IV aGVHD incidence using the IL-10 median values on day 14 and day 25 for stratification (Figure 3). The 180-day cumulative incidence of grade II-IV aGVHD according to day 14 median level of IL-10 (4.01 pg/mL) was 11.5 % in patients with day 14 IL-10 levels below the median versus 40% in patients with day 14 IL-10 levels above the median. Differences between strata calculated by log-rank tests did almost reach significance (P-value 0.07). In addition, the 180-day cumulative incidence of grade II-IV aGVHD was 8% in patients with day 25 IL-10 levels lower than median (3.94 pg/mL) versus 35% in patients with day 25 IL-10 levels higher than median. There was a significant difference between both cumulative incidence curves (P-value 0.03).

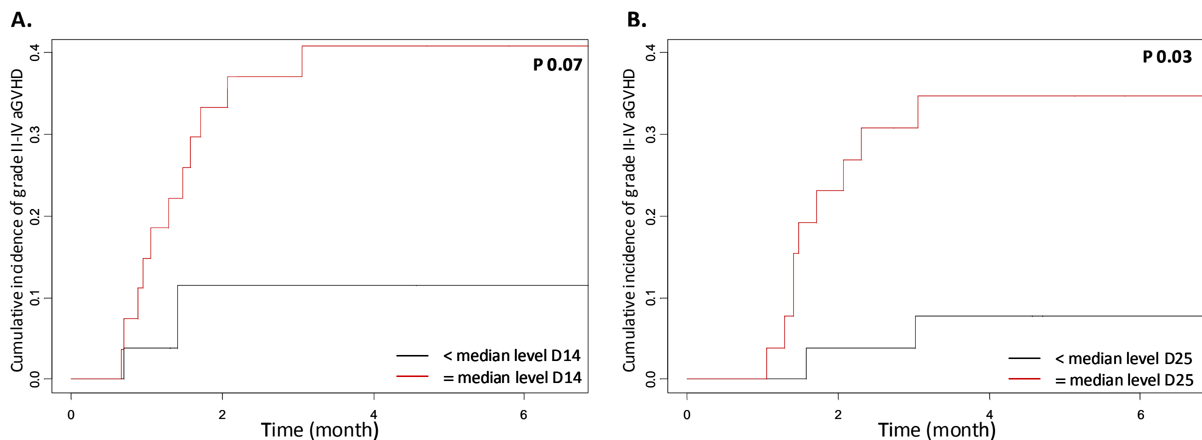


Figure 3: Estimated cumulative incidence curves of grade II-IV aGVHD according to IL-10 level on (A) the day 14 and (B) the day 25. The median IL-10 concentration on the day 14 and day 25 in the whole study population were used to segregate the 2 groups.

To further identify potential predictive factors associated with onset of grade II-IV aGVHD, a multivariate Cox model including factors presented in Table 1 was constructed. High day 14-25 median IL-10 level was the only significant predictive factor of subsequent risk of grade II-IV aGVHD (p-value 0.0014, HR 1.10).

Table 1: Factors predicting aGVHD in a multivariate Cox model. (Bold values indicate P-value <0.05)

Factors	*Grade II-IV aGVHD*	
	Hazard ratio	*P-value*
IL-10 levels at day 14 and day 25	**1.10**	**0.0014**
Patient age	1.04	0.13
Number of T-cells infused	1.77	0.18
Unrelated donor versus related donor	3.61	0.19
MSC infusion	2.28	0.19
Donor age	0.98	0.53
Female donor to male recipient versus other gender combinations	1.54	0.54
Number of CD34+ infused	1.04	0.74
Dose of total body irradiation (4 Gy versus 2 Gy)	1.01	0.99

In order to determine whether IL-10 production is associated with inflammatory process, CRP levels on day 14 and day 25 were assessed. As presented in Figure 4, CRP levels showed a similar kinetic on day 14 and 25 than those observed for IL-10. Indeed, a significant higher day 14 level of IL-10 was observed in patients who subsequently developed aGVHD compared to those without aGVHD while IL-10 concentration on day 25 was slightly elevated in aGVHD patients but the difference did not reach significance (p-value 0.14). Then, correlation between IL-10 and CRP levels was estimated using the Spearman's rank correlation test. Day 14 and day 25 IL-10 levels positively correlated with day 14 (p-value 0.05, Rs 0.53) and day 25 (p-value 0.02, Rs 0.59) CRP levels, respectively, suggesting that IL-10 production tend to be associated with inflammation in the first month post –HSCT following nonmyeloablative regimen.

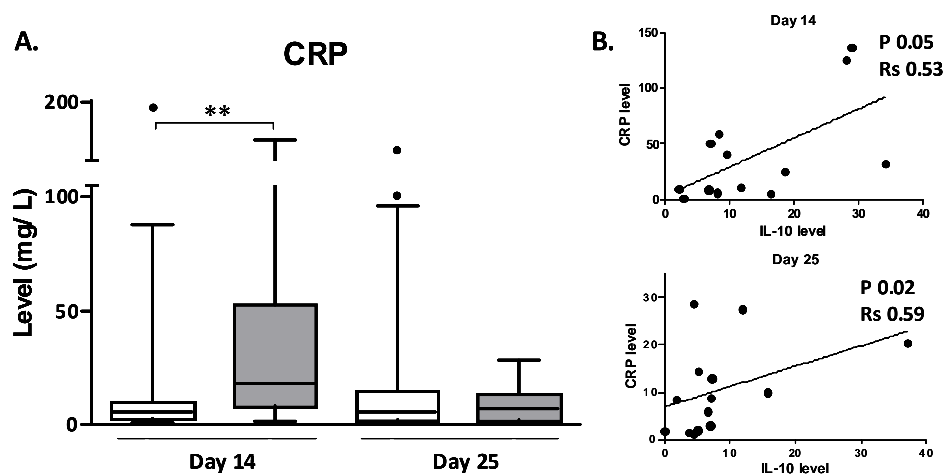


Figure 4: Association between IL-10 and CRP levels

A. Comparison of CRP serum levels on day 14 and 25 between patients with grade 0-I and grade II-IV aGVHD.
B. Correlation between day IL-10 and CRP levels on day 14 and day 25

4. DISCUSSION

Acute GVHD remains a significant cause of nonrelapse mortality after allogeneic hematopoietic cell transplantation (HCT) following myeloablative and nonmyeloablative conditioning. Current diagnostic methods can not predict subsequent aGVHD onset in patients undergoing allo-HSCT. This is why early serum biomarkers could be useful for prediction of aGVHD occurrence. Cytokines are humoral mediators that control immunological reactivity and play an important role in the development of major transplant-related complications (TRC). As aGVHD is a cytokine-mediated immune process, a preliminary study was performed in order to evaluate different cytokines levels involved in aGVHD pathophysiology as candidate biomarkers. First, levels of IL-2, IL-6, IL-7, IL-8, IL-10, IFN-γ and TNF-R1 were measured by multiplexed bead-based immunoassays (CBA) using flow cytometry, and were compared in samples of patients with or without aGVHD. For patients with grade II-IV aGVHD, samples taken on the day of aGVHD symptom onset and 15 days before onset were examined. Three cytokine levels, IL-6 ,TNF-R1 and IL-10 were found to be significantly higher at onset of aGVHD compared to patients without mild to severe aGVHD. These results are in agreement with previous studies showing an association between increased levels of these cytokines and transplant-related complications such as aGVHD (4, 16-18). Indeed, IL-6, along with TNF-α, which is indirectly evaluated by measuring TNF-R1 levels, drives the acute inflammatory response and is responsible for stimulating the liver to produce acute phase proteins (19, 20). However, we did not confirm in our cohort a significant elevation of IL-8 and IFN-γ with aGVHD occurrence as it was previously observed by some authors (7, 21, 22). Interestingly, a significant elevated IL-10 level was observed 15 days before aGVHD onset compared to samples from patients without GVHD, suggesting that IL-10 may be a predictive marker of aGVHD onset. Thereby, these results prompted us to monitor IL-10 concentration during the first month after HSCT. In a homogenous nonmyeloablative group of 65 patients, we measured whether concentration of IL-10 at day 14 and 25 allowed predicting onset of acute GVHD. When we compared IL-10 levels measured on day 14 and day 25 after HSCT between patients with and without grade II-IV aGVHD, a higher IL-10 level was observed on both time points and the difference was significant on day 14. In addition, the cumulative incidence of grade II-IV aGVHD was higher for patients with day 14 (p-value 0.07) and day 25 IL-10 (p-value 0.03) concentration superior to

median level. Moreover, after adjusting for potentially confounding factors, a high median IL-10 level on day 14-25 was the only predictive factor of subsequent aGVHD onset.

IL-10 is mainly produced by monocytes, macrophages and Th2 cells but also by natural Treg cells and Th1 cells. It is described as an immunosuppressive and immunomodulatory mediator acting first on monocytes and macrophages (review in (23). It inhibits the LPS-induced synthesis of pro-inflammatory mediators, such as IL-6, TNF-α, IL-12 (24-26) as well as antigen presentation of monocytes/macrophages by reducing expression of cell surface MHC II, co-stimulating and adhesion molecules (27, 28). IL-10 also acts on CD4+ T-cells by suppressing their proliferation and cytokines synthesis, including production of IL-2 (29) and even allows developing regulatory phenotype in vitro (30).

However, association between IL-10 levels and GVHD occurrence remains unclear. Indeed, although IL-10 has been described as a regulator and an inhibitor cytokine involved in aGVHD physiopathology (2), Blazar et al. shows that high-dose IL-10 administration in irradiated mice could enhance GVHD lethality (31). On the contrary, some authors described that a high production of IL-10 or the presence of genotype associated with high level IL-10 production are correlated with a lower risk of aGVHD (32-34). Others groups found a higher incidence of aGVHD in recipients with higher IL-10 levels (8, 35, 36). This discrepancy could be partially explained by the fact that production of many cytokines, among them IL-10, is under control of gene polymorphisms (13, 14). Moreover, it appears that dose and timing of cytokine production may be critical factors with regard to their roles in GVHD (13, 31, 37, 38). In the present study, we showed a correlation between IL-10 and CRP levels, suggesting a potential compensatory IL-10 production in response to inflammation induced by the transplant procedure.

In conclusion, in this preliminary study, we observed that an elevated level of IL-10 in the three first weeks after nonmyeloablative HSCT may be an early indicator of subsequent onset of aGVHD. IL-10 is a rather late cytokine being produced after the proinflammatory mediators with both immunoregulatory and immunostimulatory effects. However, the physiological significance of a high IL-10 level in the first month after HSCT in patients subsequently developing aGVHD as a protective fallout in response to inflammatory cytokine storm or a stimulating factor of inflammation remains to be established.

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Kinetics of IL-7 and IL-15 levels after allogeneic peripheral blood stem cell transplantation following nonmyeloablative conditioning

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Keywords : IL-7, IL-15, Hematopoietic cell transplantation, nonmyeloablative, GVHD, immunity.

Article under review in Plos One

Abstract

BACKGROUND: We analysed kinetics of IL-7 and IL-15 levels in 70 patients given peripheral blood stem cells after nonmyeloablative conditioning.

METHODS: EDTA-anticoagulated plasma and serum samples were obtained before conditioning and about once time per week after transplantation until day 100. Samples were aliquoted and stored at -80°C within 3 hours after collection until measurement of cytokines. IL-7 and IL-15 levels were measured by ELISAs.

RESULTS: Median IL-7 plasma levels remained below 6 pg/L throughout the first 100 days, although IL-7 plasma levels were significantly higher on days 7 (5.1 pg/mL, P=0.002), 14 (5.2 pg/mL, P<0.001) and 28 (5.1 pg/mL, P=0.03) (but not thereafter) than before transplantation (median value of 3.8 pg/mL). Median IL-15 serum levels were significantly higher on days 7 (12.5 pg/mL, P<0.001), 14 (10.5 pg/mL, P<0.001) and 28 (6.2 pg/mL, P<0.001) than before transplantation (median value of 2.4 pg/mL). Importantly, IL-7 and IL-15 levels on days 7 or 14 after transplantation did not predict grade II-IV acute GVHD.

CONCLUSIONS: These data could provide a rational for clinical pilot studies assessing administration of IL-7 or IL-15 in high-risk nonmyeloablative recipients with the aim of promoting immune recovery and perhaps graft-versus-tumor effects.

1. INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) following a high dose conditioning regimen has been the best treatment option for many young patients with hematological disorders. The antitumor activity of this approach is based not only on high dose chemo-radiotherapy given in the conditioning regimen but also on immune-mediated graft-versus-tumor effects [1,2]. These observations are the basis of the development of allo-HSCT following nonmyeloablative conditioning, in which eradication of malignant cells depends on graft-versus-tumor effects [3–6].

T-cell recovery after allo-HSCT following high-dose conditioning depends on both homeostatic peripheral expansion (HPE) of donor T cells contained in the graft, and T cell neo-production from donor hematopoietic stem cells (thymo-dependent pathway) [7–15]. In young patients given myeloablative allo-HSCT, most circulating T cells during the first months following HSCT are the progeny of T cells infused with the grafts [16], while neogeneration of T cells by the thymus plays an increasing role in reconstituting the T cell pool beyond day 100 after allo-HSCT [17–22]. Since HPE allow the expansion of both NK cells and non-tolerant T cells, it is generally accepted that HPE is one of the driving force of graft-versus-tumor effects.

Several studies have demonstrated that IL-7 and IL-15 are the main driving forces of HPE after allo-HSCT following high-dose conditioning [7,23]. IL-7 is a γ-common chain cytokine that is secreted by stromal cells from multiple organs including thymus, bone marrow, and lymphoid organs. IL-7 is required for human T cell development since mutations in the IL-7 receptor alpha can lead to severe combined immunodeficiency [24]. Administration of IL-7 has been shown to dramatically increase peripheral T cell numbers, primarily through augmentation of HPE [25–31]. IL-15 is another γ-common chain cytokine secreted by antigen-presenting cells, bone marrow stroma, thymic epithelium, and epithelial cells in the kidney, skin, and intestines [32]. IL-15 plays an important role in the development and function of NK cells, and of NK/T cells, and is required for optimal proliferation of CD8+ T cells and for homeostatic proliferation of CD8+ memory T cells [33–39].

While high-dose conditioning regimens typically induce a profound lymphodepletion, progressive replacement of host-derived T cells by donor-derived T cells is the rule after nonmyeloablative conditioning [40,41]. This prompted us analyzed the kinetics of IL-7 and IL-15

blood levels after allo-HSCT following a nonmyeloablative conditioning with the aim of determining whether there is a rational for boosting HPE and perhaps graft-versus-tumor effects in patients with high risk disease given grafts after nonmyeloablative conditioning by administering IL-7 and/or IL-15.

2. PATIENTS AND METHODS

2.1. Patients and donors

Data from 70 patients transplanted between March 2007 and April 2011 at the University of Liège were included in the study (Table 1).

Table 1: Patients' characteristics

	Nonmyeloablative conditioning (n=70)
Median age (range)	50 (16-73)
Gender (male/female)	48 / 22
Diagnostic (# of patients)	
Acute myeloid leukemia in CR	21
Acute lymphoblastic leukemia in CR	4
Chronic myeloid leukemia	1
Chronic lymphocytic leukemia	6
Lymphoma	16
Myelodysplatic syndrome / myeloproliferative disorder	9
Multiple myeloma	13
Donor (# of patients)	
Sibling	13
Unrelated	57
Conditioning regimen (# of patients)	
TBI 2 Gy	1
Fludarabine 90mg/m² + TBI 2 Gy	59
Fludarabine 90 mg/m² + TBI 4 Gy	10
Immunosuppressive regimen (# of patients)	
Tacrolimus + MMF	70
Co-transplantation with MSC	
Yes	23
No	44
Unknown*	3
Graft composition; median (range) x 10^6/kg	
CD34	5.4 (1.1-14.5)
CD3	314 (92-1216)

* double blind randomized study: The information of which of these 3 patients (if any) have been given MSC has been given by the director of the Cell Laboratory only to LS (the statistician); TBI, total body irradiation; MMF, mycophenolate mofetil.

All patients were given G-CSF-mobilized peripheral blood stem cells (PBSC) after low-dose [2 Gy (n=60), or 4 Gy (n=10)] total body irradiation (TBI)-based nonmyeloablative regimen. Twenty-three nonmyeloablative recipients who were given PBSC from HLA-mismatched unrelated

donors were co-transplanted with third party mesenchymal stromal cells (MSCs) as a potential way to prevent severe GVHD [42]. Further, 3 nonmyeloablative recipients were included in a double blind randomized study assessing the impact of MSC co-transplantation on transplantation outcomes.

2.2. Ethics

Written informed consent was obtained from each patient to undergo allo-HSCT and to collect, store and analyze blood samples for research purposes. The Ethics Committee of the University of Liège ("Comité d'Ethique Hospitalo-Facultaire Universitaire de Liège") approved the consent form as well as the current research study protocol (protocol #B707201112193).

2.3. Clinical management

The clinical management has been performed as previously reported [43,44]. Chimerism levels among peripheral T-cells were generally measured with PCR-based analysis of polymorphic microsatellite regions (AmpFlSTR® Identifiler®, Applied Biosystems, Lennik, Belgium) [43]. CD3 (T-cell) selection was carried out with the RosetteSepR human T-cell enrichment kit (StemCell Technologies, Vancouver, Canada)[43,44].

2.4. Cytokines levels

EDTA-anticoagulated plasma and serum samples were obtained before conditioning and about once time per week after transplantation until day 100. Samples were aliquoted and stored at -80°C within 3 hours after collection until measurement of cytokines. Kinetic courses of IL-7 production in plasma samples were evaluated before conditioning and approximately at days 7, 14, 28, 40, 60, 80 and 100 after allo-HSCT. IL-15 serum sample levels were assessed before conditioning and approximately at days 7, 14 and 28 after allo-HSCT. IL-7 and IL-15 levels were measured by ELISAs following the manufacturer's protocol (High sensitivity IL-7 and IL-15 quantikine, R&D Systems, Minneapolis, MN, USA). No sample dilution was performed for IL-15 assay. For IL-7 analysis, samples were diluted twice. Patient samples whose cytokine level were out of standard curve range, were re-assessed after dilution.

2.5. Immune recovery

Immune recovery was prospectively assessed as previously described [43,44]. Briefly, patients' peripheral white blood cells were phenotyped using 4 color flow cytometry after treatment with a red blood cell lyzing solution. The percentage of positive cells was calculated relative to total nucleated cells, after subtraction of non-specific staining. Absolute counts were obtained by multiplying the percentages of positive cells by the white blood cell counts (XE-5000 hematology analyzer, Sysmex, Kobe, Japan). Absolute lymphocytes counts (ALC) were measured directly by the XE-5000 analyzer or after microscopic review of the blood smears when the automated differential was flagged. Absolute white blood cell counts were used instead of ALC when white blood cell counts were below 150 cells $x10^9$/L.

2.6. Statistical analyses

The Mann Whitney test was used to compare counts of lymphocyte subset and cytokine levels in patients given grafts after 2 Gy or 4 Gy TBI. The Wilcoxon matched pair test was used to compare cytokines levels before and at various time points after transplantation. Generalized linear mixed models were used to analyze factors affecting immune recovery and cytokine levels after transplantation. Factors included in the models included : (1) dose of TBI (2 Gy vs 4 Gy), MSC infusion or not, number of days after allo-HSCT, number of CD3+ cells transplanted, donor type (related vs unrelated), patient age, and donor age for analyses examining lymphocyte counts; (2) dose of TBI (2 Gy vs 4 Gy), MSC infusion or not, grade II-IV acute GVHD the first 100 days after transplantation, number of CD3+ cells transplanted, donor type (related vs unrelated), patient age, and donor age, and either IL-7 or IL-15 levels on days 7-14 (median) for analyses examining lymphocyte count increments from days 14-28 (median) to days 80-100 (median); and (3) number of days after allo-HSCT, number of CD3+ cells transplanted, donor type (related vs unrelated), dose of TBI (2 Gy vs 4 Gy), ALC, CRP levels and MSC infusion or not, for analyses of cytokine levels. Incidences of acute GVHD according to the cytokines levels were assessed using cumulative incidence methods. A Cox model was constructed for determining potential factors associated with the occurrence of grade II-IV acute GVHD the first 200 days after transplantation. Factors included in the model included median day 7 and day 14 IL-7 levels, median day 7 and day 14 IL-15 levels, dose of TBI (2 Gy vs 4 Gy), donor type (related vs unrelated), female donor to male recipient versus other gender combination, MSC infusion or

not, patient age, and donor age. Spearman's correlation was used to examine the relationship between parameters. Statistical analyses were carried out with Graphpad Prism (Graphpad Software, San Diego, CA) and SAS version 9.2 for Windows (SAS Institute, Cary, NC, USA).

3. RESULTS

3.1. Immune recovery

While median CD8+ T cell levels reached the lower limit of normal values from day 60 after transplantation, median CD4+ T cell (including naïve CD4+ T cells) remained below the lower limit of normal values the first 6 months after transplantation (Figure 1).

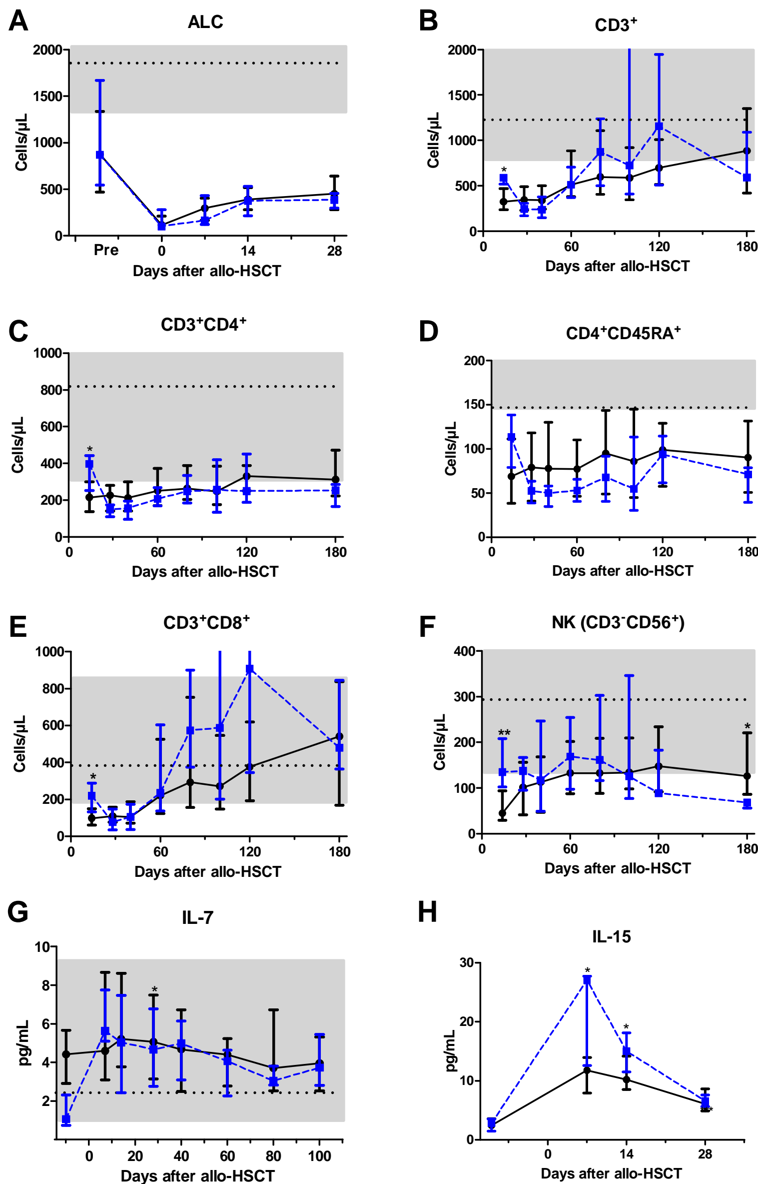

Figure 1 Median ALC (A), median MNC-subset cell counts (B-F), and median IL-7 (G) and IL-15 (H) after allogeneic hematopoietic cell transplantation following 2 Gy (continuous line) or 4 Gy (broken line) total body irradiation. The error bars shows the 25th and 75th percentiles. For ALC and MNC-subset, horizontal lines show the medians and the grey square the limit of normal value (if non truncated) in 47 healthy volunteer donors; for IL-7, horizontal line shows the medians and the grey square the limit of normal value according to the manufacturer brochure. *, P<0.05; **, P<0.01 ; ***, P<0.001.

No significant difference of T cell subset counts were observed between 2Gy and 4Gy TBI regimen. Using generalized linear mixed models taking into consideration data from day 14, 28, 40, 60, 80 and 100 for each patient, counts of CD3+ T cells (P<0.001), CD8+ T cells (P<0.001),

CD4+ T cells (P=0.024), NK cells (P<0.001) and NK/T cells (P<0.001) increased over time but not those of naïve CD4+ T cells (P=0.13). Further, high numbers of transplanted CD3+ T cells were associated with higher counts CD3+ T cells (P=0.009), CD8+ T cells (P=0.003), and CD4+ T cells (P=0.0099), while high donor age was associated with lower counts of CD3+ T cells (P=0.04), CD4+ T cells (P=0.05), and naïve CD4+ T cells (P=0.021). There was no significant association between MSC administration and lymphocyte subset counts after transplantation.

3.2. IL-7 plasma levels

Median IL-7 plasma levels remained below 6 pg/L throughout the first 100 days (the upper limit of normal range being 9.2 pg/mL (Quantikine© HS catalog number HS750)), although IL-7 plasma levels were significantly higher on days 7 (5.1 pg/mL, P=0.002), 14 (5.2 pg/mL, P<0.0001) and 28 (5.1 pg/mL, P=0.03) (but not thereafter) than before transplantation (median value of 3.8 pg/mL) (Figure 1G). Using generalized linear mixed models, low number of transplanted CD3+ T cells (P=0.003), low ALC level the day of IL-7 assessment (P<0.0001; Figure 2), and high level of CRP the day of IL-7 assessment (P=0.016) were associated with high levels of IL-7 (Table 2).

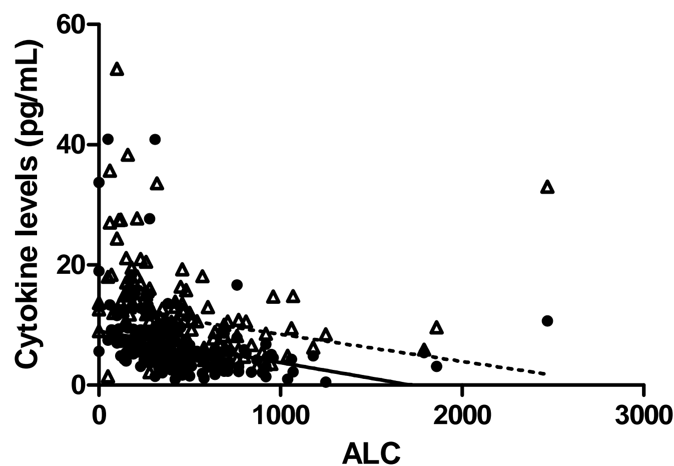

Figure 2. **Correlation between ALC and IL-7 and IL-15 levels**. Data from patients on days 7, 14 and 28 after allo-HSCT were taken together. This correlation was stronger for IL-7 (black circles and continuous line ; r=-0.54, P<0.0001) than for IL-15 (open triangles and broken lines; r=-0.46, P<0.0001).

3.3. IL-15 serum levels

Median IL-15 serum levels were significantly higher on days 7 (12.5 pg/mL, P<0.001), 14 (10.5 pg/mL, P<0.001) and 28 (6.2 pg/mL, P<0.001) than before transplantation (median value of 2.4 pg/mL) (Figure 1H). IL-15 levels on day 7 and 14 were significantly higher in 4 Gy than 2Gy TBI.

Using generalized linear mixed models, conditioning with 4 versus 2 Gy TBI (P=0.0002), having received PBSC from unrelated donors (P=0.0001), low ALC level the day of IL-15 assessment (P<0.0001; Figure 2), and high level of CRP the day of IL-15 assessment (P=0.02) were each associated with high IL-15 levels on days 7 and 14 after allo-HSCT (Table 2).

<u>Table 2</u>: Multivariable analyses of factors affecting cytokines levels on days 7 and 14 after allo-HSCT.

Cell subset	Factor(s) associated with higher levels*, †
IL-7	- Low ALC on day 7 or 14 (P<0.0001). - Low # of transplanted T cells ($CD3^+$) (P=0.003). - High CRP levels on day 7 or 14 (P=0.016).
IL-15	- 4 vs 2 Gy TBI (P=0.0002). - Unrelated donors (P=0.0001). - High CRP levels on day 7 or 14 (P=0.02). - Low ALC on day 7 or 14 (P<0.0001).

*Other factors assessed were number of days after allo-HSCT, and mesenchymal stromal cells infusion or not; † P values were determined according to generalized linear mixed models; TBI, total body irradiation.

3.4. Correlation between IL-7 and IL-15 levels and lymphocyte subset counts on days 14 or 28 after allo-HSCT

Day 14 IL-7 levels inversely correlated with day 14 counts of CD3+ T cells (R=-0.46, P=0.002), CD8+ T cells (R=-0.41, P=0.006), CD4+ T cells (R=-0.44, P=0.004), and memory CD4+ T cells (R=-0.45, P=0.003), but not with counts of naïve CD4+ T cells (R=-0.28, P=0.07), NK/T cells (R=-0.04, P=0.8) nor NK cells (R=-0.14, P=0.4). There was a weak association between day 14 IL-7 and IL-15 levels (R=0.27, P=0.049). Further, day 14 IL-15 levels correlated with day 14 counts of NK cells (R=-0.32, P=0.039) and of NK/T cells (R=-0.32, P=0.037), but not with those of other T cell subsets.

Day 28 IL-7 levels inversely correlated with day 28 counts of CD3+ T cells (R=-0.47, P<0.001), CD8+ T cells (R=-0.41, P=0.002), CD4+ T cells (R=-0.39, P=0.002), naïve CD4+ T cells (R=-0.40, P=0.002), and memory CD4+ T cells (R=-0.38, P=0.004), but not with counts of NK/T cells (R=-0.17, P=0.2) nor NK cells (R=-0.02, P=0.9). There was no significant association either between day 28 IL-7 and IL-15 levels (R=0.07, P=0.6). Further, day 28 IL-15 levels correlated with day 28 counts of NK cells (R=-0.32, P=0.015) but not with those of T cell subsets.

To further assess the potential association between early IL-7 or IL-15 levels on immune recovery, we analysed whether there was a relationship between median cytokine levels on days 7 and 14 and the difference of lymphocyte subset counts between days 80-100 (median) and days 14-28 (median). Interestingly, in multivariate analyses, early IL-7 levels did not correlate with any lymphocyte subset increment from days 14-28 to day 80-100 after transplantation, while high IL-15 levels early after transplantation correlated with a lower increment of NK cells over time (P=0.04).

3.5. IL-7 and IL-15 levels did not predict for subsequent acute GVHD

As shown in the Figure 3, no statistically significant associations between cytokines levels on days 7 or 14 after transplantation and occurrence of grade II-IV acute GVHD were observed.

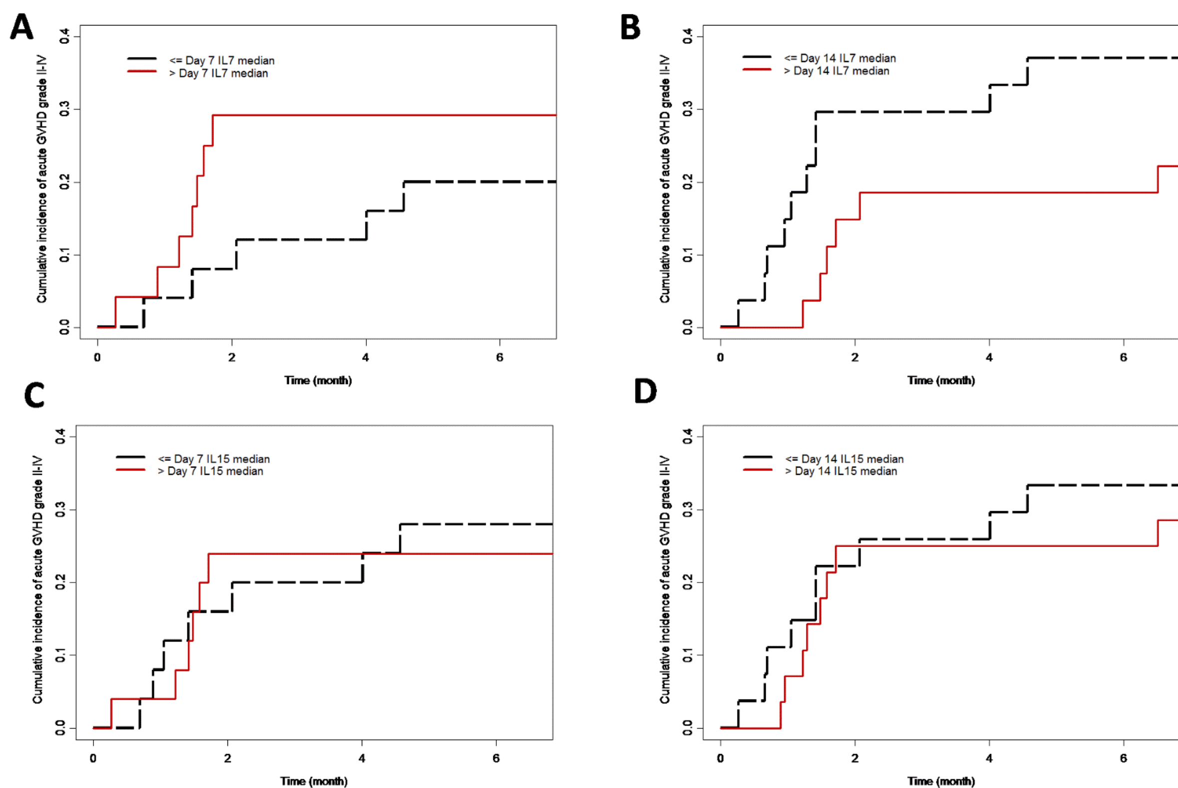

Figure 3 A) Cumulative incidence of grade II-IV acute GVHD according to day 7 IL-7 plasma levels among nonmyeloablative recipients (P=0.4). B) Cumulative incidence of grade II-IV acute GVHD according to day 14 IL-7 plasma levels among nonmyeloablative recipients (P=0.18). C) Cumulative incidence of grade II-IV acute GVHD according to day 7 IL-15 serum levels among nonmyeloablative recipients (P=0.8). D) Cumulative incidence of grade II-IV acute GVHD according to day 14 IL-15 serum levels among nonmyeloablative recipients (P=0.6).

Specifically, the 180-day cumulative incidence of grade II-IV acute GVHD was 29% in patients with day 7 IL-7 levels > median (5.1 pg/mL) versus 20% in patients with day 7 IL-7 levels ≤ median (P=0.38) (Figure 3A). Similarly, the 180-day cumulative incidence of grade II-IV acute GVHD was 19% in patients with day 14 IL-7 levels > median (5.2 pg/mL) versus 37% in patients with day 14 IL-7 levels ≤ median (P=0.18) (Figure 3B).

The 180-day cumulative incidence of grade II-IV acute GVHD was 24% in patients with day 7 IL-15 levels > median (12.5 pg/mL) versus 28% in patients with day 7 IL-15 levels ≤ median (P=0.8) (Figure 3C). Similarly, the 180-day cumulative incidence of grade II-IV acute GVHD was 25% in patients with day 14 IL-15 levels > median (10.5 pg/mL) versus 33% in patients with day 14 IL-15 levels ≤ median (P=0.8) (Figure 3D).

Finally, in a multivariate Cox model, neither median IL-7 levels (P=0.17 with a trend for an inverse correlation) on days 7-14 nor median IL-15 levels (P=0.21 with a trend for a positive correlation) on days 7-14 correlated with occurrence of grade II-IV acute GVHD the first 200 days after transplantation.

4. DISCUSSION

Following allo-HSCT, eradication of residual tumor cells depends in part (in case of high-dose conditioning) or mainly (in case of nonmyeloablative conditioning) on immune-mediated graft-versus-tumor effects [1,2,4]. Prior studies have demonstrated a close relationship between T cell reconstitution and graft-versus-tumor effects after allo-HSCT [4,14,45–47]. Given that HPE allows the expansion of potentially alloreactive T cell clones, it has been generally accepted that HPE plays a major role in graft-versus-tumor effects, but could also cause or favor acute GVHD. This prompted us to investigate the kinetics of IL-7 and IL-15 levels in a cohort of 70 patients given grafts after truly nonmyeloablative conditioning.

First, patients given grafts after nonmyeloablative conditioning had only a modest (<2 fold) increase of IL-7 levels after transplantation, that persisted up to day 21. This is probably due to the fact that nonmyeloablative patients experienced relatively mild lymphopenia (and thus continue to consume the IL-7 produced by stromal cells). Indeed, as observed by other groups of investigators [48–50], there was a strong inverse correlation between IL-7 levels and absolute lymphocyte counts [49,50], as well as a strong inverse correlation between IL-7 levels and T cell subsets on days 14 and 28 after transplantation. Other factors associated with IL-7 levels included high CRP levels, and low numbers of transplanted T cells. Levels of IL-7 in current nonmyeloablative recipients where lower to what was observed by Thiant et al. in a cohort of 45 patients given grafts after fludarabine + 2 Gy TBI (n=18) or more intense but still reduced-intensity conditioning (n=27) [50], and where much lower than what was observed by Dean et al. in patients given grafts after sequential chemotherapy followed by a chemotherapy/fludarabine-based reduced-intensity conditioning [51]. This apparent discrepancy is probably explained the fact than median ALC counts on day 0 were 110 (range, 10-5440) cells/µl in current patient versus 0 (range, 0-322) cells/µL in the Dean et al. study, while median counts of CD3+ T cells were 0 (range, 0-1900) cells/µL at the time of transplantation in Thiant et al. study.

Il-15 levels were lower in nonmyeloablative patients conditioned with 2 Gy TBI than in those conditioned with 4 Gy TBI. As observed by Thiant et al. [49,50], there was a correlation between IL-7 and IL-15 levels on day 14 (but not on day 28) after transplantation, and an

inverse correlation between IL-15 levels and NK cell counts. Other factors affecting IL-15 levels included high CRP levels.

Several observations demonstrate that immune recovery depended mainly on HPE the first year after nonmyeloablative conditioning regimen in current patients. Firstly, there was a strong correlation between the number of infused T cells and high counts of CD4 and CD8+ T cells, as previously observed [43,52]. Secondly, thymic function was minimal during the first 100 days after allo-HSCT given that levels of naïve CD4+ T cells did not significantly increase the first 100 days after transplantation despite that some naïve T cells can undergo HPE and keep their naïve phenotype. Third, there was a correlation between high donor age and low counts of CD3+ T cells (P=0.04), CD4+ T cells (P=0.05), and naïve CD4+ T cells (P=0.021), as previously observed in patients given grafts after nonmyeloablative conditioning[53]. Despite that, we failed to find any significant association between IL-7 and/or IL-15 levels early after transplantation and increment of T cell subset counts from days 14-28 to day 80-100, even after adjusting for potentially confounding cofactors.

A number of previous studies have demonstrated that high levels of IL-7 [49–51] and/or IL-15 [49,50] early after transplantation correlated with subsequent occurrence of grade II-IV acute GVHD, while others study failed to find such an association [48,54]. The largest study including data from 153 consecutive allogeneic transplant recipients given grafts after high-dose conditioning and ATG observed no correlation between IL-7 levels early after transplantation and acute GVHD, while, interestingly, there was an inverse correlation between IL-15 levels early after transplantation and grade II-IV acute GVHD [55]. In the current study, we did not observe any association between levels of IL-7 or IL-15 early after allo-HSCT and grade II-IV acute GVHD. The same was true after adjusting the analyses for potentially confounding cofactors.

In summary, our data evidenced immune recovery by HPE following nonmyeloablative transplantation despite that IL-7 and IL-15 levels remained relatively low after transplantation. These data could provide a rational for clinical pilot studies assessing administration of IL-7 or IL-15 in high-risk nonmyeloablative recipients with the aim of promoting immune recovery and perhaps graft-versus-tumor effects.

Acknowledgements

Grants: MdB and MH are Télévie Research Fellow, and MPM and FB are Senior Research Associate at the National Fund for Scientific Research (FNRS) Belgium. The study was in part supported by funds from the FNRS, the Belgian Foundation against Cancer (FBC), the anti-cancer and the Leon Fredericq foundations from the ULg, the CHU of Liège, and by the Terry Fox foundation.

The authors are grateful to O. Dengis for excellent technical support.

Disclosure

The authors do not have potential conflict of interest.

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DISCUSSION AND PERSPECTIVES

Numerous advances have been made in the field of medicine the last century, particularly since the coming age of whole-genome sequencing. Genomics provides new tools to improve understanding of disease mechanisms and implication of genetic background in the development of pathologies. However, cellular genome is a static entity that could not solely explain the complexity of a pathology process. Therefore, emergence of proteomics offers complementary information to genomics through the knowledge of protein biological functions directly involved in pathophysiology, yielding new insight into disease and therapy. The ultimate goal of proteomics is to identify and quantify proteins and their variants in any cell type under condition of interest. Indeed, proteins of an entity may be present in different forms, in different amounts and at different times, affected by a large amount of factors.
Development of two-dimensional gel-based method and mass spectrometry instrumentation as well as integration of biostatistics and bioinformatics tools such as gene and protein databases have considerably contributed to the understanding of biological networks by increasing the quantity of new acquired information. Therefore, application of these technologies for the discovery of new biomarkers for diagnosis, stratification of patients for specific treatment, or therapy monitoring become very attractive with the further expectation of developing a personalized medicine (Hanash, 2003). However, although proteomic technologies allow a rapid screening of proteins in complex samples, identification of new diagnostic biomarkers or new protein targets for drugs in the clinical routine is a complex framework (Rifai et al., 2006). The biomarker discovery process consists of a succession of crucial steps: study design, sample handling and storage, sample preparation, quantitative and/or qualitative protein evaluation, data analysis and comparative statistical analysis.
In the present work, we used up-to-date proteomic approaches to find new biomarkers for the diagnosis and early detection of acute GVHD (aGVHD), a common complication of the allogeneic HSCT. Challenges and limitations of our work are discussed in this section.

1. The clinical question to answer

Emergence of HSCT as an immunotherapy provides a new option to cure and improve survival of patients affected by haematological malignancies and congenital immunodeficiency syndrome. However, in the first HSCT experimental models, acute GVHD (aGVHD) rapidly become a major issue that significantly increases non-relapse mortality and limits application of this promising therapy. Later, improvements made in patient selection and novel approaches of

conditioning regimen and intensive prophylaxis immunosuppressive drugs make it possible to reduce aGVHD-related mortality without compromising the efficacy of the treatment. Despite this progress, moderate to severe aGVHD occurs in approximately 40% of all recipients of allogeneic HSCT making it a major cause of non relapse transplant morbidity and mortality. Current diagnosis relies on the presence and observation of clinical manifestations confirmed by histological examination of organ biopsies. This procedure is time-consuming, poorly specific, and discommoding for the patients. In addition, no diagnostic tools are available for the identification of patients at higher risk of developing aGVHD or patients which are unresponsive to the first line therapy. Thus, there is a clinical need to determine early, rapid and specific aGVHD plasma biomarkers to predict future occurrence of GVHD before clinical signs and to monitor progression of the disease during treatment.

Until now, no plasma biomarkers are available in clinical biology laboratory for aGVHD diagnosis despite of the large amount of studies. Indeed, such an investigation presents several challenges:

1) aGVHD is not an isolated disorder as it occurs in the context of an immunotherapy which involves a large amount of variable factors. Indeed, from a patient to another, differences in underlying disease to cure, conditioning regimen, source and compatibility of donor cells, immunosuppressive drugs, age or genetic background impacting on cytokine production make the population to analyse very heterogeneous. In addition, advances in HSCT setting to reduce toxicity-related-conditioning by applying RIC or nonmyeloablative conditioning introduce a later form of aGVHD occurring after 100 days post-HSCT. Although late-onset aGVHD present similar features than classical aGVHD, the initiation phase of this aGVHD may be independent of tissue damaged induced by conditioning regimen, as classically described (Ferrara et al., 2006; Ferrara et al., 2009). These disparities make challenging the search of specific and early biomarkers common for each case of aGVHD.

2) aGVHD is a complex pathology which is originated from a classical immunologic reaction in response to the recognition of “non self” antigens. It can be considered in a framework of three distinct sequential phases of immune system interactions involving

both cellular effectors (T-cells, macrophages, NK cells) and soluble factors (e.g. cytokines, chemokines), proteins secreted in very low abundance (Ferrara et al. 2009). However, besides being a systemic immunologic complication, aGVHD affects specific organ systems; skin, gastrointestinal tract, and liver. According to the severity of the pathology, clinical features are variable involving one, two or the three target organs, responsible for the heterogeneity in the aGVHD population. Finally, occurrence of aGVHD is a time-dependent factor after HSCT, which is influenced by interindividual variability as well as settings of the HSCT procedure (e.g myeloablative versus nonmyeloablative regimen).

3) Besides onset of aGVHD, numerous other side-effects arise in recipients after HSCT such as bacterial, fungal and viral infections, coagulation disorders, liver or renal dysfunctions which result from pancytopenia, immunodeficiency and toxicities of the conditioning regimen and immunosuppressive strategies. Presence of these confounding disorders after HSCT, that could share similar features with aGVHD, complicates the discovery of highly specific predictive and diagnostic biomarkers of aGVHD.

2. Choice of the biological fluid

Besides the definition of the clinical question and selection of adequate cases and controls to examine, an important step of biomarker study design is the determination of the most adequate type of samples to analyse. Proteomic technologies have the advantage to allow the analysis of a large variety of sample types, biological fluids or tissues. In the framework of the discovery of new diagnostics biomarkers, sample has to provide a maximum of information relating to pathology, to be easily accessible without discomfort for patient, sufficiently stable and easily transposable in clinical routine. As aGVHD is a systemic disease associated with organ tissue damages, the dynamic nature of the circulatory system and the ease of sampling make blood a logical choice. Indeed, serum and plasma as circulating fluids are the most informative specimen representing the state of an entire organism making it one of the most complex components of the human proteome (Thadikkaran et al., 2005). Therefore, some practical considerations have to be taken in to account.

Firstly, it is important to limit preanalytical variables related to sampling, which can have an impact on the determination of analytes and can affect result outcomes and reproducibility (Rai

et al., 2006). Thus, in this study, we take care to develop and follow standard operating procedures (SOPs) during sampling (time between collection and frozen, centrifugation settings, type of collection tube) as well as along the entire experimental workflow to assure reproducibility (De Bock et al., 2010).

Secondly, the major issue of plasma proteome analysis is the dynamic range of protein concentrations which is extended over 11 orders of magnitude. Despite the improvement of the detection limit of novel proteomic methods that can reach the low femtomole or attomole range, the sensitivity of proteomic methods remains limited by the nature of the sample (Addona et al., 2009). Indeed, the detection of disease-related biomarkers present at low concentrations (e.g. cytokines) is hampered by the "masking" signal effect caused by a number of highly abundant proteins (e.g. albumin and IgG). To deal with this problem, prefractionation of samples before proteomic analysis is an essential step of the proteomic experimental workflow in order to decomplexify serum protein content. Sample preparation methods have to be reproducible, high-throughput, cost-effective and preserve quantitative information.

In this work, we tested three different approaches of sample pretreatment based on complementary properties; precipitation by an organic solvent, combination of metal affinity with restricted access materials and combinatorial peptide libraries based on the compression of protein concentration range (ProteoMiner®) (De Bock et al., 2010). These methods were tested on serum and plasma using 1D-SDS-PAGE and SELDI-TOF-MS allowing rapid evaluation of protein information as well as efficiency to deplete high abundance proteins. We evaluated them in terms of reproducibility, ability to increase the number of detectable information, easiness to use and high-throughput in order to determine the best one to apply in our biomarker discovery process. All approaches were found reproducible although ProteoMiner technology provided more information, particularly when applied in plasma samples, with a good efficiency for depletion of high abundance proteins (De Bock et al., 2010).

3. Proteomic strategies for plasma protein analysis

Discovery of disease-related biomarkers using proteomic technologies consists of the differential protein expression analysis comparing diseased and adequate control samples, avoiding "contamination" by other diseases or confounding conditions.

Since emergence of proteomics, great technical efforts have been made in terms of accuracy, sensitivity and reproducibility. Indeed, the fraction of the proteome identified in discovery

proteomic studies has increased over time but the analysis of the complete proteome remains challenging, expensive and slow. So, there is currently no single method capable of routinely analysing all the components of a proteome (Patterson et al., 2003).

Moreover, the low throughput capacity of current proteomic strategies (2D gel-based and 2D-LC-MS/MS-based shotgun methods) is a major problem, limiting the analysis of few samples per day. Particularly, proteomic analysis using shotgun MS approaches involves multi-dimensional separation steps which is very time consuming. To deal with this issue, samples can be pooled but this solution decreases the power of statistical analysis, especially with the high-dimensional proteomic data acquired. Moreover, proteomic analysis using pooled samples do not allow the removal of inadequate samples from the analysis in the case of misclassification of patients into different groups. As far as we know, SELDI-TOF-MS is the only proteomic technology allowing sensitive analysis of complex native protein samples with high-throughput capacity. However, its inability to readily identify analysed peptide/proteins often limits its application to pattern profiling. Candidate biomarkers discovered by SELDI-TOF-MS can be further identified after purification but this process is labour-intensive and time-consuming. Thus, combination of complementary platforms should be considered as a solution to increase plasma proteome coverage and deal with the limitations of different proteomic technologies. Therefore, to overcome limitations of proteomic technologies and improve the proteome coverage of plasma samples in our study, comparison of protein profiles was performed using a combination of three complementary proteomic approaches: 2D-DIGE, SELDI-TOF-MS and 2D-LC-MS^{E}.

SELDI-TOF-MS and 2D-DIGE approaches allow detection of intact proteins as well as specific cleavage products or PTMs in a mass range of 1.5 - 30 kDa and 15 - 200 kDa, respectively. Additionally, LC-MS^{E} technology can simultaneously quantify and identify a large amount of proteins. However, this technique requires protein digestion before analysis that leads to the loss of information related to specific cleavage products. Compared to 2D-DIGE and LC-MS^{E} approaches, the high throughput ability of the SELDI-TOF-MS system allows hundreds of samples to be screened for discovery of disease biomarkers in a relatively short time period, increasing the power of statistical analysis.

Prior to sample analysis, methods of sample preparation were applied to decomplexify plasma samples and facilitate access of low abundance proteins. One approach based on

immunodepletion of the 14 most abundant plasma proteins prior the LC-MS^E analysis was used while combinatorial hexapeptide ligand library (ProteoMiner®) was applied before 2D-DIGE and SELDI-TOF-MS analysis.

4. Discovery of new aGVHD biomarkers

Proteomic analysis is based on the assumption that the development of pathological states leads to changes in protein expression which should be detected. Therefore, to detect proteins differentially expressed with the occurrence of aGVHD, we compared plasma protein profile from patients undergoing HSCT developing aGVHD or not (control). Moreover, samples taken on day of aGVHD diagnosis and 15 days before aGVHD onset were analysed to evaluate intra-individual progression of the pathology as well as to determine whether early markers can be detected before symptom appearance. Being aware of the numerous variable factors of the studied population, control and aGVHD patients were matched for intensity of conditioning, age, gender and day of sampling to minimize bias. Moreover, SELDI-TOF-MS and 2D-LC-MS^E analysis were performed on a homogeneous group of patients undergoing HSCT following nonmyeloablative conditioning in order to decrease variability due to conditioning regimen.

As resumed in Table 1, combination of the three approaches provided us a panel of proteins differentially expressed between patients developing aGVHD or not, comprising proteins primarily involved in inflammation and coagulation process as well as in lipid metabolism. Interestingly, each proteomic approach provided complementary information while some differentially protein levels such as plasminogen or fibrinogen chain were found redundant between analyses, demonstrating their reliability. Moreover, the differential expression of our proposed biomarkers were further confirmed in a larger cohort of samples (n=56) using targeted methods (ELISA, Western Blot and LC-MS/MS). In addition, being aware of the limit of detection and quantification of proteomic technologies for the analysis of complex samples, we assessed cytokine levels (in the pg/mL range of protein concentration) in patients with or without aGVHD using immunoassays to complement our biomarker panel. We found that IL-10 levels, a regulatory cytokine related to inflammation, at day 14 and 25 after HSCT following nonmyeloablative regimen were an early indicator of subsequent onset of grade II-IV aGVHD. On the contrary, monitoring of IL-7 and IL-15 levels, cytokines involved in the immune reconstitution process after HSCT, were not able to predict subsequent occurrence of grade II-IV aGVHD.

Table 1: Summary of approaches used to discover biomarkers

Methods		Biomarkers	Abbreviations
NON TARGETED	2D-DIGE	Coagulation factor XIII B chain	F13B
		Fibrinogen beta chain	FGB
		Plasminogen	PLG
		Serum amyloid A4	SAA4
	2D-LC-MS^E	C-reactive protein	CRP
		Fibrinogen beta chain	FGB
		Histidine rich glycoprotein	HRG
		Plasminogen	PLG
	SELDI-TOF-MS	Apolipoprotein A1	APOA1
		Hepcidin	HAMP
		Serum amyloid 1	SAA1
TARGETED	ELISA	Prothrombin fragments 1 and 2	F 1+2
	CBA/ ELISA	Interleukin-10	IL-10

As presented in Figure 1, the proteins that we discovered as aGVHD biomarkers are mainly implicated in two interrelated biological processes: inflammation and coagulation processes (Esmon, 2005). Moreover, changes of Apo A1 and SAA4 levels also suggest an alteration of lipid metabolism regarding aGVHD onset. It can be observed that the levels of positive acute phase reactants of inflammation are elevated with the occurrence of aGVHD while coagulation factor levels are decreased. The alteration of these protein levels are in agreement with previous papers describing the complex pathophysiology of GVHD (Antin et al., 1992; Ferrara et al., 2009; Socie et al., 2009). Indeed, the complex pathophysiology of GVHD suggests that plasma proteins involved in multiple processes such as T-cell alloreactivity, inflammation, tissue damage and repair might be altered with the disease. Moreover, many reports described thrombotic and hemostatic disorders after HSCT (Matsumoto et al. 2004; Pihusch 2004; Petrolla et al. 2010; Pinomaki et al. 2010; Han et al. 2011).

Inflammation and coagulation factors have host-protective functions in case of endothelial damages. Indeed, inflammation and coagulation are processes mediated by the appearance of intercellular adhesion molecules on endothelia, and various inflammatory mediators released by tissue cells and leucocytes in response to tissue aggression.

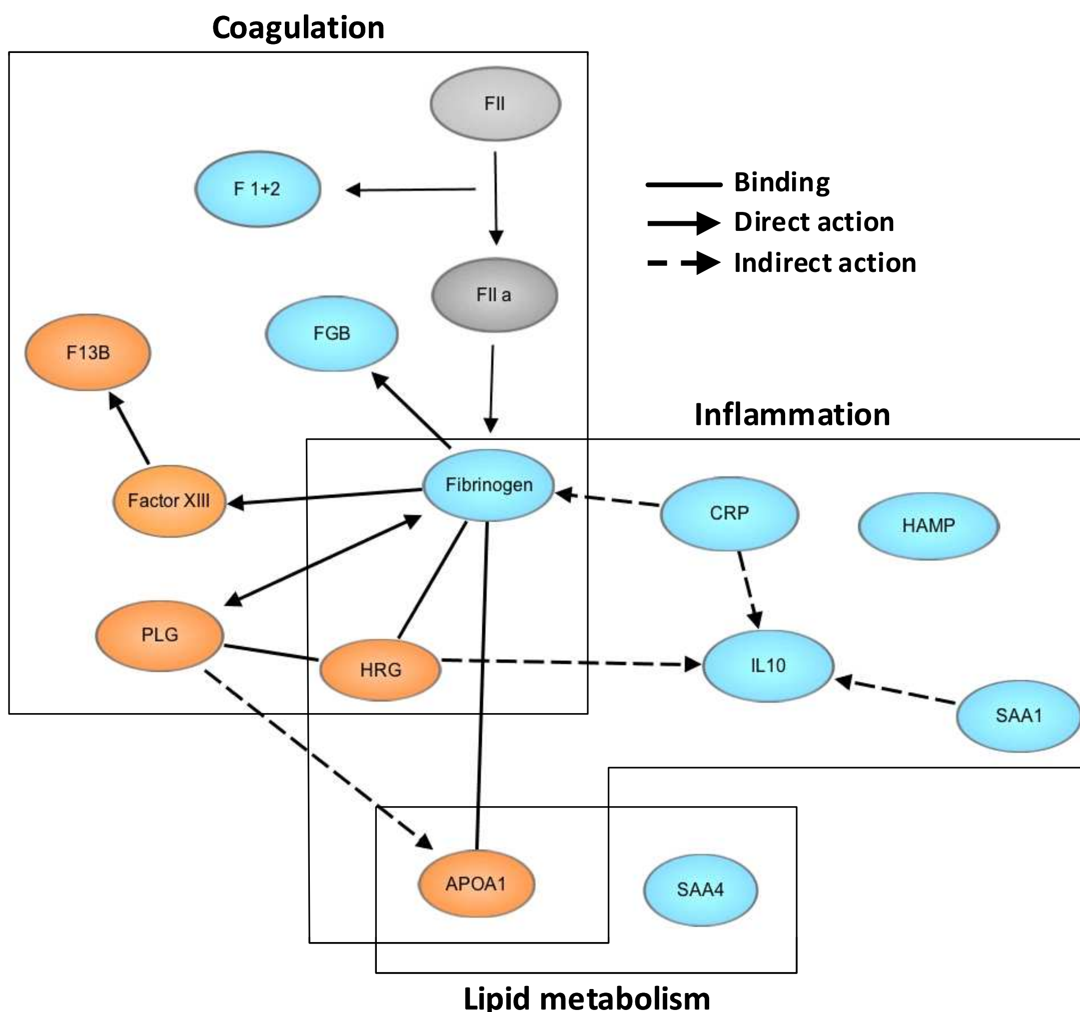

Figure 1: Schematic representation of discovered biomarker interactions generated by Ingenuity Pathway Analysis software. Proteins are classified according to the biological process in which they are involved. Colors indicate level changes in aGVHD samples compared to control (blue upregulation; orange downregulation; grey protein level not determined in our study). FII: Prothrombin. FIIa: Thrombin

As widely described particularly in the case of myeloablative conditiong, aGVHD is initiated by tissue damages mainly caused by the underlying disease and the conditioning-related toxicity. Endothelial cell impairment leads to tissue factor and cytokine release, and subsequent antigen-presenting cell activation (e.g. macrophages and dendritic cells). Intestinal endothelium injuries are particularly eminent in the amplification of the immune process due to its direct contact with bacterial products such as lipopolysaccharides (LPS) that stimulate macrophages. Consequently, elevation of proinflammatory proteins levels such as IL-6 and TNF-α levels (as observed in our results) that are secreted by activated immune cells, induces production of acute phase reactants mainly by the liver, such as CRP and SAA (Baumann et al., 1994). IL-6 as a potent inducer of hepcidin, might also explain the elevation of plasma hepcidin concentration (Nemeth et al. 2003). Moreover, Wu et al., recently described that macrophages also produced

hepcidin in response to LPS (Wu et al., 2012). Increase of hepcidin levels, an inhibitor of iron absorption, might also result from the disruption of iron homeostasis related to T-lymphocyte-inflicted tissue damage in the aGVHD process (Deeg et al., 2009). Thus, it is logical that we observe higher levels of these proteins in samples from aGVHD patients compared to patients without GVHD. In addition, IL-6 and TNF-α are important mediator of inflammation-induced coagulation (van der Poll et al. 1990; van der Poll et al. 1994). Indeed, they promote fibrin generation in severe inflammatory state, both systemically and locally.

In aGVHD, the "cytokine storm" and APCs activation favour complex interaction between incompatible donor and recipient immune cells which results in donor T-cell activation, the main mediators of aGVHD. Subsequently, alloreactive Th1-cells mainly release pro-inflammatory cytokines (mainly IL-2) while Th2-cells secrete regulatory cytokines such as IL-4 and IL-10. Thus, increase of IL-10 levels might result from a compensatory mechanism in response to systemic inflammation.

Then, IL-2 induced T-cell expansion leads to subsequent recruitment of cytotoxic effectors in different sites such as skin, gastrointestinal tract and liver tissue injuries, amplifying previous endothelium injuries. It results in a systemic and severe inflammatory response accompanied by excessive coagulation activation which leads to consumption of clotting factors and widespread depositions of fibrin. Indeed, we observed an increased levels of prothrombin fragments (F1+2), deriving from prothrombin activation and a decreased levels of coagulation factor XIII which demonstrate an activation of the coagulation cascade. In the same time, plasminogen levels, the precursor of plasmin that acts to dissolve the fibrin of blood clot, decrease. The activation of these processes leads to both thrombotic and hyperfibrinolytic disorders. It was previously described by Pihusch et al. that aGVHD with gastrointestinal damage presents an increased risk of bleeding and is associated with a decreased level of factor XIII, a protein stabilizing the fibrin clot (Pihusch et al. 2002). This could be the result of a higher consumption of this protein at the site of damaged tissue. In parallel of this thrombotic process, plasminogen levels were found decreased during aGVHD probably due to its higher consumption in response to the excessive release of plasminogen activator by injured epithelium. This phenomena leads to hyperfibrinolysis. Moreoever, levels of HRG, a binding factor of plasminogen and fibrinogen as well as a negative acute phase reactant, also decrease with aGVHD onset due to acute state of inflammation. Thus, depletion of HRG could contribute

to the induction of coagulation and fibrinolysis cascades in GVHD (Jones et al., 2005). Our results showing decreased HRG levels in aGVHD samples are in agreement with those described by Mauz-Körholz *et al* (Mauz-Korholz et al. 1995). In addition, fibrinogen is a central protein of inflammation and coagulation (Figure 1). Although it is an acute phase reactant increasing in inflammatory situations (Hantgan et al. 2001), the increase in fibrinogen level was somewhat less significant than the increase of CRP. This suggests that inflammation-dependent stimulation of fibrinogen release is counterbalanced by its consumption through coagulation activation. Moreover, in consequence of hyperfibrinolytic status, fibrinogen beta chain fragment levels as potential fibrin degradation products increase.

Proteins levels of lipid metabolism were also altered with aGVHD occurrence. Indeed, apolipoprotein A1 is a protein involved in lipid metabolism by participating in the reverse transport of cholesterol from tissues to the liver. However, decrease of Apo A1 levels might be rather explained by its role as a negative acute phase reactant. Indeed, HDL associated with Apo A-I is a negative acute phase reactant which was found to decrease by at least 25% during acute inflammation (Burger et al., 2002). Indeed, it plays an inhibitory role by interacting with activated T cells and interfering with monocyte activation responsible for IL-1 and TNF-alpha release (Hyka et al., 2001).

The gene of SAA4, a protein of the serum amyloid A family, is constitutively expressed and its protein product is a constituent of normal, non acute phase high-density lipoprotein (HDL). Thus, role of SAA4 is not well understood but it does not seem to be associated with acute phase inflammation as SAA1 and SAA2 (Whitehead et al., 1992; Steel et al., 1993). Indeed, we did not find a correlation between SAA4 and SAA1 and CRP levels. As reported previously (Upragarin et al., 2005), expression of SAA4 can be induced in human monocyte/macrophage cell lines (Urieli-Shoval et al., 1994) as well as in histologically normal human tissues such as small and large intestine or skin epidermidis (Urieli-Shoval et al., 1998). This might explain its increased levels with aGVHD occurrence as a consequence of cell damages.

In addition to protein level changes observed at time of aGVHD onset, it is worth noting that some biomarkers levels already varied before symptom development. Indeed, protein levels evaluated in samples taken 15 days before GVHD onset were found already modified compared to control samples, suggesting that early signs of aGVHD could be detected at the subclinical

level. However, it remains to be determined if these early changes reflect the first signs of aGVHD or if there are related to preexistent lesions and an inflammatory context which are responsible of aGVHD onset. These results suggest that aGVHD occurrence could be predicted before clinical manifestations.

Finally, as a single protein biomarker usually failed to detect disease with good sensibility and specificity, a combination of biomarkers was generated. Multivariate logistic regression was applied to identify an optimal combination of biomarkers that can distinguish control from aGVHD samples. As presented in the Figure 2, area under the ROC curve of the composite biomarker panel (AUC 94.7) is higher than AUC calculated for each individual marker included in the panel (AUC< 73.4) (Figure 2). This result clearly demonstrates that a panel of biomarkers provides a higher discriminative power than one single disease-related biomarker, particularly for the diagnosis of complex diseases such as aGVHD.

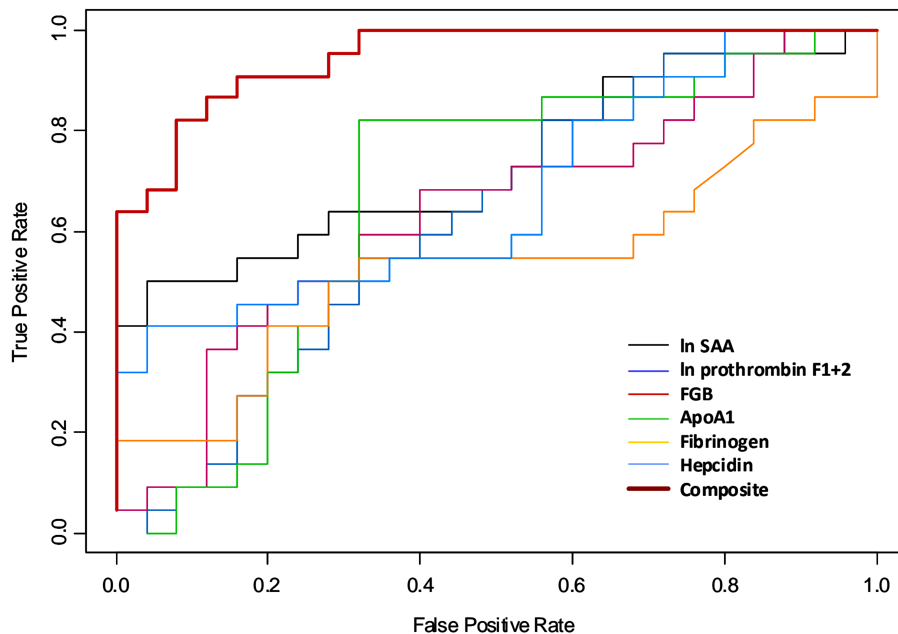

Figure 2: ROC curves of the 6 best discriminative individual markers compared to the ROC curve of the combination of these markers

In addition, due to missing values for day 14 and 25 IL-10 levels in our cohort of 56 patients, this biomarkers was not be taken into account in the model presented in Figure 2. However, a predictive model using multivariate logistic regression was constructed based on the values of 30 samples (18 controls and 12 aGVHD) in order to include IL-10 D14 and IL-10 D25.

Interestingly, the multivariate model was able to generate a composite biomarker panel including SAA and day 14 IL10 levels, with an AUC of 84.8 (Figure 3), demonstrating the good discriminative ability of the combination of those two markers. This result suggests that evaluation of IL-10 levels in the first month after HSCT could be an interesting indicator to early detect patients with higher risk of developing aGVHD and could be then helpful to improve aGVHD diagnosis. It is obvious that this should be confirmed in a higher number of samples

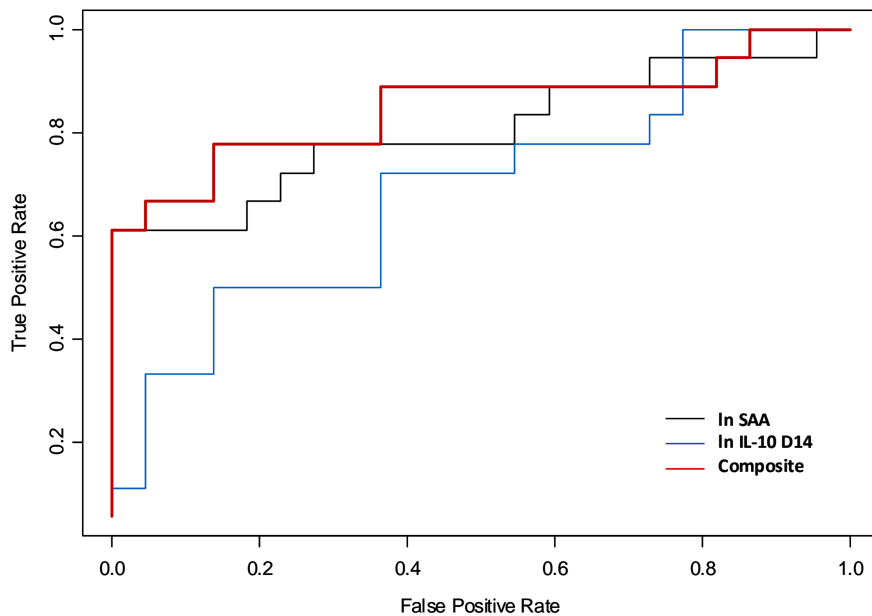

Figure 3: ROC curves of the individual markers included in the panel compared to the ROC curve of the biomarker panel composite

5. Limitations and perspectives

1) The main limitation of this work is the low number of patients in our studied cohort. Indeed, investigation of a complex immunological process such as aGVHD that has to be distinguished from other numerous confounding complications in a so heterogeneous population requires a high number of samples. However, despite the low number of samples, we were able to identify a panel of biomarkers discriminating control and aGVHD with good sensitivity and specificity. Our discovery study design was in accordance with other studies recently performed by Paczesny, Ferrara et al. as well as Weissinger et al. groups (Kaiser et al., 2004; Weissinger et al., 2007; Paczesny et al.,

2009; Paczesny et al., 2010; Ferrara et al., 2011). Indeed, their discovery studies also included a low number of samples (below 45 samples) including heterogeneous population. But they could further validate their biomarkers on larger training and validation set of patients. In our case, it is obvious that a validation step is needed. However, aGVHD remains a rare disease with low incidence in the whole population. So, to obtain a large cohort, sample collection should be done on a larger period of time and include several transplant centres. It will also be interesting to monitor these biomarkers during aGVHD treatment to determine whether it is likely to rapidly identify patients that respond or not to the first-line corticosteroid therapy. Indeed, unresponsiveness to corticosteroid treatment is associated with increased mortality (Deeg, 2007).

2) Biomarkers revealed in this work are mostly proteins implicated in acute phase response of inflammation and coagulation cascade that are non specific processes also altered in other post-HSCT complications, such as septicaemia. The discovered markers in this study also exhibit level changes in patients undergoing HSCT with septicaemia. However, despite of the numerous studies that have been undertaken to determine aGVHD biomarkers (Paczesny et al., 2009; Chen et al., 2012; Levine et al., 2012; Paczesny, 2012), only very few groups tested their markers in patients with confounding post-HSCT complications. Recently, Paczesny and al. identified a panel of biomarkers that correctly discriminate patients with and without aGVHD after a proteomic analysis based on antibody arrays. However, the proteins included in this panel (IL-2Rα, TNF-R1, IL-8 and HGF) were also associated with other complications such as bacterial infection, veno-occlusive disease (VOD) or idiopathic pneumonia syndrome (IPS). Another proteomic analyses performed on urine by Weissinger et al. using CE-MS provide a aGVHD-specific profile based on a pattern of polypeptides that correctly classify patients with or without aGVHD. However, in their validation set, incorrectly positive-classified samples revealed bacterial infections in combination with fever higher than 38°C as the major reason for false-positive results (Weissinger et al., 2007). This demonstrates the difficulties to distinguish aGVHD process from bacterial infection. Indeed, as recently commented by Serody, there is a close relationship between infectious complications

and gastrointestinal aGVHD (Serody, 2012). However, in recent studies performed by Ferrara, Paczesny et al. (Paczesny et al., 2010; Ferrara et al., 2011), they discovered specific biomarkers of GI and skin aGVHD. In a validation analysis, their biomarkers could discriminate aGVHD from non GVHD etiologies, comprising bacterial infection of GI tract and drug hypersensitivity. Therefore, as performed by Ferrara and Paczesny, discovery of systemic aGVHD biomarkers should be complemented by organ-specific biomarkers to increase specificity of aGVHD diagnosis. Moreover, aGVHD following myeloablative or nonmyeloablative should be investigated separately as the existence of late-onset aGVHD after nonmyeloablative may show some differences in the initiation of aGVHD, potentially impacting on the nature of early biomarkers.

3) From a technical point of view, despite the evolution of proteomic platforms in terms of sensitivity and efforts made to cover the wide dynamic range of protein content, detection of very low abundance proteins in complex fluids such as serum and plasma remains a great challenge in clinical proteomics. Indeed, in our study, plasma samples were analysed using three complementary proteomic approaches after applying two different methods of sample preparation, immunodepletion and combinatorial peptide libraries. However, detected proteins were ranged from high to middle abundance concentrations. Although that we applied the ProteoMiner® sample preparation methods that exhibited good reproducibility and showed efficiency for depletion of high abundance protein, low abundance proteins in plasma remain undetectable. Moreover, despite the combination of immunodepletion with subsequent 2D-orthogonal separation before LC-MS^E analysis, the less abundant proteins were found in the ng/ml concentration. This is not yet the deep proteome.

   Currently, multidimensional LC separation represents one of the most common strategies coupled with MS for proteomic analysis (Mauri et al., 2009; Di Palma et al., 2012). So, the most promising sample preparation methods for proteomic analysis should be based on a multiple combination of offline and/or online orthogonal strategies in order to serially fractionate sample until reaching deep proteome (Wang et al., 2010; Zhang et al., 2010; Khan et al., 2011). However, such strategies may have the

disadvantage to increase number of fractions to be analysed and time of analysis as well as compromise reproducibility.

Another option for biomarker discovery of aGVHD that could be considered is the proteomic analysis of less complex proteome such as cellular proteome. Indeed, a recent transcriptomic study was performed on T regulatory cell population in order to determine biomarkers of aGVHD and cGVHD (Ukena et al., 2012). Therefore, proteomic analysis of specific subsets of immune cells involved in aGVHD should be considered for the identification of more specific markers and for the better understanding of the cell involvement in aGVHD mechanism.

6. Conclusions

In this work, we have discovered a new panel of biomarkers comprising proteins involved in inflammation and coagulation processes that may help for aGVHD diagnosis. It demonstrates that proteomic technologies are useful tools for rapid identification of several disease markers, contributing to improvement of disease diagnosis even if it is obvious that these biomarkers have to be validated on a larger cohort of samples. In addition, we propose that IL-10 levels could be monitored in the first weeks after HSCT in order to early identify patients at higher risk of developing aGVHD.

With this aim in view, the predictive potential of our plasma markers should be validated. Then, the most appropriate time points for their measurements should be determined for early diagnosis and early patient management including response to treatment.

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APPENDIX

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2010, Article ID 906082, 14 pages
doi:10.1155/2010/906082

Review Article

Challenges for Biomarker Discovery in Body Fluids Using SELDI-TOF-MS

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Received 27 June 2009; Accepted 1 September 2009

Academic Editor: Pieter C. Dorrestein

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Protein profiling using SELDI-TOF-MS has gained over the past few years an increasing interest in the field of biomarker discovery. The technology presents great potential if some parameters, such as sample handling, SELDI settings, and data analysis, are strictly controlled. Practical considerations to set up a robust and sensitive strategy for biomarker discovery are presented. This paper also reviews biological fluids generally available including a description of their peculiar properties and the preanalytical challenges inherent to sample collection and storage. Finally, some new insights for biomarker identification and validation challenges are provided.

1. Introduction

The objective of biomarker discovery is to identify specific protein markers susceptible to improve early diagnosis survey therapeutic outcomes and facilitate the development of novel drug candidates [1, 2]. The methodology relies on differential protein expression profiling. The fundamental approach is based on the assumption that the pathology of concern will affect some physiological processes causing changes in the protein expression levels. Proteins generating similar signals in both sample groups are ignored while significantly up- and downregulated proteins become potential biomarkers. Differential expression profiling requires both a sensitive technology to discern any tiny differences and a high-throughput system in order to process large series of samples required to reach statistical significance. Protein differential display techniques such as two-dimensional gel electrophoresis (2-DE), one- or two-dimensional liquid chromatographic (LC-MS), or surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) are regarded as the most powerful tools for establishing fingerprint profiles [3–6].

Many reports regarding the application of the SELDI-TOF-MS technology have been published since its introduction in 1993 [7] and its first use for disease detection [8]. One of the key features of SELDI-TOF-MS is its ability to provide rapid protein expression profiles from a variety of biological samples with minimal requirements for purification and separation of proteins prior to mass spectrometry. SELDI-TOF-MS profiling studies revealed that biological fluids contain many proteins with low molecular weight (<15 kDa) not resolved on conventional 2D gels [6, 9].

As can be seen in Figure 1, the SELDI technique consists in surface arrays involving various chromatographic models based on both classic chemistries (normal phase, hydrophobic, cation- and anion-exchange surfaces) and specifically affinity-coated surfaces (immobilized metal affinity capture : IMAC). After the binding phase of the sample to these surfaces, the unbound proteins are washed out while retained

molecules are overlaid with an energy-absorbing matrix. In the final step, mass spectra are recorded using a laser for the ionization and a TOF mass spectrometer for its resolving power.

Recent interest in the field has yielded a large number of candidate biomarkers in various diseases [10–35]. However, the small size and poor design of some studies drove validation of these biomarkers quite challenging [36–41].

In the context of clinical proteomic using SELDI-TOF-MS, many recent reviews discussed newly identified disease biomarkers [13, 21, 22, 24, 27, 30, 35, 42–44]. The present review focuses on technical challenges encountered with the SELDI-TOF-MS technology taking into account new insights coming from the last three years. Critical steps that should be undertaken to avoid any bias, to maximize reproducibility and detection sensitivity, with the final aim to find relevant, specific, and robust biomarkers are addressed [45, 46]. For prospective studies, current knowledge on the different biological fluid sources available for SELDI-TOF-MS experiments is described presenting their respective advantages and limitations.

2. Study Design

A successful biomarker research program starts with a careful study design and the preparation of a detailed protocol. Many manuscripts report encountered problems, emphasizing the importance of Standard Operating Procedures (SOPs), clinical protocols, instrument tuning, and stabilization [37–40, 47–63]. Only critical points will be discussed in this review.

In the early phase of biomarker discovery, the clinical question addressed has to be defined in the disease(s) context collecting adequate control samples. Indeed, it can be criticized that in many published studies, patients were compared to healthy subjects rather than to patients presenting similar diseases or clinical signs.

Experimental workflow and technologies have to be selected with great care. The avoidance of bias is not trivial and must be addressed throughout the whole study, from its design to the data analysis and interpretation (cf. Figure 2). Current proteomics and genomics technologies are extremely sensitive and can detect very small changes in expression levels. Some of these changes may arise from biological differences related to disease or pharmacological treatment. They could also result from the heterogeneity of the patient panels tested across multiples sites, the inherent biological complexity, and the diversity of sample types. Small differences in sample collection, processing, and analytical techniques could have some impact on the outcomes of the study. As a consequence, clinical data may be site-, study-, population-, or sample-dependent, without any actual clinical relevance [53, 62, 64–66]. The key factor for maximizing reproducibility in biomarker research is to identify and minimize all potential sources of preanalytical and analytical bias [53, 55] (Table 1). Adherence to strict guidelines and SOPs is critical to reach the highest operating standards for data quality and reproducibility [37, 38, 47, 50, 51, 53, 55, 62, 63, 67]. SOPs also facilitate the validation of biomarkers by other groups using different sets of samples.

3. Sample Handling and Preparation

Besides the instrumentation and the methodologies related to chromatography-mass spectrometry analysis, the nature, quality, and number of clinical samples to process are key elements to be considered for any proteomic approaches.

3.1. Selection of Body Fluid. In order to provide positive answer to any precise clinical question, the investigator has to make a choice among the most relevant biological target samples (body fluid, tissue, etc.). Many criteria must be considered at this level, that is, availability, easiness of collection, stability, composition, proximity with disease location, patient discomfort, ethics, and so forth.

For biomarker discovery using SELDI-TOF-MS, a great variety of biological sample types can be used; major applications concern biofluids (plasma, serum, urine, saliva, cerebrospinal liquid, bronchoalveolar wash out, nipple aspirate fluid, tears, amniotic fluid) [1, 10–14, 18, 19, 21, 22, 24, 28, 42, 48, 49, 68–77], and tissues or cell extracts [78, 79]. Among them, serum, plasma, urine, and saliva are the most popular. Many variables related to collection, storage, and conditions for sample preparations have to be carefully considered. These parameters have been commented in literature according to the nature of the biofluids [48, 49, 54, 57, 61, 67, 73, 74, 80–84]. In this section of the review, consensus opinions derived from these recent studies are provided. Advantages and drawbacks of the most popular fluids are presented in Table 2 as well as formulated recommendations. When reviewing a series of studies [47–49, 51, 54, 57, 58, 60, 61, 67, 68, 73, 81–83, 85] general guidelines can be forwarded. Optimal serum clotting arises after 60 minutes at room temperature. After clot formation, samples can be transported or stored on wet ice for 3 hours before centrifugation. Aliquots must be prepared and stored at -80°C. For plasma collection, anticoagulant EDTA is preferred and its processing should be realized as soon as possible after sampling, ideally within the first hour. Although storage at low temperature promotes peptides and proteins stability, one should not recommend storing plasma samples at 4°C due to the cold activation of platelets. Prior any freezing, plasma can be depleted in platelets by using a filtration step; aliquots are then freezed at -80°C.

According to the “HUPO PPP Specimens Committee” recommendations, plasma appears preferable to serum because it contains less peptides of degradation and consequently presents less variability [57, 81, 86]. In order to avoid the presence of platelet related peptides, the authors also recommend to use platelet-poor plasma obtained by centrifugation followed by a filtration step. However, the choice of serum could be justified when studying diseases related to coagulation abnormalities. Furthermore, it is often more available in sample banks for retrospective studies.

A controversial parameter is the addition of protease inhibitors (PIs) to the samples. Some authors found that the

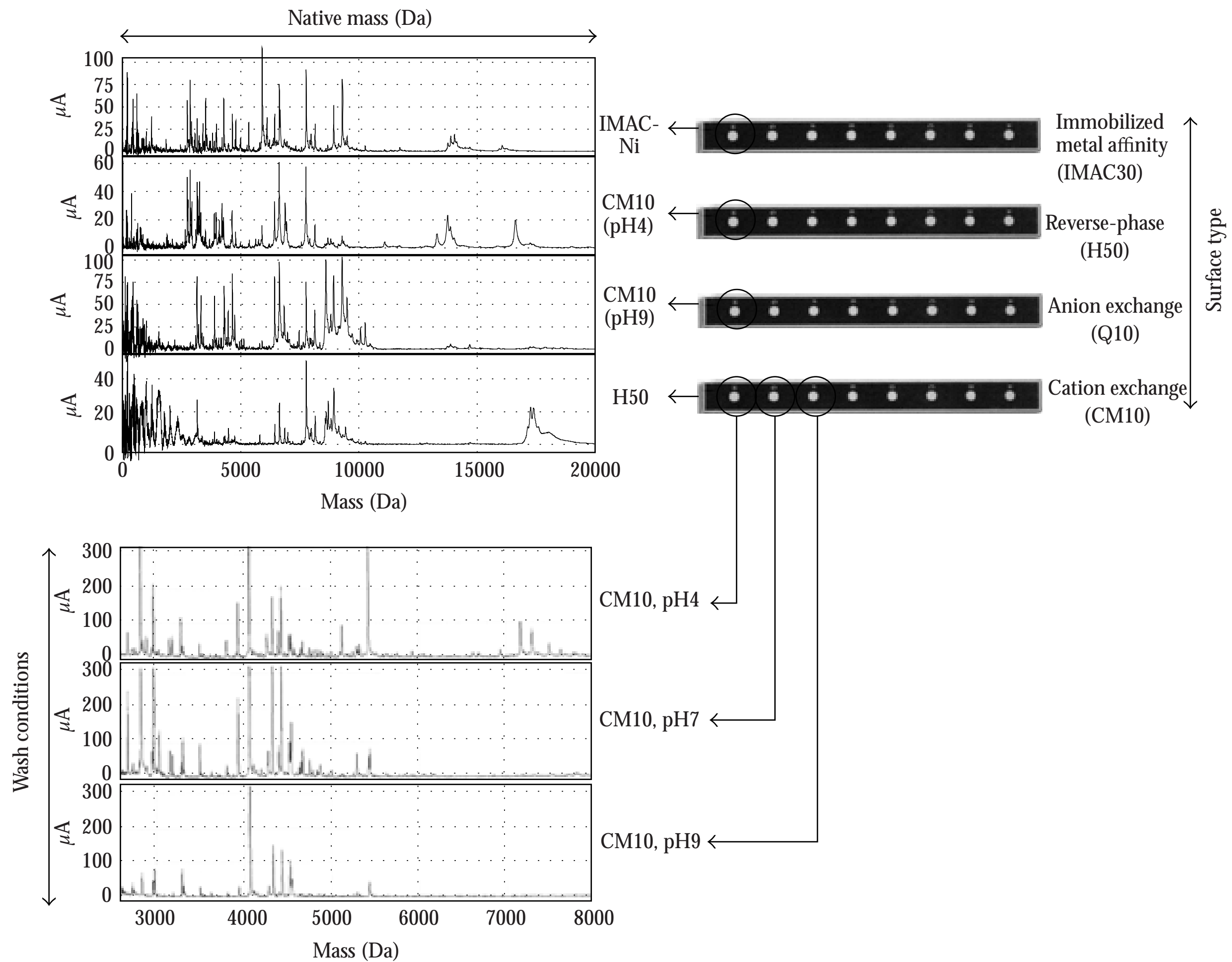

Figure 1: Effects of different ProteinChip array surfaces and wash conditions. The combination of ProteinChip array surface types and wash conditions maximize the potential for protein biomarker discovery.

addition of a PI cocktail induces significant differences in protein profiles when compared to crude samples [58, 83]. Whenever directly introduced during phlebotomy, PI allows fluid stabilization for at least 2 hours at room temperature by reducing proteolysis damages. However, PI presents some additional drawbacks such as the presence of highly concentrated components in the cocktail which can compete later on for protein array interactions.

Another important factor to decrease the risk of variation, bias, and errors is the communication between researchers and medical staff. One generally considers that 70% of the errors are due to human intervention (mostly due to communication problems) while only 30% appear instrumental related errors [87]. The mode of specimen collection (veni-puncture or arterial puncture), the site of collection, the position of the patient, or the tourniquet technique can influence the concentration of certain blood constituents [58]. Hemolysis also causes significant changes in blood proteome specimens [67]. It is generally advised to discard those kinds of samples, but when the disease studied involves spontaneous hemolysis, this cannot reasonably be done.

Less commonly used, filter papers were also described to collect blood [68]. This mode of collection has the advantage that only few drops of blood are needed (particularly interesting for neonatal and repeated screening). Moreover, it does not require specific medical support for sampling, which could be promising for multiple collects realized by the patient at home, in the perspective of a treatment follow-up, for instance. Stability and reproducibility of this collection mode remain to be studied.

Saliva and urine have more recently presented an interest in biomarker discovery. Their collection is simple, noninvasive, and cheap and can be easily repeated. However, like blood specimen, such factors have to be taken into consideration to improve reproducibility of sample collection. Saliva protein composition varies with circadian rhythm, diet, age, gender, and physiological status [86]. It is also affected by the method of sample collection (stimulated versus non-stimulated saliva production) [60]. Food ingestion increases the proteolysis activity and then collection before lunch rather than after is recommended [73]. The addition of PI can reduce but not completely eliminate the impact of the proteolysis [60]. It will stabilize, qualitatively and

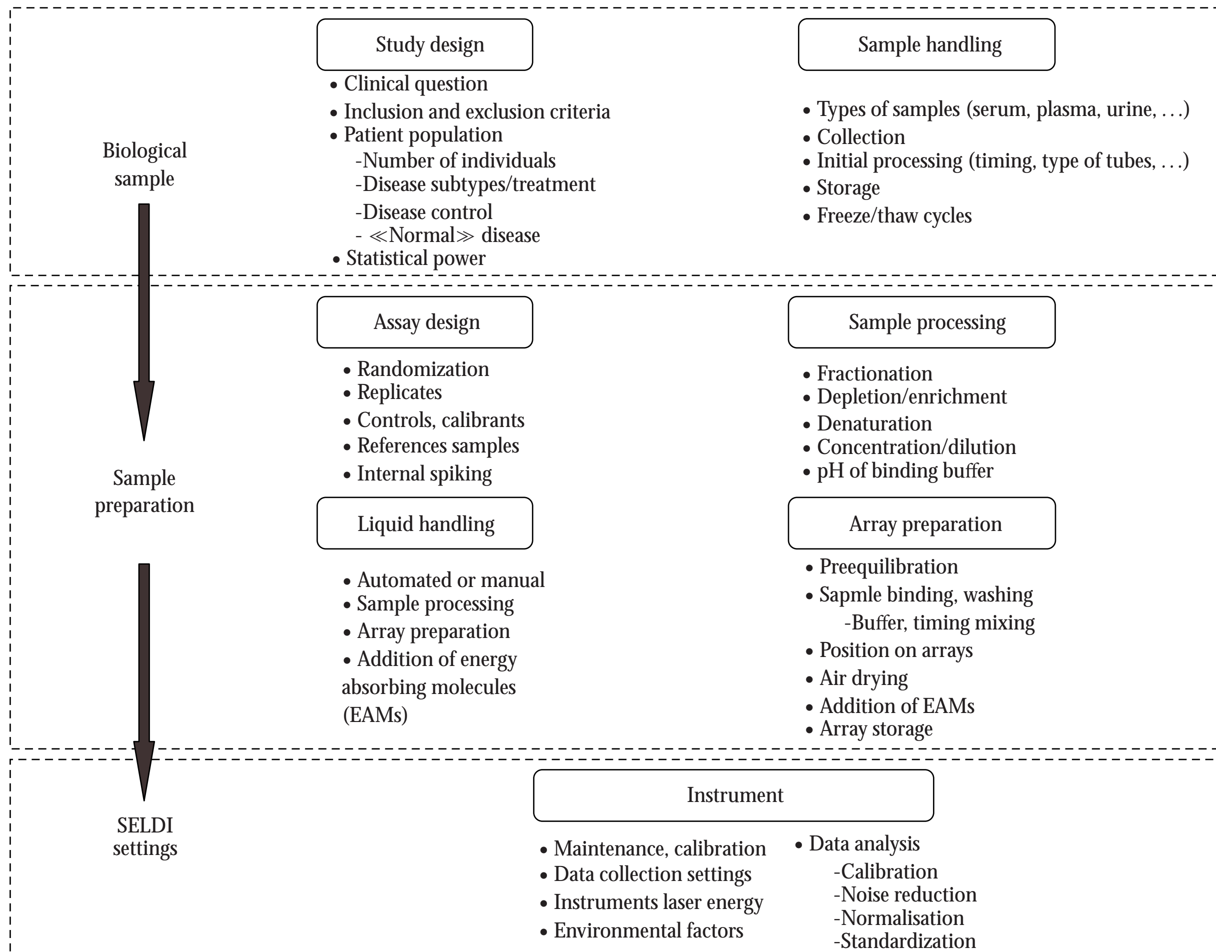

Figure 2: Experimental variables that can affect proteomics data. Most of the steps shown are involved in all proteomics workflows, but SELDI technology performs many of them on a single platform.

quantitatively, the saliva proteome for up to 48 hours [73]. Regarding storage conditions, it is preferred to store the saliva specimens at −80°C rather than at −20°C where the preservation of the protein content could not be guaranteed for more than 1 month. Interestingly, repeated freeze-thaw cycles (4/5) do not seem to significantly alter saliva protein profile [74].

Urine has the advantage that it can be obtained in large volume. It is mainly an aqueous solution (95% of water) of waste electrolytes and metabolites, organic components (urea, uric acid), and proteins at low concentrations in healthy individuals (150 mg/day). Urine proteome variation depends mainly on plasma composition due to its role as blood content regulator and on the integrity of the glomerular filtration step leading to a large intra and intersubjects variability. Protein and salt concentrations can vary along the day for a same subject (first void compared to midstream urine samples) [80, 88]. Progressive degradation of urine proteome due to proteolytic activity can be prevented by PI addition only up to 2 hours of storage [54]. As already mentioned for blood and saliva, up to 5 freeze/thaw cycles do not significantly affect urine proteome profile. Storage at −80°C is still requested.

Other fluids such cerebrospinal fluid, nipple aspirates, tears, synovial fluid, bronchoalveolar lavage, follicular, and amniotic fluids have already been explored by SELDI-TOF-MS [15, 20, 33, 34, 69, 75, 89, 90]. These fluids are generally used to study well-localized diseases. Despite the presence in such fluids of some plasma proteins, their implication to study systemic diseases is not recommended and difficult to apply in routine diagnosis due to risk and discomfort related to collection.

3.2. Sample Processing. One of the most challenging aspects in studying body fluids protein profiles remains the detection of the deep proteome [91]. The protein concentration dynamic range detectable by means of MALDI-TOF or SELDI-TOF-MS is about 2 orders of magnitude, whereas the range in blood reaches about 10 orders of magnitude [91, 92]. As protein binding onto chromatographic surface depends on its affinity, its concentration, but also on the surface binding capacity, one can imagine that the competition between different proteins for binding sites is

Table 1: Factors that impact preanalytical and analytical bias.

Preanalytical bias	
Patients information	Age, gender, ethnicity Disease subtype and/or severity Medical background Health background Smoking status, alcohol intake, diet, other risk factors Drug treatments Patient position (seated/standing/lying), daily moment of collection Type of control (healthy or disease) Location of sample collection (single or multisite) Study inclusion and exclusion criteria
Sample characteristics	Number of individuals Type (blood, serum, plasma, urine, cerebrospinal fluid, cell lysate, etc.) Source (banked or prospectively collected)
Sample-handling procedures	Collection protocols (initial processing, procedure, timing, type of anticoagulant, type of tubes, number of sites, etc.) Storage procedures (time, aliquoting, storage materials, temperature, freeze-thaw cycles, etc.)
Analytical bias	
Sample-Processing procedures	Fractionation and depletion methods Processing steps (denaturation, buffer components, delipidation, etc.) Liquid handling methods (automated or manual, technique, equipment, etc.)
Experimental protocols	Array types Sample pH and dilution factor Quantity of sample loading and position on arrays Sample binding, washing and drying procedures Matrix addition (type and method) Instruments settings Number of instruments, locations Environmental factors (temperature, humidity percentage)
Data analysis methods	Spectrum processing (baseline subtraction, normalization, alignment, noise reduction, etc.) Peak labelling Feature selection, statistical analysis Classification approaches

very complex. A highly abundant protein with low affinity for the chip surface and a low abundant protein with high affinity may give similar peak intensities in the final SELDI mass spectrum. Furthermore, protein steric hindrance can also affect the SELDI profiles.

Several fractionation procedures are now available to decrease the sample protein concentration dynamic range [85, 93–102].

A major inconvenience for sample fractionation is the resulting low sample throughput capacity, due to a significant increase of the duration of analysis and to a risk of poor reproducibility affecting data treatment. Use of automatized technologies can improve the reproducibility and decrease the total analysis time. Additionally the same proteins can be presented in different fractions challenging the comparison of their abundance between samples.

Several methods have been proposed for fractionation such as centrifugal ultrafiltration, precipitation by organic solvents, electrophoresis, chromatography (on-column or on-magnetic beads), or subcellular localization. The choice will be made based on the nature of the sample to be analysed and the protein properties (molecular weight, localization, abundance, etc.). All these sample preparation methods have already been discussed by other reviewers [85, 94, 101, 103, 104]. Recently, with the growing interest in studying posttranslational modifications new methodologies set up to isolate rare amino acid-containing peptides (cys, met, trp, his) or PTM peptides (phosphopeptides, glycopeptides) have been developed [25]. One of the most widely used approach

Table 2: Advantages and limitations of body fluids particularly useful for biomarker discovery.

Body fluids	Advantages	Limitations	Recommendations
Serum	(i) established sample banks often composed of serum aliquots (retrospective studies),	(i) presence of various products derived from coagulation cascade,	(i) use standardized collection protocol,
	(ii) proteins and peptides that "survive" to the clotting procedure exhibit a stability that can be exploited in routine clinical applications.	(ii) biomarker with poor stability during coagulation process will not be detected in serum,	(ii) keep sample during 1 hour at RT to allow clotting process before centrifugation,
		(iii) possible influence of the disease on coagulation process.	(iii) preserve on ice after clotting. Aliquoting and freezing (-80°C) cannot be done immediately.
Plasma	(i) more rapidly processed than serum (interesting for emergency diagnosis),	(i) interference with chip surface (i.e. heparin tube),	(i) use standardized collection protocol,
	(ii) larger final volume of fluid after processing than with serum,	(ii) sample dilution in citrate tube,	(ii) carefully choose the type of anticoagulants (EDTA tubes are preferable),
	(iii) more stable than serum due to the inhibition of coagulation cascade.	(iii) possible interference of EDTA with protein binding on IMAC surface,	(iii) use platelet-poor plasma,
		(iv) SELDI-TOF spectra less rich in peaks number and intensity than serum.	(iv) centrifuge, aliquot and freeze (-80°C) as soon as possible. If not possible, keep at RT to avoid cold platelet activation.
Dry blood	(i) medical staff not needed for collection,	(i) elution step to recover sample from filter paper.	(i) keep dry specimens at RT for 3–4 hours in horizontal position,
	(ii) low blood volume necessary,		(ii) store at -20°C.
	(iii) easy storage and transport.		
Saliva	(i) easy and noninvasive sampling,	(i) low volume collected,	(i) always collect with the same method (stimulated or not) and at the same moment of the day,
	(ii) medical staff not needed for collection,	(ii) presence of many proteases and unspecific materials such as food residues or microorganisms,	(ii) centrifuge to remove insoluble material, aliquot and freeze at -80°C.
		(iii) level of certain plasma proteins are not reflected in saliva.	
Urine	(i) easy and noninvasive collection,	(i) fluctuation of protein concentration overtime and according to renal integrity,	(i) use standardized collection protocol,
	(ii) medical staff not needed for collection,	(ii) presence of salts and proteins in low concentration.	(ii) concentrate the samples,
	(iii) obtained in large volume.		(iii) centrifuge, aliquot and freeze at -80°C,
			(iv) normalization with creatinine content.

for highly abundant proteins removal in serum and plasma is their depletion using antibodies. Despite the depletion of the nine most abundant proteins from serum or plasma samples, overall published results were quite disappointing [105]. This sensitivity problem is most probably inherent to the too low concentration of the peptidome constituents. Moreover, some of the abundant proteins act as carrier explaining the codepletion of almost 3000 species (peptides and proteins) as observed by several groups [106].

A new fractionation approach has been recently developed by Righetti and Boschetti [107]. It implied a solid-phase combinatorial library of hexapeptides on which millions of copies of a unique ligand are graft on a bead. This technique, enabling the dilution of abundant protein by rapid saturation of its ligand, concentrates components of the deep proteome which could not reach saturation. This method presents the advantage to reduce the dynamic range between the most and less abundant proteins and peptides. It has also been showed that despite compression of the dynamic range, this technology used for differential studies was only applicable for proteins or peptides which do not reach saturation (low and medium abundance proteins) [107]. Many studies conducted on different types of samples report good reproducibility and important gain in the number of low abundant species by comparison with analyses performed on corresponding crude samples [96, 98, 99, 108–111], which make this approach very promising to investigate the deep proteome.

4. SELDI Settings

In order to highlight candidate protein biomarkers, several chromatographic surfaces must be screened. The choice of the protein chip array chemistry and the nature of the matrix depend on whether the application requires general profiling or requires a specific protein assay. Different array types and binding conditions may generate complementary protein profiles for the same sample [7]. The use of relevant quality controls (QCs) is highly recommended and even mandatory in such applications [37–40, 47–63]. QCs should be well-characterized pools of samples processed alongside the experimental samples in order to monitor instrument performances, optimize mass spectrometry settings (laser energy, etc.), compare target protein profiles to those of historical reference samples, and to calculate coefficient of variation for peak intensities as a measure of reproducibility.

It is important to point that the resolution and mass accuracy provided by this kind of instrument are rather low compared to high-resolution mass spectrometers (i.e., Q-TOF, FT-MS, etc.). Using SELDI-TOF-MS, one could not expect to accurately determined m/z values or peak intensities on complex mixtures. Indeed, low resolution causes peaks overlap making abundance and mass assignment difficult. This means that only large differences in peak intensities are to be considered and that peaks of interest have to be identified with more accurate mass spectrometers. Beside those instrumental weaknesses, on the contrary to other mass spectrometers, SELDI-TOF-MS can be used for high throughput analysis.

During SELDI settings, numerous sources of spectra variability have to be taken into account.

Several events, such as matrix crystallization, ion suppression, and in-source decay occurring during mass spectra acquisition strongly influence the peak intensities. These are commented in more details below.

4.1. Matrix Crystallization. Differences in reagents, handling of material, room temperature, and level of humidity may all influence the (co)crystallization step of matrix molecule with sample causing interday fluctuation. The structure and nature of the target surface may also affect peak intensities. These parameters must be highly controlled and standardized for each study protocol. During the crystallization process, a competition phenomenon can occur between proteins for crystal inclusion. Easily embedded proteins will be present at higher concentrations in the matrix and consequently more efficiently desorbed and ionized [47]. To improve sample-to-sample reproducibility of MALDI ion yield and to increase the precision of peptide quantification, some authors use nitrocellulose in order to improve the homogeneity of the matrix/analyte crystallization [55, 112, 113]. This operation might also be helpful for SELDI-TOF-MS measurements.

4.2. Ion Suppression. Depending on sample composition, ion suppression is another factor that significantly contributes to the variability observed in SELDI-TOF-MS spectra [47, 50, 55]. Indeed, during ionization, analytes compete for protons that are transferred from matrix molecules. If a protonated analyte collides with an unprotonated one which has higher gas-phase basicity, it may pass its proton to the collision partner. Therefore, the presence of an analyte may reduce the signal intensity of another. This phenomenon is called "ion suppression effect." In a complex protein mixture like serum, where highly abundant proteins constitute a large proportion of the total protein content, it is possible that such peaks override signals from low abundant peptides. This phenomenon, obviously difficult to prevent in complex samples, would be more easily controlled on mixtures issued from fractionation.

4.3. In-Source-Decay. Another source of variations is the fragmentation of proteins or peptides during mass spectrometric process. Fragmentation occurring before the first field-free region is called in-source decay (ISD); it is responsible for consecutive series of ions [114].

Ekblad et al. showed that ISD generates quite additional spectral peaks in the spectrum of proteins contained in serum samples when compared with the data collected for pure reference proteins [114]. One obviously creates ISD favourable conditions when optimizing the analytical conditions by maximizing the total peak count, particularly when using a high laser beam which would increase the thermal ions energy and consequently the number of collisions between ions. Hopefully, in-source fragmentation remains

quite limited [114]. Dijkstra et al. developed a method which deconvolutes the spectrum by appropriately associating peaks belonging to the same protein [50]. To take benefit of this procedure, highly efficient sample fractionation is recommended.

4.4. Miscellaneous. Other phenomena susceptible to affect SELDI spectra must be considered. Common mechanisms accounting for the arising of multiple peaks in mass spectra include, for example, the formation of salt adducts and multiply charged ions [50]. Chemical reactions using energy from the laser may take place between sample protein molecules, matrix molecules, or molecules from the washing buffers generating intermolecular complexes known as "ions cluster" [50]. The formation of these complexes increases the number of spectrum peaks causing artefacts (i.e., satellite peak at +206 Da corresponds to a SPA adduct). Moreover, the performance of the SELDI-TOF-MS may change over time due to possible fluctuation in the laser intensity and/or detector sensitivity.

All these difficulties can be addressed only by substantial reduction in sample complexity and the application of a rigorous standardization program of the entire analytical process. This involves optimized acquisition protocols (i.e., avoiding too high laser intensity), a fully operational and calibrated instrument and the use of suitable QC samples, similar in nature and complexity to the studied samples.

5. Data Analysis

5.1. Spectrum Processing. Another important methodological source of artefacts is the data analysis of protein profiles. The data preprocessing (calibration, baseline correction, normalization, peak detection, and peak alignment) represents a key step for SELDI analysis [115–117].

Spectra are generally normalized in order to equalize or minimize differential effect due to external variation [59, 115, 116, 118, 119]. The widely used total ion current (TIC) gives a clear indication of the impacts of technical variables such as laser and detector performances, matrix application, and sample amounts. TIC normalisation relies on the assumption that the technical parameters are mostly responsible for the largest differences observed between samples. But Cairns et al. showed that TIC may also potentially remove some pertinent biological information [115]. They suggest to examine whether normalisation factors vary systematically between study groups and they recommend to specify the applied methodology (local or global normalisation, matrix signal excluded or not). The ideal normalisation procedure would be to resort to some internal spiking method.

5.2. Classification Approaches. One important aspect in SELDI-TOF-MS data analysis is to avoid false discovery of protein peaks, for which the discriminative power results from random variation. A general criticism concerns the use of inadequate algorithms for data analysis and the problem issued from over-adjustment in combination of high-dimensional data with a low number of cases. Those artefacts could be prevented by analysing a sufficient number of samples, by resorting to overfitting-resistant algorithms, by an appropriate validation of the resultant model, and by using optimal spectra processing techniques (calibration, exclusion of spectral regions affected by high noise, peak alignment, and normalization). Two others remarks can be formulated from literature reports: (1) multiple biomarkers have generally a better predictive value than individual markers, and (2) positive-predictive values of peptide patterns are often insufficient to be recognized as early markers when they concern low-frequency diseases in the population [38, 53].

The most commonly used bioinformatics approaches are decision tree-based ones and support vector machines [120, 121]. Authors generally emphasized on the need for validated model selection using cross validation loop and permutation testing to develop generalized classifier able to correctly predict classification of new samples [122].

6. Biomarkers: From Identification to Clinical Application

Identification of candidate biomarkers, while not strictly necessary for diagnostic purpose, can be regarded as extremely satisfying in helping to data interpretation and better understanding the disease. As often criticized, the SELDI-TOF-MS technology does not provide peptide/protein identification. In order to succeed in the identification by sequencing (Q-TOF, TOF-TOF, ion-trap, etc.) or peptide fingerprinting (MALDI-TOF), enrichment and purification of the biomarker of interest is often needed, which is laborious and time consuming. To solve in part this weakness, new ProteinChip interface coupled to tandem mass spectrometer was recently developed allowing direct sequencing of peptides <6000 Da [124]. In all cases, identifications must be corroborated using antibody-based detection (i.e., Western blot or ELISA) or antibody pull-down with subsequent detection by SELDI-TOF-MS.

It should be noted that the concentration range of widely used biomarkers in plasma samples is remarkably wide and differ from the high milligram until low nanogram per liter range. For example, serum albumin, within a normal concentration range of 35–50 mg/mL, is measured as an indication of severe liver disease [125] or malnutrition [126], whereas IL-6 normally varies in a range of 0–5 pg/mL, is measured as a sensitive indicator of inflammation or infection [127].

Until now, most of the markers identified after an SELDI-TOF-MS study could not yet be considered as very specific of a given disease but they are rather representative of disease's consequences like inflammation or immune response. The most frequently identified proteins so far are haptoglobulin, transthyretin, apolipoproteins, serum amyloid, or complement factors present at μg/mL to mg/mL [13, 19, 23–25, 28, 38]. Although individual acute-phase reactions proteins are not satisfactory diagnosis biomarkers, their combined use with other serum biomarkers may enable more sensitive and specific diagnosis (cf., Figure 3). This phenomenon has recently been termed "host response protein amplification

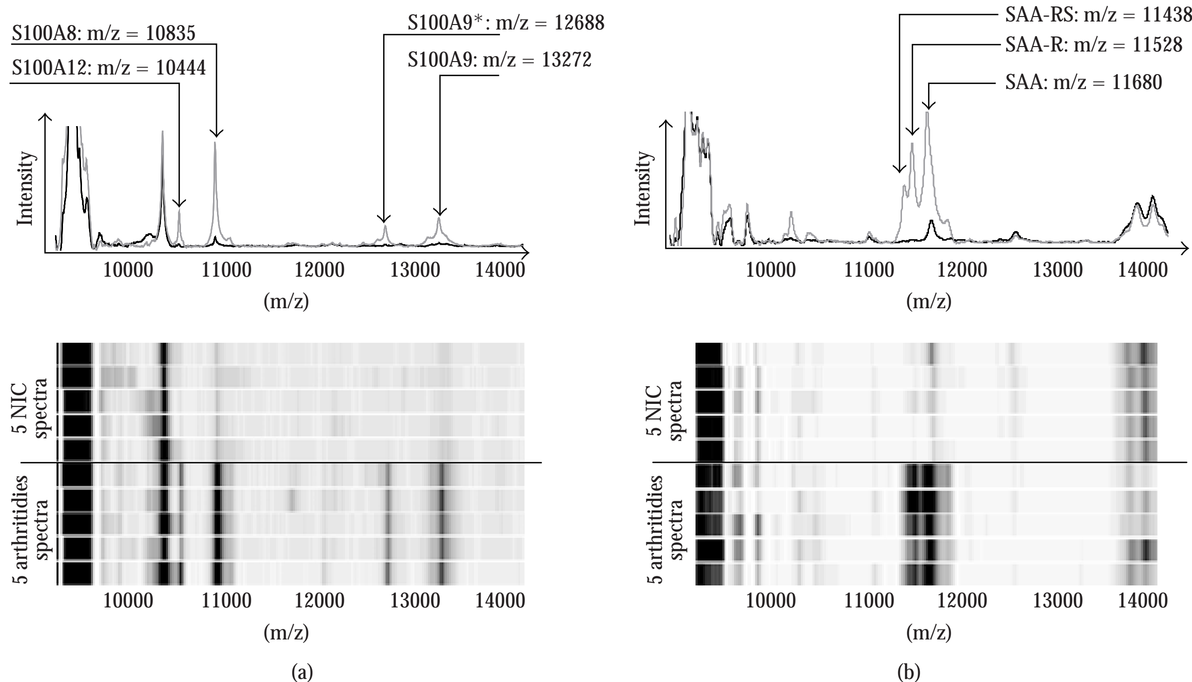

FIGURE 3: Protein mass spectra collected on CM10 and IMAC-Cu^{2+} ProteinChip arrays with serum samples provided by five patients with arthritidies (including rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis) and five noninflammatory controls (NIC) (including osteoarthritis). (a) The inflammatory-related proteins S100A8, S100A12, S100A9, and one of its variant S100A9* are arthritis biomarkers detected on CM10 arrays. (b) On IMAC-Cu^{2+} ProteinChip arrays, SAA and its 2 variants (SAA-R and SAA-RS) are illustrated, reproduced from [19].

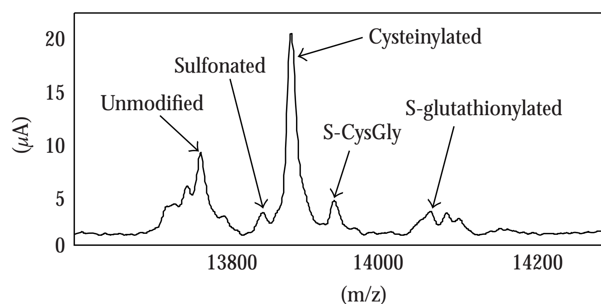

FIGURE 4: Modified transthyretin forms observed in ovarian cancer sample, adapted from [123].

cascade” [122]. Acute-phase proteins could also be directly produced by the disease tissue.

The moderate specificity of SELDI-discovered biomarkers could be explained by its low sensibility. To date, SELDI-TOF-MS has not yet identified any protein marker present at ng/mL level. This probably indicates that the lowest detect limit of this technology is around μg/mL as considered by Diamandis [128]. To overcome this limited detection sensitivity, the serum (or plasma) proteins can be fractionated (cf., Section 3.2) before SELDI-TOF-MS analysis. Fractions could then be loaded on different arrays using complementary binding conditions.

Moreover, the decisive advantage of the mass spectrometry technologies is the capacity to detect protein variants, protein fragments, and posttranscriptional modifications (PTMs), which is usually not possible with affinity-based technologies. It is now recognized that those components may be disease-specific and can be considered as potential biomarkers (i.e., modified transthyretin forms in ovarian cancer in Figure 4 and in familial amyloidotic polyneuropathy) [123, 129, 130].

In the last two years, lots of applications using SELDI-TOF-MS were published for diagnostic of cancers [25, 42, 44, 131], especially breast [10, 17], prostate [21, 132, 133], and colorectal cancer [24]. Other recent papers concerning infectious diseases [22], neurodegenerative disorders [35], renal diseases [26, 134], and chronic inflammatory diseases [19, 135] also demonstrated the great potential of the technique.

SELDI-TOF-MS technology was also used to predict response to therapy, particularly in cancers. Röcken and Whelan described in detail the use of SELDI-TOF-MS to not only predict responses to cancer therapy but also demonstrate its interest in the follow-up of metastasis disease progression and in the development of drug resistance [44, 136]. Recently platelet factor 4 (PF4) appeared to be a biomarker for Infliximab nonresponse in Crohn’s disease and rheumatoid arthritis [29, 137].

For most of these studies, a validation phase should assess the validity of the described potential biomarkers against a larger and more heterogeneous population of patients. The robustness of the candidate markers has to be tested against

a level of biological variability that more accurately reflects the variability in the target population.

Unfortunately, several groups failed to validate the biomarker discovered in their pilot study, such as McLerran et al. [39, 40], and others [36, 66]. McLerran et al. described preanalytical bias. They concluded that their first study samples most likely had biases in the sample selection. Another validation performed by Engwegen et al. using distinct patient populations confirmed that SAA peak clusters are associated to renal cell carcinoma. However, some other markers could not be validated [36]. Such examples demonstrate the importance to strictly control parameters such as storage, clotting, time of analysis, instrument performances, sample selection, and statistical classification method.

The urgent need for SOPs in clinical proteomics research is therefore absolutely mandatory reflecting a growing trend in the field [19, 53, 62, 63]. Interaction between researchers, clinicians, and statisticians is also a key element for the success.

Altogether, these applications of SELDI-TOF-MS technology illustrate its capability for discrimination and follow-up of a multitude of diseases using different body fluids as well as certain therapeutic response prediction. It is worth mentioning that FDA approved recently the first diagnostic tool (named OVA1) issued from SELDI proteomic researches. It is made of the combination of 5 markers for ovarian cancer diagnostic.

7. Concluding Remarks

Taking into account herein and previously described recommendations, SELDI-TOF-MS offers very exciting opportunities to discover not only diagnostic but also prognostic and mechanistic markers for a number of major diseases.

To face the general criticism, standardized procedures and recommendations to minimize bias are now followed by most of the users. However, some challenges still remain, as for all other proteomic approaches, due in part to the complexity and the wide dynamic range of the samples. Sample fractionation and/or enrichment procedure, such as peptide ligand affinity beads, will certainly be the solution to visualise the deep proteome. In addition, improvements in mass spectrometry instrumental performances could be expected (higher resolution, reducing adduct formation, and ion suppression), contributing further to more reliable and faster biomarkers discovery.

Abbreviations

2-DE: Two-dimensional electrophoresis
CSF: Cerebrospinal fluid
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme-Linked Immunosorbent Assay
HUPO PPP *Specimens Committee*: Plasma Proteome Project
IMAC: Immobilized Metal Affinity Capture
ISD: In-Source Decay
LC-MS: Liquid Chromatography—Mass Spectrometry
MALDI-TOF: Matrix-Assisted Laser Desorption/Ionisation—Time-Of-Flight
PI: Protease inhibitor
PTM: Post-translational modifications
PSA: Prostate specific antigen
QC: Quality control
SELDI-TOF-MS: Surface-Enhanced Laser Desorption/Ionisation—Time-Of-Flight—Mass Spectrometry
SOP: Standard Operating Procedures
SPA: Sinapic acid
SVM: Support Vector Machine
TIC: Total Ion Current.

Acknowledgment

The authors thank Jean De Graeve for his advices. M. Fillet is a Reasearch Associate; M.P. Merville and E. Louis are Senior Research Associates at the FNRS (National Fund for Scientific Research, Belgium).

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